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Inhibition of PP2A in a Mono and Co-Culture Model of Hepatocellular Carcinoma: Implications on the Regulation of the Matrix Metalloproteinase System and the Formation of the Metastatic Niche



A thesis submitted to Trinity College Dublin,
The University of Dublin

In the fulfilment of the requirement
For the degree of
Doctor of Philosophy
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By

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Declaration

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Summary of Methods and Results

Okadaic acid alteration in Hep3B cells Phosphoproteome: Cell proliferation was measured by MTT assay. Okadaic acid (OA; 40 nM) increased Hep3B cells cell proliferation after 12, 24 and 48 h compared to untreated control (P<0.05). Migration and angiogenesis assay were assessed using scratch and HUVEC tube formation assays following exposure to OA (40 nM). OA increased Hep3B cell migration by 43.7 ± 4.3% compared to untreated control (P<0.05), while FTY-720+OA reduced cell invasion compared to OA alone by 18.7 ± 4.9% (P<0.05). OA increased HUVEC tube formation ability while FTY-720 and FTY-720+OA impaired it. Phosphoprotein enrichment and resulting mass spectrometry analysis identified 281 basal phosphorylated proteins in Hep3B cells, while a further 180 proteins were identified following exposure to OA (40 nM). Cellular process (GO:0009987), metabolic process (GO:0008152) and cellular organisation (GO:0016043) were the major ontologies of the OA phosphorylated proteins identified using PANTHER gene ontology. DAVID protein ontology identified protein types with acetylated proteins, phosphoproteins (P=1.04E-28), cytoplasmic proteins, nucleotide-binding proteins, ubiquitin conjugation proteins and ATP-binding proteins as significant (P<0.05). WebGestalt software identified gene expression (P=9.61E-17), plasma membrane oestrogen signalling (P=5.9E-16), PAR-1 mediated thrombin signalling (P=1.85E-15), EGF signalling (P=1.89E-15) and mTOR signalling (P=1.85E-15) as the major pathways altered. Motif-X was used to identify phosphorylation motifs in OA phosphorylated proteins. Serine phosphorylation motif **S**E was identified as the dominant phosphorylation motif. A threonine CDK-like motif *TK/*T*K** and a putative Src kinase tyrosine motif *YGQH** were also identified. Phosphorylated moesin, eif3H, VASP, ROCK2 and HYOU1 were all identified by Western blot. Ingenuity Pathway Analysis (IPA) identified RNA post-translational modification (P=8.67E-07), cellular growth and proliferation (P=2.93E-06), cell death and survival (P=3.48E-06), protein

synthesis (P=4.45E-0.6) and cell morphology (P=6.78E-05) as the top biological processes within the OA-phosphorylated data set. The top upstream regulators identified were Myc (P=4.59E-11), sirolimus (mTOR signalling; P=1.65E-07) insulin receptor signalling (P=2.19E-06) and 1,2-dithiol-3-thione (P=6.48E-0.6). Myc was identified as the major upstream regulator *in silico*, furthermore OA (40 nM) was found to increase c-Myc protein expression in Hep3B cells.

Serine/threonine phosphatase regulation of Matrix Metalloproteinases: OA (40 nM) increased expression of MMP-9 and TIMP-1 mRNA expression by 1.90 ± 0.34 and 1.74 ± 0.31 fold respectively in Hep3B cells, while increasing MMP-9 1.3 ± 0.2 fold and decreasing TIMP-1 by 0.7 ± 0.1 fold in LX-2 cells (P<0.05). MMP-9 and TIMP-1 expression were not altered by tautomycetin (TMC; 10nM) or cyclosporin (CSA; 10 nM) in either cell line. OA augmented IL-8 mRNA expression by 1.57 ± 0.24 and 1.9 ± 0.31 fold compared to control in Hep3B and LX-2 cells (P<0.05). OA, TMC, or CSA altered none of the other inflammation/growth factor releasers irrespective of cell line. OA (40 nM) elicited a time dependent increase in expression of MMP-9 mRNA that was detectable after 4 h (P<0.05). OA increased secreted MMP-9 abundance to 413.9 ± 118 pg/ml from a basal level of 171.6 ± 35.88 pg/ml in Hep3B cells (P<0.05), and from 123.4 ± 14.43 pg/ml to 298.2 ± 24.83 pg/ml in LX-2 cells (P<0.05). TMC and CSA had no effect on secreted MMP-9 abundance in either cell line studied. Transfection with PPP2CA siRNA increased detectable MMP-9 activity by ~1 fold and increased MMP-9 abundance in conditioned media by 581.6 ± 105.1 pg/ml compared to media from mock transfected cells (P<0.05). In contrast, knockdown of PPP2R1A and PPP1CA did not alter detectable MMP-9 activity or abundance in Hep3B cells. Knockdown of PPP2CA, PPP2R1A and PPP1CA had no effect on detectable MMP-2 activity. Transient transfection of Hep3B cells with MMP-9 promoter constructs containing targeted mutation of the AP-1 motifs revealed that okadaic acid absolutely required the proximal AP-1 $_{(-79)}$ site, while mutation of

the AP-$1_{(-533)}$ binding motif reduced the response by 1.16 ± 0.93 fold (P<0.05). Mutation of the NF-$\kappa B_{(-600)}$ binding site was not required for MMP-9 induction by OA. Transfection with a CIP2A over expression plasmid increased total MMP-9 abundance from 361.3 ± 61.86 pg/ml to 882.8 ± 90.37 pg/ml and increased detectable MMP-activity compared to mock transfected and pDNA.3.1 transfected cells (P<0.05). Exposure of Hep3B cells to OA (40 nM) for 24 h decreased PP2A activity by ~80% compared to untreated control cells (P < 0.05). Overexpression of CIP2A attenuated PP2A activity by ~65% compared to mock transfected controls (P < 0.05; Figure 6B). siRNA knockdown of PPP2CA inhibited PP2A activity by ~70% compared to mock and non-template siRNA transfected cells (P<0.05). Exposure of Hep3B cells to OA (40 nM) for 24 h increased cell invasion through by 3.7 ± 1.1 fold, while over expression of CIP2A increased Hep3B cell migration by 3.22 ± 0.96 fold (P<0.05).

PP2A regulation of TIMP-1: In Hep3B, Huh-7 and LX-2 cells exposed to PMA (50 ng/ml) and LPS (50 ng/ml), increased TIMP-1 mRNA expression and protein abundance in all cell lines studied (P<0.05). TNF-α (10 ng/ml) increased TIMP-1 mRNA expression and abundance in LX-2 cells only. OA (40 nM) increased TIMP-1 mRNA expression and protein abundance in Hep3B cells and Huh-7 cells, while decreasing in LX-2 cells (P<0.05). OA-mediated effects in all cell lines were found to be concentration dependent. Transfection of Hep3B, Huh-7 and LX-2 cells with PPP2CA siRNA decreased mRNA expression of TIMP-1 by 0.59 ± 0.08, 0.53 ± 0.06 and 0.64 ± 0.1 fold respectively compared to mock transfected cells (P<0.05). Knockdown of PPP2CA decreased TIMP-1 abundance in conditioned media from Hep3B, Huh-7 and LX-2 cell lines (P<0.05). CIP2A overexpression in Hep3B cells decreased TIMP-1 abundance in conditioned media by 0.65 ± 0.09 fold, while transfection with a SET overexpression plasmid attenuated it by 0.83 ± 0.02 fold (P<0.05). Interestingly, when OA (40 nM) is added 24 h post transfection, TIMP-1 abundance in condition media was higher in

CIP2A and SET transfected cells compared to their corresponding counterpart in the absence of OA ($P<0.05$). Hep3B cells exposed to NF-κB III inhibitor (10 μM) or BAY 11-7085 (10 μM, NF-κB and/or IK- κB inhibitor) reduced TIMP-1 levels by 0.43 ± 0.1 and 0.45 ± 0.04 fold respectively in conditioned media but not the OA increase ($P<0.05$). LY-294002 (10 μM; PI3K inhibitor) prevented OA from increasing TIMP-1 abundance in Hep3B cells, while PD-032590 (10 nM; MEK inhibitor) attenuated the response ($P<0.05$ 5.8D). Interestingly, SP600125 (10 μM; c-Jun inhibitor) in combination with OA not only inhibited the ability of OA to increase TIMP-1 abundance in conditioned medium from Hep3B cells, but also elicited an additional inhibitory effect (~0.5 fold; $P<0.05$). Using bioinformatics, we compared three potential TIMP-1 promoter sequences and identified 75 putative transcription factor-binding sites across the three promoter sequences. Plasmids were synthesised -890 upstream from the TIMP-1 transcription start site and mutated at transcription factor binding sites CREB $_{(-231)}$, AP-1 $_{(-351)}$ and a PEA3 $_{(-460)}$ respectively. In transient transfected Hep3B cells, OA (40 nM) increased TIMP-1 -890/+96 and -738/+96 promoter activity by 1.28 ± 0.12 and 1.44 ± 0.14 fold respectively ($P<0.05$). With regard to the -890/+96 promoter construct, mutation of the AP-1 motif at position -351 and the CREB motif at position -231 attenuate the OA by 78% and 95% respectively ($P<0.05$). Mutation of the PEA3 motif at position -460 had no effect on the OA response compared to the -890/+96 construct ($P<0.05$). In Hep3B cells transfected with the -102/+96 promoter construct, OA increased promoter activity by 1.1 ± 0.13 fold ($P<0.05$). This effect was attenuated by 91% on mutation of the AP-1 binding site (Figure 5.11; $P < 0.05$). Co-transfection with CIP2A plasmid decreased TIMP-1 promoter activity by 0.56 ± 0.07 fold compared to non-CIP2A overexpressing cells ($P<0.05$). Interestingly, truncation of the -890/+96 construct by 152 bp (-738/+96 construct) obliterated the ability of CIP2A to decrease TIMP-1 promoter activity ($P<0.05$). Mutation of AP-1 $_{(-351)}$ or PEA3 $_{(-460)}$ binding motifs had no effect on CIP2A mediated TIMP-

1 promoter activity. However, mutation of the CREB $_{(-231)}$ binding motif negated the effect of CIP2A ($P<0.05$).

Macrophage induced alteration in Hep3B PP2A activity: Hep3B cells were exposed to condition media from Mϕ, M1 and M2 polarised THP-1 cells or co-cultured with pre-polarised THP-1 cells for 24 h. Hep3B cells exposed to conditioned media from Mϕ and M1macrophages had reduced proliferation by 55.08 ± 6.16%, and 67.4 ± 3.8% respectively ($P<0.05$). Conditioned media from M2 macrophages did not alter Hep3B proliferation. Hep3B cells co-cultured with Mϕ macrophages had a 38.22 ± 5.1% decrease in cell proliferation, M1 macrophages decreased proliferation by 31.48 ± 3.8%, while M2 macrophages increased Hep3B proliferation by 35.6 ± 6.3% ($P<0.05$). PP2A activity in Hep3B cells co-cultured with Mϕ cells was 25 ± 1.48% lower than in monocultured Hep3B cells ($P<0.05$). Co-culture with M1 macrophages did not alter PP2A activity in Hep3B cells, co-culture with M2 cells reduced activity by 29± 2.58% compared to Hep3B cells alone ($P<0.05$). Western blot determined macrophage co-culture did not alter PP2Ac protein expression in Hep3B cells. Hep3B CIP2A mRNA expression increased by 1.26 ± 0.14 fold ($P<0.05$) when co-cultured with THP-1 derived M2 macrophages. Co-culture with Mϕ, M1 and M2 macrophages increased Hep3B CIP2A protein expression. Co-culture with Mϕ, M1 and M2 macrophages increased Hep3B MMP-9 activity by 1.52 ± 0.25, 1.65 ± 0.17 and 3.66 ± 0.63 fold respectively ($P<0.05$). M2 macrophages co-culture increased Hep3B mRNA expression of VEGFA, IL-8 and IL-10 5.8 ± 0.98, 2.37 ± 0.74 and 1.88 ± 0.53 fold respectively ($P<0.05$). LDHA mRNA expression was increased by 1.02 ± 0.39 fold in Hep3B cells co-cultured with M2 macrophages ($P<0.05$). HIF1A mRNA expression was increased by 2.53 ± 0.59 fold in Hep3B cells co-cultured with M2 macrophage ($P<0.05$). Lactate concentration increased in Hep3B cells exposed to OA (40 nM) for 24 from 7.72 ± 0.6 mM to 14.82 ± 1.2 mM ($P<0.05$). Overexpression of CIP2A increased lactate production to 13.06 ± 1.02 mM from 7.06 ± 0.16, similar to that found in Hep3B cells co-cultured with M2 macrophages ($P<0.05$). PP2A activity was decreased by 70 ± 4.05% in M1 polarised macrophages, and increased in M2 by 67 ± 4.4% compared to in Mϕ macrophages, while M2 macrophages co-

cultured with Hep3B cells had a similar PP2A activity as that found in M1 macrophages ($P<0.05$). M2 macrophages co-cultured with Hep3B cells had increased CIP2A expression by 3.87 ± 0.99 compared to M2 macrophages in monoculture ($P<0.05$). Hep3B cells co-cultured with M2 macrophages had increased invasion by 2.14 ± 0.31 while exposure to lactic acid (15 mM) increased invasion by 2.15 ± 0.19 fold ($P<0.05$).

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Publications and Presentations

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Ward, MP and Spiers, JP. Protein Phosphatase 2A (PP2A) Regulation of Markers of Extracellular Matrix Remodelling in HCC Cells: Functional Consequences for Tumour Invasion. *In prepartion*

Ward, MP, Botting, CH, Lucitt, MB and Spiers, JP. Okadaic Acid Triggered Alterations in the Phosphoproteome of Hep3B cells: Functional Consequences of PP2A Inhibition in Liver Cancer. *In prepartion*

Ward, MP and Spiers, JP. Upregulation of CIP2A in HCC Cells Following Co-culture with Macrophages. *In prepartion*

Abstracts:

Ward, MP, Botting, CH, Lucitt, MB and Spiers, JP. Identification of Phosphoproteins Regulated by PP2A Inhibition in Human Hepatocellular Carcinoma. British Pharmacological Society, BPS Winter Meeting, London, UK, December 2014.

Ward, MP and Spiers, JP. Serine.Threonine Protein Phosphatase Regulation of Matrix Metalloproteinase-9 in Hepatocellular Carcinoma. 17th World Congress of Basic and Clinical Pharmacology. Cape Town, South Africa, July 2014.

Ward, MP and Spiers, JP. Modulation of MMP-9 and TIMP-1 expression by Protein Phosphatases in Hep3B and LX-2 cell lines. British Pharmacological Society, BPS Winter Meeting, London, UK, December 2013.

Ward, MP and Spiers, JP. Induction of MMP-9 by Okadaic Acid in MRC-5 Fibroblasts. 16th Annual Meeting of the Institute of Molecular Medicine, Trinity College Dublin, November 2013.

Oral Presentation

Ward, MP and Spiers, JP. Increased Cancerous Inhibitor of PP2A (CIP2A) Expression in Hepatocellular Carcinoma Cells Following Co-Culture With Anti-Inflammatory Macrophages. British Pharmacological Society, BPS Winter Meeting, London, UK, December 2015.

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Abbreviations

12^{PP2A}	SET protein
ANP32A	Acidic leucine phosphoprotein 32
AP-1	activator protein-1
ARE	AU-rich element
ARG-1	Arginase 1
BMI	Body mass index
C/EBP	CCAAT/Enhancer binding protein
Ca^{2+}-CaM	Calcium calmodulin
CAF	Cancer associated fibroblasts
CDK	Cyclin dependent kinase
CIP2A	Cancerous inhibitor of protein phosphatase 2A
CK2	Caesin kinase II
CML	Chronic myeloid leukaemia
CPD1	Cerebella developmental-regulated protein 1
CREB	Cyclic AMP response element
CSA	Cyclosporin A
CTLs	Cytotoxic T lymphocytes
Cys	Cysteine
DARPP	Cyclic AMP-regulates phosphoprotein
DDT	Dithiotreitol
DMEM	Dulbecco's modified eagle medium
DMS	Dimethylsphingosine
DMSO	Dimethylsulphoxide
DNMT1	DNA methyltransferase 1
ECM	Extracellular matrix
EGF	Epidermal growth factor

eIF	eukaryotic translation initiation factor family
ELAVL1	ELAV like RNA binding protein 1
EMT	Epithelial-mesenchymal transition
EPA	Erythroid potentiating activity
ERK	Extracellular-regulated kinase
FGF2	Fibroblast growth factor 2
FTY-720	Fingolimod
GPI	Glycosylphosphatidylinositol
GS	Glycogen synthase
HBV	Hepatitis B virus
HBx	X gene of HBV protein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEAT	Huntington/elongation/A-subunit/TOR
HEP3B	Human epithelial hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible growth factor
Hpx	Hemopexin
HRP	Horseradish peroxidase
HSPA4	Heat shock □0 kDa protein 4
Huh□7	Hepatocellular carcinoma (cell line)
HUVEC	Human umbilical vein endothelial cells
I-1	Inhibitor 1
I-2	Inhibitor 2
IDT	Integrated DNA technologies
IGF-2	Insulin-like growth factor 2
IL	Interleukin
INF-$_{\gamma}$	Interferon gamma
INHAT	Inhibitor of acetyltransferase

IPA	Ingenuity pathway analysis
IPP5	Protein phosphatase 1 inhibitor 5
IRS1	Insulin receptor substrate 1
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LDH-A	Lactate dehydrogenase A
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MMP-9	Matrix metalloproteinase 9
MMP-7	Matrilysin-1
MSN	Moesin
MT-MMP	Membrane-type matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyltrazolium
NASH	Non-alcoholic steatohepatitis
NFAT	Nuclear factor of activated T cell
NFκB	Nuclear factor kappa B
NOS2	Nitric oxide synthase 2
OA	Okadaic acid
OXFOS	Oxidative phosphorylation
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPA	Phosphatase activator
PVDF	Hybond-P hydrophobic polyvinylidene difluoride
R subunit	Regulatory subunit
ROS	Reactive oxygen species
S	Serine
SMART	Simple modular architecture research tool

SV40	Simian virus 40
T	Threonine
TAM	Tumour Associated macrophage
TFBS	Transcription factor binding sites
TGF-β	Transforming growth factor β
T_H1	Type 1 $CD4^+$ T cells
T_H2	Type 2 1 $CD4^+$ T cells
TIMP-1	Tissue inhibitor of matrix metalloproteinase 1
TIMP-2	Tissue inhibitor of MMP-2
TKi	Tyrosine kinase inhibitor
TMC	Tautomycin
TME	Tumour Microenvironment
TNF-α	Tumour necrosis factor- α
VEGF	Vascular endothelial growth factor
WB	Western blotted
WCL	Whole cell fractions
XPB	Xeroderma pigmentosum type B
Y	Tyrosine

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Chapter 1.

1. Introduction

1.1 Cancer

Cancer is among the leading cause of death in the world with a reported 14 million new cases every year and 8.6 million cancer related deaths in 2012 [1]. With new cases expected to rise by over 70% in the next decade, cancer is quickly becoming an even greater burden on our stressed health services. However, the word cancer does not refer to a single disease, but to a collection of malignancies with diverse characteristics. The progression of cancer requires alterations, which may occur at a genetic or epigenetic level. Inherited mutations may act as a predisposition to cancer formation. However, the majority of cancers form via the accumulation of mutations over a period of years resulting from environmental stresses.

Fifteen years ago, scientists Hanahan and Weinberg defined six "Hallmarks of cancer", key alterations, which allow human cancers to develop [2]. These crucial changes include self-sufficiency in growth signals, insensitivity to growth inhibitory signals, ability to escape programmed cell death (apoptosis), infinite replicative potential, sustained angiogenesis, and tissue invasion and metastasis [2]. These features are considered commonalities in all cancers. Recently, the same authors revised these hallmarks and suggested two additions: reprogramming of energy metabolism and evading destruction by the immune system [3]. In addition to cancer cells, tumours exhibit another dimension of complexity: they contain a repertoire of recruited, seemingly normal cells that contribute to the acquisition of hallmark traits by creating the "tumour microenvironment". Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer.

1.2 Hepatocellular Carcinoma

1.2.1 Incidents and Risk Factors of Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the second highest rate of cancer related deaths in the world [4]. With this, there is an increasing interest in the advancement of new diagnostic and pharmacological treatment for HCC. Occurrence varies across the globe, with highest rates in Asia, followed by Western Africa, with intermediate risk in the Western countries. The recent rise in incidence reported in the West was thought to be attributed to increased immigration from Hepatitis B virus (HBV) endemic countries and the increased rate in chronic Hepatitis C virus (HCV) infection [5]. However, this is now known to be only partially demeritorious. Although the incidence of HCC rises with increasing age, reaching its peak in those aged above 65 years [6, 7] and is more common in men [5], over the past two decades there has been a shift in incidence towards a younger age demographic due to vertical transmission of Hepatitis B and the increase in non-viral incidence of primary HCC [8]. The increase risk and occurrence of HCC in western countries coincides with the increase in obesity related diseases such as Diabetes Mellitus, non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver disease (NAFLD), liver cirrhosis as a result of heavy alcohol consumption, aflatoxin exposure, and an ever increasing aging population [9-11].

1.2.2 Hepatitis B Virus

Hepatitis B virus (HBV) is the most common risk factor for the development of HCC, a fact particularly relevant to HBV endemic areas, where vertical transmission is responsible for the majority of HBV acquirement [12]. In such patients, HCC can develop before the onset of cirrhosis as a result of the direct oncogenic effects of HBV. With the

increasing global implementation of HBV immunisation, incidents of HBV and subsequently HCC diagnosis are falling in areas previous identified as HBV hot spots [7]. Recent studies have shed light on the significance of HBV genotypes in HCC development, with evidence to suggest that in Asia, genotype C, independent of the viral load, predisposes to HCC, whilst in the Western nations it is genotype D [13]. Several factors are implicated in the pathogenesis of HBV related HCC, which includes the repeated liver damage by HBV causing necroinflammation and consequent regeneration of hepatocytes. The other is the direct oncogenic property of HBV, which integrates itself into the hepatocyte DNA, producing cis- or transactivation of the cellular oncogenes [6, 14].

1.2.3 Life Cycle of Hepatitis B Virus

The virus has a unique life cycle that produces large viral loads during active replication but is not directly cytopathic. Liver injury occurs as a result of repeated attempted by the host's immune system to control the infection by reducing inflammation, apoptosis and regeneration. This in part facilitates the accumulation of random mutations and epigenetic alterations that form the premise for HCC neoplasm formation [15]. For viral replication to occur, the synthesis and maintenance of a covalently closed circular DNA (ccc-DNA) form of the viral genome in the nucleus of host cells is required for the HBV infection. HBV virions infect hepatocytes by binding to cellular surface receptors, the identity of which remain unknown. After membrane fusion, the viral inner cores are transported into the nucleus, where the viral DNA turns into a cccDNA, and is transcribed by the host RNA polymerase II into a series of viral mRNAs. These transcripts are transported out to the cytoplasm, where they are translated into different viral proteins [16]. Viral inner cores are then assembled in the cytosol, with a single molecule of pre-genomic RNA and a viral DNA polymerase packed with core proteins into each particle. The synthesis of the viral genome takes place in

newly assembled capsids by reverse transcription of the pre-genomic RNA. HBV genome replication requires a reverse transcription step, which is error-prone due to the lack of proofreading ability of the viral reverse transcriptase. However, the compactness of the HBV genome prevents large degrees of genetic variability from occurring. As a result, the mutation rate of HBV genome is higher than those of other DNA viruses but lower than those of retroviruses [17]. Viral genome integration in the host genome is thought to result from the integration of viral double stranded linear DNA in the host's genome, harbouring breakpoints involving topoisomerase I [18]. The X gene encodes the HBx protein, and interferes with transcription, signal transduction, cell cycle progress, protein degradation, apoptosis and chromosomal stability in the host [19]. HBx protein inhibits activity of interferon-β [19], and also activates NF-κB [20]. This suppression of interferon signalling and activation of pro-inflammatory NF-κB signalling by proteins within the virus contributes to the formation of neoplastic masses and progression of HCC following viral replication.

1.2.4 Hepatitis C Virus

Like HBV, Hepatitis C virus (HCV) has a major impact on public health; it infects around 170-200 million people in the world, with an estimated global incidence of three to four million new infections per year [21]. Most patients infected with HCV are unaware of their exposure and remain asymptomatic during the initial stages of the infection; making early diagnosis during the acute phase (first six months after infection) unlikely. While some of these infections will spontaneously resolve, the majority will progress to chronic HCV [22]. The infection by HCV may be unresolved in approximately 85% of the infected individuals, representing an important cause of liver cirrhosis and hepatocellular carcinoma. However, successful antiviral therapy in patients with HCV-related cirrhosis may decrease HCC risk moderately among patients treated with interferon [23] and with the advancement in HCV therapies

such as Sofosbuvir and others, HCV-related HCC has the potential to all but be eradicated [24].

1.2.5 Hepatitis C Virus Life Cycle

How HCV infection leads to oncogenic transformation within the liver is not fully clear. The Hepatitis C virus contains a core protein which is involved in binding viral RNA, regulating HCV RNA translation, making homotypic interactions for particle assembly and interacting with the glycoproteins to generate a complete virion [25, 26]. The core protein has the ability to bind to p53 and suppress its tumour suppressing capabilities [27]. As a result, DNA damage occurs, which stimulates the production of reactive oxygen species (ROS) within the hepatocytes [28]. In HCV infection, ROS are also produced by NADPH oxidase and xanthine oxidase in neutrophils and macrophages [29]. The production of ROS and the influx of pro-inflammatory cytokines such as TNF-α are thought to activate various oncogenic signalling pathways, which initiate Hepatitis C virus induced HCC.

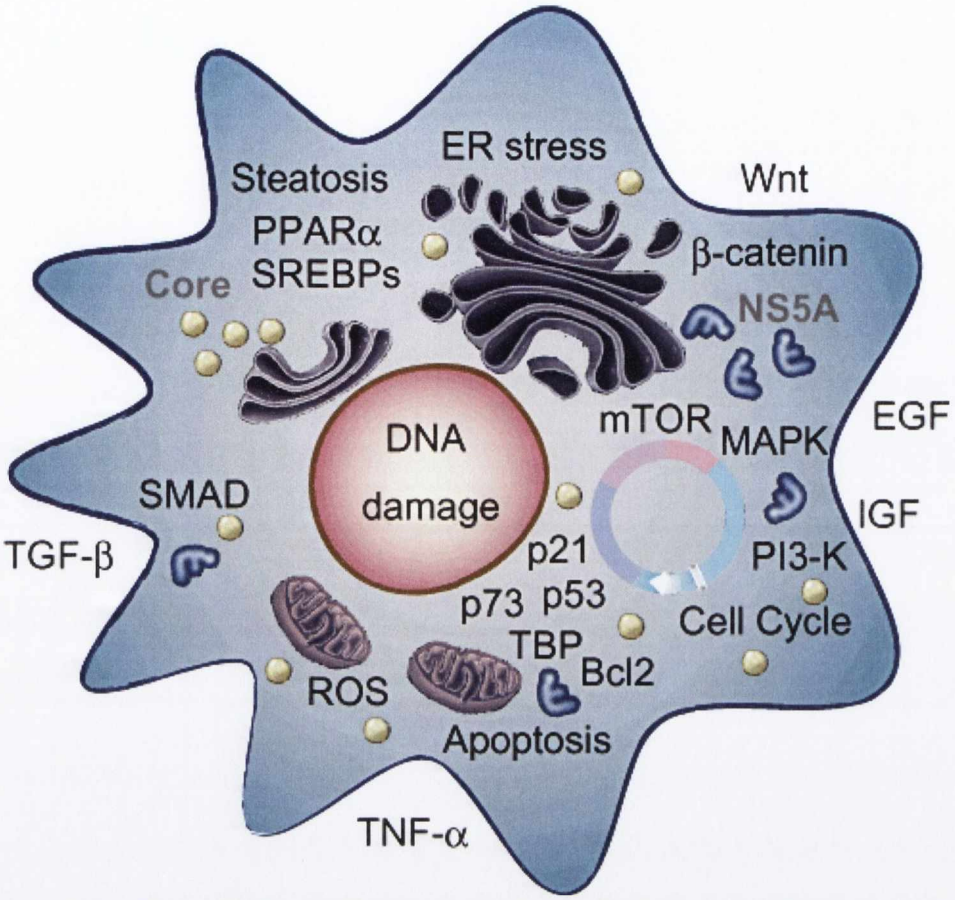


Figure 1.1 Signalling pathways and mediators implicated in HCC associated with chronic hepatitis C virus infection. Taken form Yamashita *et al.* [30].

Abbreviations: EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin- like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3- K, phosphatidylinositol 3- kinase; PPAR, peroxisome proliferator- activated receptor; ROS, reactive oxygen species; SREBP, sterol- regulatory element binding protein; TBP, TATA binding protein.

1.2.5 Non-Viral Causes of Hepatocellular Carcinoma

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)-associated HCC is reported as the leading cause of non viral based HCC in adults in the Western world and Japan [31, 32]. This is due in part to diet and the increasing levels of diabetes mellitus and resulting liver damage from the disease [33]. NASH represents the advanced disease progression stage in the spectrum of NAFLD and as much as 20% of NASH may progress to cirrhosis as well as end stage complications such as HCC [34]. In a recent study, the incidence of HCC in diabetic patients was shown to be as high as double the incidence in non-diabetic patients [35]. A raised body mass index (BMI) has itself been associated with HCC development with several studies reporting a significant relationship between a BMI ≥ 30 and HCC [36, 37]. NAFLD and obesity is now the leading cause of chronic liver disease in the USA [38].

Aflatoxin B1, a potent carcinogen produced by *Aspergillus flavus* and *A. parasiticus*, promotes HCC through altered p53 function [39]. Aflatoxin B1 exposure has been linked with mutation of A or G to T transversion at the third position of codon 249 of the p53 gene (249ser) in HCC [40].

1.2.6 Molecular Signalling and Pathogenesis of Hepatocellular Carcinoma

1.2.6.1 WNT and Hedgehog Signalling

There is an increased interest in exploring the molecular pathogenesis of HCC with the aim of developing newer targeted molecular therapies. As discussed previous, various aetiologies result in HCC, however all share a commonality in that the liver is exposed to stress and trauma. There is no dominant signalling pathway in HCC, but instead many pathways working synergistically to create the perfect malignancy. The activation of several oncogenic pathways has been proposed. Wnt signalling in particular plays an important role in maintaining liver rejuvenation[41]. Beta-cadherin acts as an intracellular signal transducer in the Wnt signalling pathway with [41-44]. A recent study demonstrate that over-expression of Sonic Hedgehog protein is found in 60% of 115 human HCC samples analysed [45]. Moreover, tumourigenic activation of hedgehog signalling can mediate c-Myc overexpression, which plays an important role in hepatocarcinogenesis [46].

1.2.6.2 Growth Factor Signalling

IGF2 (insulin-like growth factor 2 is overexpressed in up to 30% of HCC samples [47], with mutations in the IGF2 receptor, IGF2R, being demonstrated in over 60% of dysplastic hepatic nodules and HCC [48]. EGFR deregulation is also found in HCC; however, its expression within HCC varies in the literature [47, 49]. Hepatocyte growth factor (HGF) is a potent molecule in hepatocyte regeneration after injury and is secreted by stellate cells and exerts its actions by binding to the c-MET receptor [50, 51]. Overexpression of c-MET and HGF are common events in HCC [52, 53]. Unfortunately, the correlations between their expression and clinical features do not indicate that c-MET overexpression has any prognostic impact on recurrence or survival of patients with HCC undergoing surgical resection. However, 27.9% of

patients positive for c-MET overexpression could be considered candidates for treatment with a c-MET inhibitor [53]. Hence, the role of these molecules in the molecular pathogenesis of HCC needs further investigation.

1.2.6.3 AKT Signalling

Activation of the P13/Akt pathway and impairment of the Akt regulator, phosphatase and tensin homolog (PTEN), occurs in approximately 50% of HCC [54]. PTEN is a known tumour suppressor and its mRNA expression is decreased in HCC compared to surrounding non-cancerous tissue [55]. Loss of PTEN has also been found to associated with high tumour grade and intrahepatic metastasis, correlating with p-Akt, p-mTOR and MMP-9 overexpression, with an inverse correlation with loss of PTEN and the increase in MMP-9 expression [56]. The role of Akt in HCC is still uncertain; however, one study reported that the presence of phosphorylated Akt in resected HCCs from 135 patients conferred a 12-fold increased risk of early recurrence [57]. Secondly, resistance to sorafenib in HCC cell lines is thought to develop through the over-activation of PI3/Akt signalling [58].

1.2.6.4 p53

The derangement of apoptotic pathways such as p53 mutations accounts for up to 40% of HCC [59, 60]. Mutations in p53 decrease HBx (X gene of HBV protein) mediated p53 binding to XPB (xeroderma pigmentosum type B), which is involved in both nucleotide excision repair and in transcription as a basic transcription factor [61]. Infection with HBV and HCV cause inflammation with the release of free radicals, chemokines and cytokines resulting in DNA damage, cell proliferation, fibrosis and angiogenesis. The p53 pathway is a key responder to inflammatory stress and thus loss of p53 function may also promote HCC progression through inflammatory processes [62].

1.2.6.5 VEGF Signalling

Hepatocarcinogenesis, resulting from autocrine/ paracrine secretion of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), or angiopoietin-2 or high-level amplification of VEGFA gene also plays a role in the promotion of HCC [63-65]. These growth factors and cytokines induce angiogenic signalling through a variety of mechanisms, including activation of the RAF/MEK/ERK, PI3K/AKT/mTOR and Janus kinase (JAK)/signal transducer and activator of transcription pathway [66].

1.3 Serine/Threonine Protein Phosphatases

1.3.1 Protein Phosphatases in Eukaryotes

Reversible protein phosphorylation is one of the most important post-translational modifications of proteins in eukaryotic organisms. Two types of enzymes, known as protein kinases (PKs) and protein phosphatases (PPs), are responsible for this reversible reaction and constitute approximately 2-4% of the genes in a typical eukaryotic genome [67, 68]. Protein kinases are enzymes that phosphorylate proteins by chemically adding phosphate groups to specific amino acids such as serine (S), threonine (T) and tyrosine (Y) residues. Protein phosphatases regulate this kinase dependent phosphorylation by facilitating the removal of the phosphate group by enzymatic actions.

To date, 518 protein kinases have been identified in the human genome: protein serine/threonine kinases (PSK)-385, protein tyrosine kinases (PTK)-90 and protein tyrosine like proteins-43 [69]. The exquisite specificity of signalling and the reversible nature of phosphorylation seem to suggest that there would be a similar numbers of protein phosphatases in the human genome. Unpredictably however, only 107 putative protein tyrosine phosphatases (PTPs) and approximately 30 protein serine/threonine phosphatases (PSPs) have been identified [70]. While the numbers of PTKs roughly match that of PTPs, the number of catalytically active PSPs is considerably less than PSKs.

PSPs are currently divided into three structurally unrelated families (Figure 1.2). The PPM family comprises Mg^{2+}-dependent enzymes, including protein phosphatase PP2C. The FCP family only contains CTD phosphatase and the FCP phosphatase, which are also Mg^{2+} dependent. All other protein serine/threonine phosphatases are classified in the PPP family, consisting of the subfamilies PP1, PP2A (including PP4 and PP6), PP2B, and PP5, which all have a structurally related core and a similar catalytic mechanism.

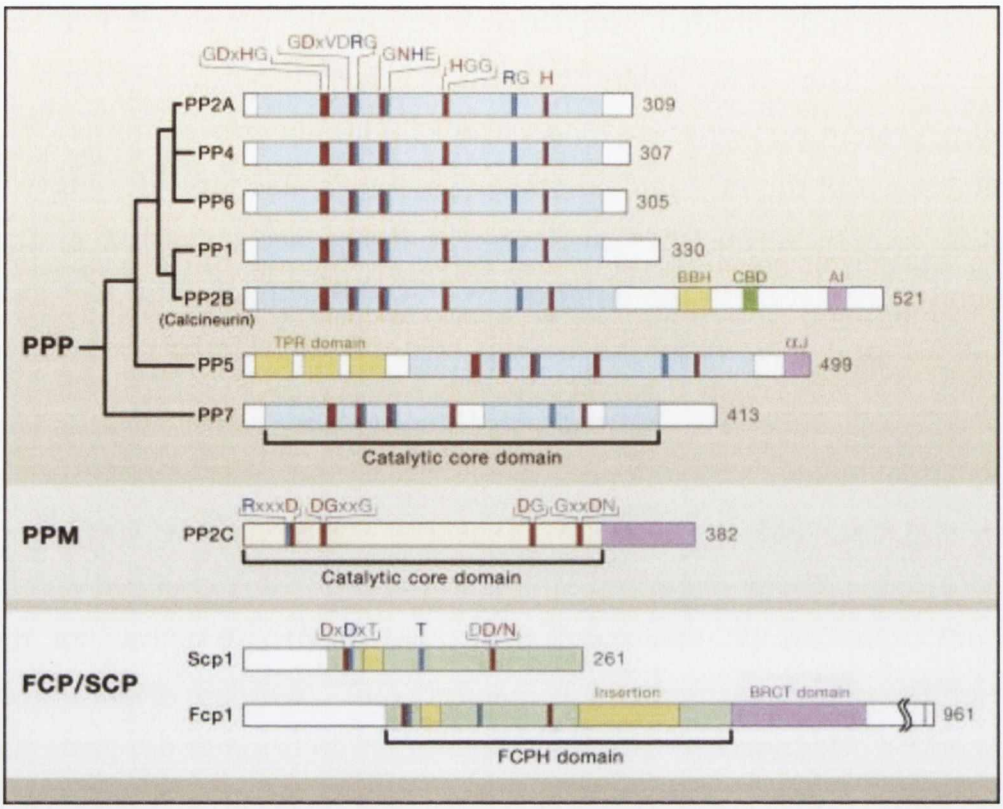


Figure 1.2 Classifications of Serine/Threonine Protein Phosphatases.

Serine/threonine protein phosphatases (PSPs) can be categorised into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases FCP (TFIIF-associating component of RNA polymerase II CTD phosphatase) and SCP (small CTD phosphatase) [71].

1.3.2 Small Molecule Inhibitors of Serine/ Threonine Phosphatases

Many natural product extracts have been found to be potent inhibitors of serine/threonine protein phosphatases. Marine toxins such as okadaic acid (produced by marine sponge dinoflagellates) and calyculin A (isolated from *Discodermia calyx*) were among the first serine/threonine phosphatase inhibitors identified [72, 73]. Okadaic acid is a polyether fatty acid and is best regarded as a small molecular weight inhibitor of PP1 and PP2A, with the IC_{50} for PP2A being 50–500-fold less than that for PP1 [74, 75]. Other inhibitors such as tautomycin, tautomycetin and fostriecin are isolated from bacteria, were found to have a greater potency towards PP1. However, in actuality, it appears that most of the mentioned inhibitors act not only on PP1 and PP2A, but also PP4, PP5 and PP6 [74, 76]. For the purpose of this study it must be regarded that these pharmacological inhibitors are the preeminent tools for studying phosphatases. Nevertheless, their promiscuity and ability to inhibit other members of the serine/threonine family of phosphatases must be considered.

1.3.3 Protein Phosphatase 1

PP1 is ubiquitously expressed in eukaryotic cells and is implicated in meiosis and cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganisation, and in the regulation of membrane receptors and channels [77-79]. Proportionate to its myriad of functions, the many forms of PP1 collectively exhibit broad substrate specificity. However, each PP1 enzyme is thought to display stringent substrate specificity and elicits specific biological responses depending on its precise location within the cell. Two types of PP1 inhibitors are protein inhibitors and natural small molecular toxins. The toxins such as microcystin, okadaic acid (OA), calyculin A and tautomycin (TMC) inhibit PP1 activity potently, but not specifically [74]. *In vitro*, PP1 is selectively inhibited by the three acid and heat-stable protein inhibitors inhibitor-1 (I-1), dopamine and cyclic AMP-regulated phosphoprotein of molecular weight 32,000 (DARPP-32) and inhibitor-2 (I-2) [80, 81]. In contrast to I-2, phosphorylation of I-1 and DARPP-32 by PKA on Thr35 and Thr34, respectively, is essential to convert them into potent inhibitors of PP1 [82].

1.3.4 Structure of PP1

PP1 exists as a dimeric enzyme consisting of a catalytic subunit and a regulatory subunit (R subunit). The catalytic subunit of PP1 is highly conserved among all eukaryotes, with approximately 70% or greater protein sequence identity between various species. These sequences support a conserved fold and a similarly positioned active site for all members of the PPP family. The catalytic subunit adopts a compact α/β fold, with a β sandwich wedged between two α-helical domains [83, 84]. Two metal ions identified in the active site of are Mn^{2+} and Fe^{2+} and are located at the three-way joint between the β sandwich and the two helical domains. Coordination of the two metal ions is provided by three histidines, two aspartic acids, and one asparagine [84]. These residues

are highly conserved in all members of the PPP family suggesting a common mechanism of metal-catalysed reaction in the protein family. The two metal ions are thought to bind and activate a water molecule, which initiates a nucleophilic attack on the phosphorous atom [83, 84]. Three shallow surface grooves roughly follow the domain boundaries and converge at the catalytic centre, forming a Y-shaped surface feature where tumour-inducing toxins such as microcystin and okadaic acid associate tightly with the active site through interactions with amino acids on the surface loops of the catalytic centre [85].

At least 200 putative R subunits (phosphatase interacting proteins) have been identified so far in eukaryotes, highlighting their complexity [77, 86, 87]. Many of the PP1 regulatory proteins contain a specific RVxF motif that is required for binding to the catalytic subunit [88]. For a more detailed description of the RVxF motif, please see Wakula *et al.*, 2003 [88]. Functionally, the R subunits may target the PP1 catalytic subunit to specific subcellular compartments, modulate substrate specificity, or serve as substrates themselves. Thus, the interactions between the catalytic subunit and specific R subunits are central to the function of PP1.

1.3.5 Role of PP1 in the Cell

Within humans, four isoforms of PP1 exist: PP1α, PP1β, PP1γ1 and PP1γ2. PP1 catalytic isoforms do not exist freely within cells, but rather associate with regulatory subunits to form distinct multimeric holoenzymes [87]. As previously stated, PP1 regulatory subunits function as signalling modules by regulating the enzymatic activity or targeting of catalytic subunits to specific substrates. This ability gives rise to PP1 being found to have a crucial role in various cellular homeostatic events. PP1 is involved in cell cycle progression, especially during mitosis and mitotic exit, and dysregulation of PP1 activity causes mitotic arrest or deficient cytokinesis in mammals (Figure 1.3)[89]. PP1 is associated with multiple mitotic structures, including chromosomes, centrosomes and spindles, and is implicated as a major phosphatase acting on phosphoproteins at mitotic exits [90]. However, the specific regulatory or targeting subunits required for this activity are not well understood.

PP1 conservation across species is believed to arise due to its strict regulation and reversal of starvation induced metabolic shifts [91]. PP1 controls glycogen metabolism in mammals through regulation of AMP-activated protein kinases AMPK in response to stress and glucose deprivation [92]. Additionally, PP1 is one of the key components of the insulin-signalling pathway, which is responsible for bridging the initial insulin-simulated phosphorylation cascade with the ultimate dephosphorylation of insulin sensitive substrates[93, 94]. Dephosphorylations catalysed by PP1 activate glycogen synthase (GS) and simultaneously inactivate phosphorylase A and phosphorylase kinase promoting glycogen synthesis within the liver. It also has a role in skeletal muscle insulin resistance in type 2 diabetes [95]. Insulin receptor substrate-1 (IRS1) plays a key role in insulin signalling, the function of which is regulated by both phosphorylation and dephosphorylation of tyrosine and serine/threonine residues. PP1

regulatory subunit 12A (PPP1R12A) is a novel endogenous insulin-stimulated interaction partner of IRS1 indicating a potential role for PP1 subunit rearrangement in insulin signalling [94].

1.3.6 PP1 and Cancer

With regards cancer, the role of PP1 has not been studied with the same level of interest as that of PP2A. The PP1 catalytic subunit (PPP1CA) dephosphorylates Retina Blastoma protein (pRb) and attenuates/increased ceramide accumulation induced by RAS [96, 97], which are key processes in tumourigenesis. In bladder cancer, its role as a biomarker for monitoring disease progression has been investigated. A positive correlation was found between bladder cancer grade and sensitivity, indeed for grade 1 and grade 2 tumours, PPP1CA and other evaluated markers were superior to cytology in diagnosing disease progression [98]. In cervical cancer, knockdown of PP1 prevented cells from migrating and invading through inhibition of Cdc42 signalling [99]. PP1 has been found to be involved in the migration of cancer cells through electrical gradient [99]. Consistent with this, protein phosphatase 1 inhibitor 5 (IPP5), an inhibitory protein of PP1 acts as a tumour suppressor in cervical cancer; overexpression of mutant IPP5 in HeLa cells caused G2/M arrest both *in vitro* and *in vivo* via inhibition of ERK activation [100]. Furthermore, protein phosphatase 1, regulatory subunit 7 is significantly up regulated in metastatic cervical cancer after radiotherapy [101]. Thus taken collectively, whether PP1 has a positive or negative role in cancer remains to be elucidated further.

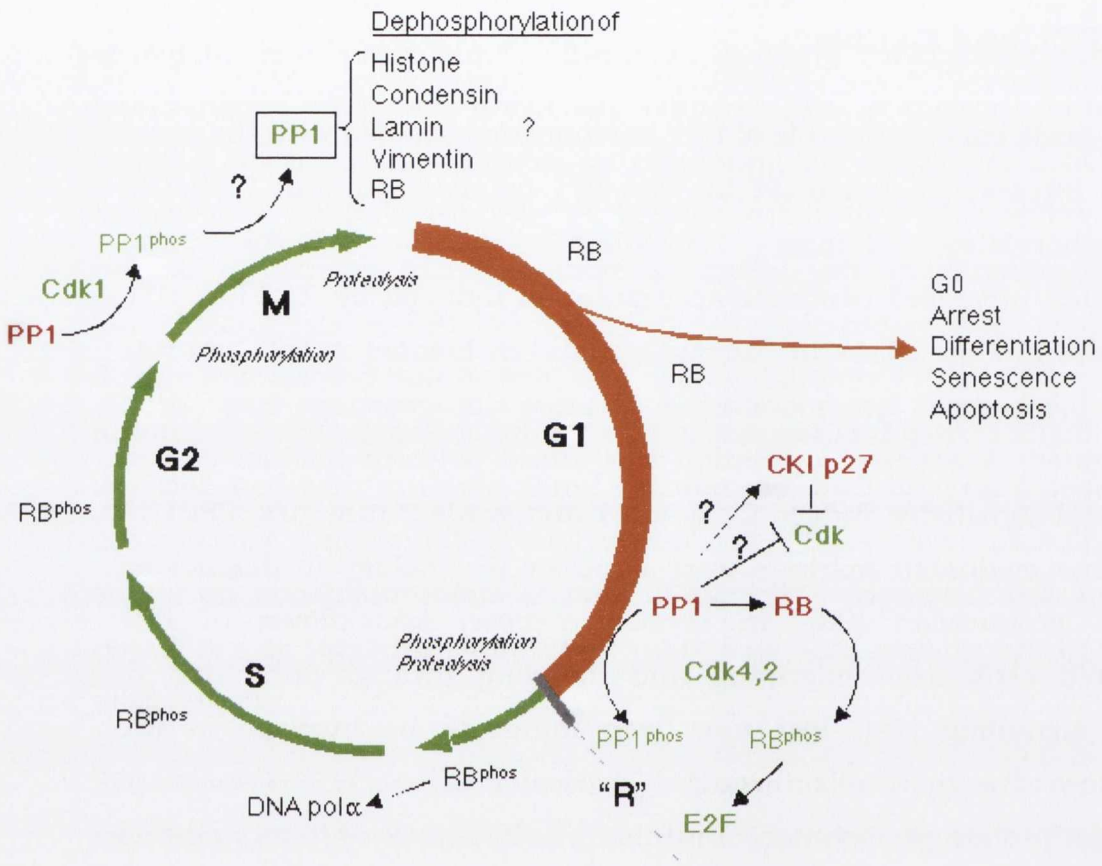


Figure 1.3 Role of PP1 in cell cycle progression.

PP1 undergoes inhibitory phosphorylation by Cdk1 at G2/M; later, PP1 is required to exit from mitosis and G1 entry which is due to the necessary dephosphorylation of several proteins including RB. It is not known whether the mitotic requirement for PP1 activity involves just other PP1 isoforms or reactivation by another phosphatase. The exact roles of the different PP1 isoforms remain to be determined. After return to G1, PP1 would have several opportunities to activate RB and thereby maintain cells in G1: by directly dephosphorylating RB, inhibiting any of the Cdks or stabilising CKIs such as $p27^{Kip1}$. Figure take from [102].

1.3.7 Protein Phosphatase 2B

Protein phosphatase 2B (PP2B) or Calcineurin is a eukaryotic Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase [103]. PP2B was first detected nearly 40 years as a column fraction that inhibited the calmodulin-dependent cyclic nucleotide phosphodiesterase [104]. PP2B is abundantly expressed in the brain and broadly distributed in non-neural tissues as well [105]. It has several functions with the cell including, controlling intracellular Ca^{2+} signalling, neurodevelopment and memory formation, immune response, apoptosis, cardiac hypertrophy, signal transduction, and muscle development [106-108]. PP2B also participates in gene regulation and external signal-mediated biological responses in many organisms and cell types.

1.3.8 Structure of PP2B

PP2B is a dimeric enzyme composed of a catalytic (A subunit) and regulatory subunit (B) that is well conserved from yeast to man [109]. The active site of calcineurin is located on the A subunit, which in mammals, has a molecular weight of 57–60 kDa depending on the isoform and species it is isolated from [110, 111]. The regulatory subunit (calcineurin B) is smaller at 19-kDa. There is strict conservation throughout all eukaryotic organisms such that all calcineurin A genes encode for a polypeptide consisting of a catalytic domain homologous to other serine/threonine protein phosphatases and three regulatory domains at the COOH terminus that distinguish calcineurin from other family members (Figure 1.4) [107]. These domains have been identified as the calcineurin B binding domain [112], the calmodulin-binding domain [113] and the "autoinhibitory" domain [114]. The autoinhibitory domain binds in the cleft of the active site in the absence of Ca^{2+}/calmodulin, rendering it inactive [115]. As a result, PP2B only gains phosphatase activity upon association with Ca^{2+}-calmodulin (Ca^{2+}-CaM) [116].

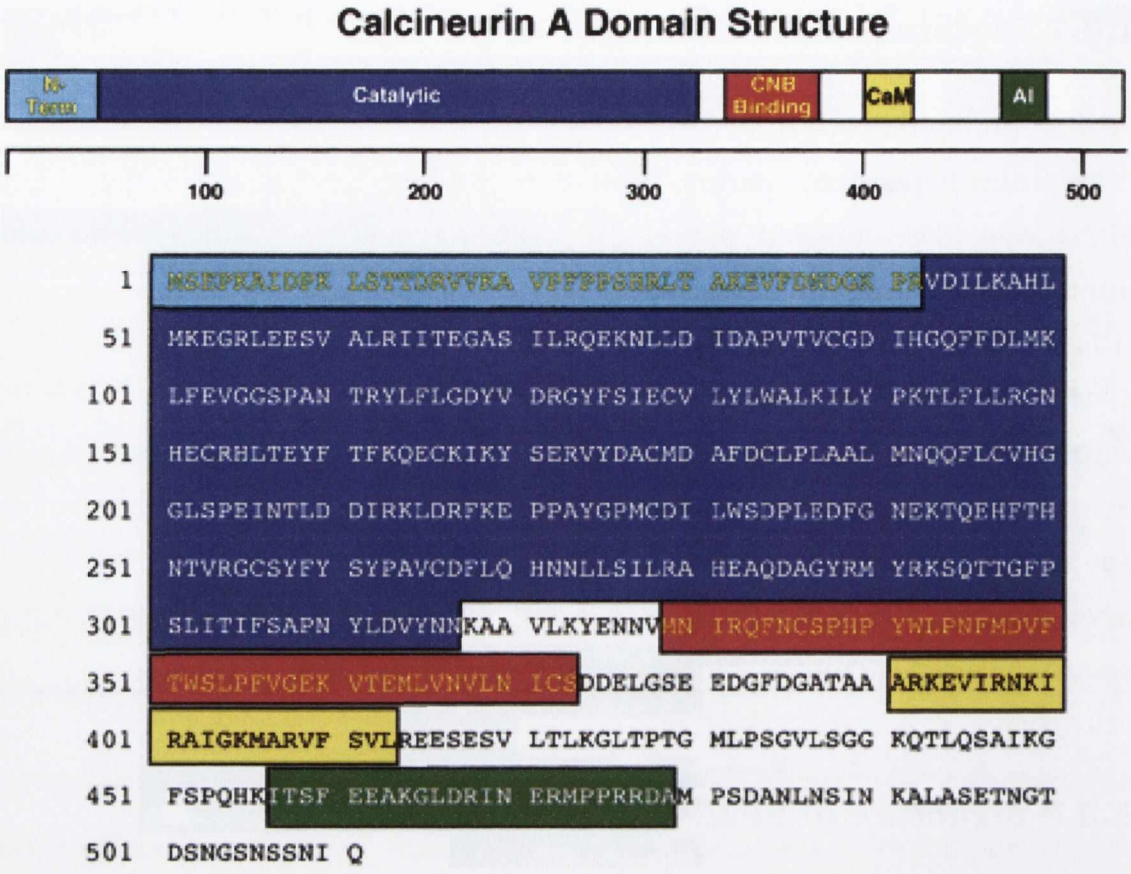


Figure 1.4 Primary sequence and domain structure of PP2B.

The amino acid sequence represents the rat PP2B α-isoform. CaM, calmodulin; CNB, Calcineurin B; AI, autoinhibitory. This image is taken from Rusnak *et al.* [107] while domain structure sequences are from Kissinger *et al.* [115]

1.3.9 Role of PP2B in the Cell

PP2B controls intracellular Ca^{2+} signalling, neurodevelopment and memory formation, immune response, apoptosis, cardiac hypertrophy, signal transduction, and muscle development [106-108]. It also participates in gene regulation and external signal-mediated biological responses in many organisms and cell types. PP2B is central in cAMP-induced dephosphorylation and translocation of the sodium/bile transporter NTCP. Clinically, PP2B is target for the transplant immunosuppressant Cyclosporin [117]. Its immunomodulatory effects are mediated through attenuation of T cell activation through NFAT (transcription factor nuclear factor of activated T cell) and IL-2 signalling [118]. Binding of cyclosporin is thought to inhibit calcineurin-mediated dephosphorylation of NFAT, ultimately resulting in the suppression of T cell activation and immunosuppression [119]. Cyclosporin directly interacts with residues from the calcineurin-B and BBH domains of PP2B [120].

1.3.10 PP2B and Cancer

With regards cancer, again little or nothing is known about the role PP2B plays in oncogenesis or tumour progression. PP2B promotes cervical cancer cell proliferation by directly interacting and enhancing c-Jun protein stability and activity [121]. This was confirmed in a second study showing that dephosphorylation of c-Jun is increased in primary tumour samples, which correlates with enhanced expression of total PP2B. In contrast, PP2B is down-regulated in malignant squamous carcinomas [122]. This inconsistency likely reflects the small number of samples used in either studies or differences in tumour pathology/origin.

1.3.11 Protein Phosphatase 2A

PP2A, like PP1 and PP2B, functions as a multimeric enzyme found ubiquitously in all eukaryotes (Figure 1.5), constituting up to 1% of the total cellular proteome [123]. Between eukaryotes, the amino acid sequence is highly conserved (78-99% sequence homology) [124]. At a metabolic level PP2A controls the enzymatic activity of proteins involved in glycolysis, lipid metabolism and nucleotide metabolism [125-127]. PP2A is essential for embryonic survival as ablation of its catalytic subunit and subsequent loss of function is fatal in mice and drosophila cell lines [128, 129]. This is not surprising as PP2A is intricately involved in regulating the cell cycle, DNA replication, transcription, translation, signal transduction, cell proliferation, cytoskeleton rearrangement, cell motility and apoptosis [128, 130, 131]. PP2A has been found to play a central role in cell transformation in cancer [132, 133].

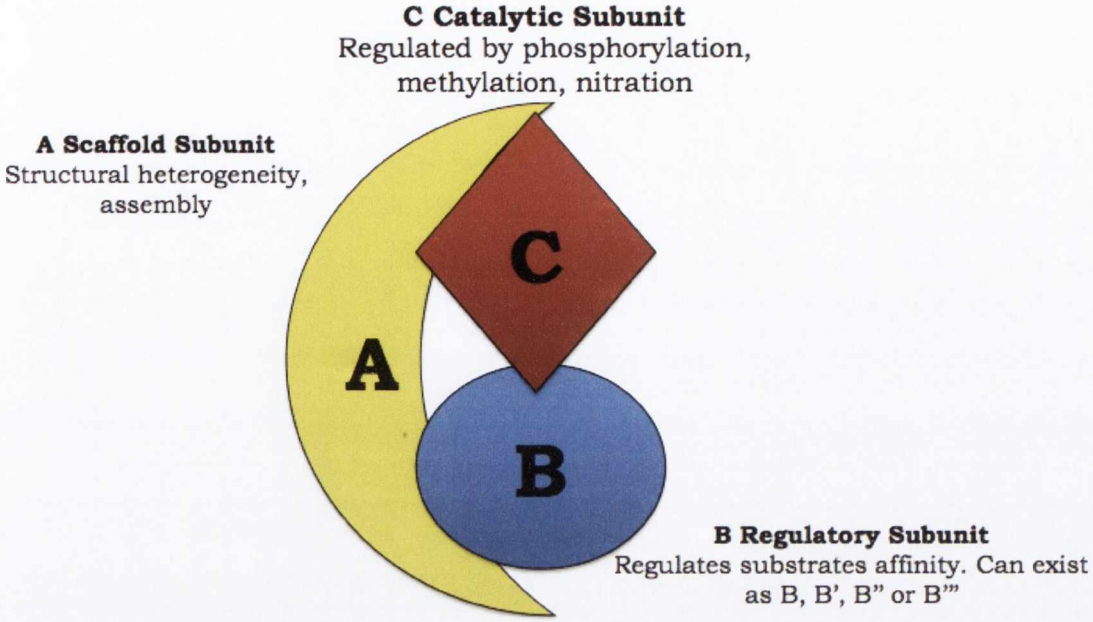


Figure 1.5: Protein Phosphatase 2A schematic representation.

PP2A functions as a trimeric holoenzyme with a scaffolding subunit (A), regulatory subunits (B and a catalytic subunit (C). A provides structural integrity; B regulatory subunits regulate substrate specificity and C catalyses dephosphorylation reactions.

1.3.12 Structure of PP2A

Although PP2A is commonly described as a single phosphatase, this notion is oversimplified. PP2A exits as a trimeric enzyme (Figure 1.5) composed of a catalytic subunit, regulatory B subunit and scaffold subunit. The structure of PP2A is much more complex than the binding of three protein subunits as multiple isoforms of each subunit exist (Table 1.1). It is through combination of these subunits that differing activity and diverse substrate specificity is achieved [71].

1.3.12.1 Catalytic subunit

The catalytic subunit, PP2Ac, is a 37kDA protein and shares 86% identical homology between yeast and man [124]. It also shares 50% amino acid similarity with PP1 and 40% with PP2B. However, various genes encode the different structural component of PP2A. PP2Ac exists in two isoforms, PP2Acα and PP2Acβ, with are 97% identical in sequence [134]. The cα isoform is encoded for by PPP2CA, while cβ is encoded for by PPP2CB. Cα is more abundant than cβ due to having strong promoter activity, faster kinetics and due to differences in the rate of mRNA turnover [135]. However, although two isoforms exists, the formation of the PP2A holoenzyme is not isoform dependent [134].

The levels of PP2Ac are tightly regulated within cells at the translational post-translational level, which makes ectopic expression extremely difficult [136]. However, catalytic activity of PP2Ac is reduced through tyrosine kinase mediated phosphorylation of Tyr307 [137] and reversible methylation at leu309 [138]. The latter is particularly interesting as methylation of PP2Ac indirectly modulates its activity by altering subunit composition of the holoenzyme [139].

1.3.12.2 Scaffolding Subunit

The scaffolding subunit of PP2A (PP2Aa) is encoded by two distinct genes, PPP2R1A (aα) and PPP2R1B (bβ). Both are ubiquitously

expressed and share 86% sequence similarity [140]. In about 90% of PP2A assemblies, PP2Aaα is the predominate isoform present in the holoenzyme. The scaffolding subunit is also referred to as the PR65 subunit. PP2Aa acts as a structural assembly base to assist the catalytic subunit and facilitate interaction with the regulatory subunits when it is in the heterodimeric form. Importantly, the two isoforms differ in their ability to interact with the numerous regulatory B subunits [141]. In the dimeric configuration it acts as a regulator by changing the catalytic specificity of PP2Ac [142]..

Structurally, PP2Aa is comprised of a HEAT sequence (Huntington/elongation/A-subunit/TOR) having 15 tandem repeats of 39 amino acid each [143]. The HEAT repeat is suggested to mediate the protein-protein interactions that keep the holoenzyme together [144]. Each repeat is composed of two α-helices that are connected by inter- and intra-repeat loops. The catalytic subunit of PP2A binds to the repeats 11-15 and the regulatory subunits bind to repeats 1-10 within the HEAT sequence (Fig 1.6). The scaffold subunit bends accordingly to form a base and shows a horseshoe like structure. This flexibility in bending is mediated through the HEAT repeat which allows for the recruitment of the regulatory subunit and PP2A substrates [145]. The PP2Ac/PP2Aa dimer has been suggested to exist freely *in vivo* in the absence of a third "regulatory" subunit [146, 147]; although this has not yet been formally proven. Experiments using RNAi in Drosophila suggest that a trimeric holoenzyme complex is required for stable existence *in vivo* [129], while disruption of the complex by depleting single subunits resulted in degradation of the other holoenzyme components.

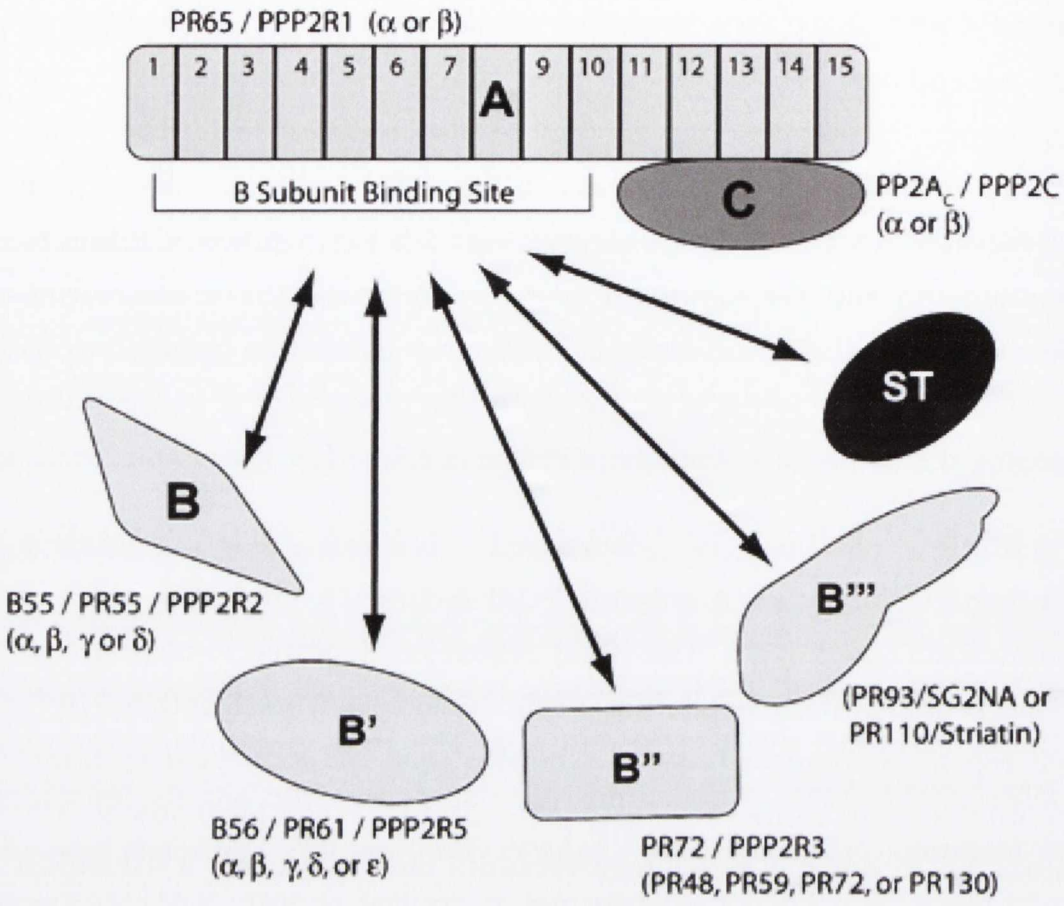


Figure 1.6: PP2A holoenzyme composition.

Binding of the B and C subunits of PP2A to the 15-heat repeats on the A subunit. Figure taken from Arroyo *et al.* [148]. A is representative of the scaffolding subunit; B (B', B" and B"') is regulatory subunits while C is the catalytic subunit.

1.3.12.3 Regulatory Subunits

The regulatory B subunits of PP2A are structurally diverse and are encoded by a minimum of 26 different transcripts and splice variants, which are encoded by 15 different genes in man [149]. The B subunit of PP2A is thought to be the master regulatory of PP2A due to its diverse nature and potential to allow PP2A to exist in over 100 different conformations.

The regulatory proteins are classified into four different families: B (B55/PR55), B' (B56/PR61), B" (PR48/PR72/PR130) and B"' (PR93/PR110) [150]; the numbers within the names represent the approximate molecular weight of each of the proteins in kDa. Each of the four families of B subunits is distinctively different from one another.

(I) B

B55 exists as 4 different isoforms (α, β, γ and δ) [151]. In mammals, B55 modulates cytoskeletal dynamics and nuclear translocation. Fibroblasts, in which B55 is knocked-out, lack the ability to dephosphorylate vimentin, leading to increased cell migration [152].

(II) B' subunit

The B'56 subunit is the most abundant member of the B subunit family and has 5 known isoforms (α, β, γ, δ, and ε), which have the unique ability to be independently phosphorylated [153]. B'56 members regulate growth factor signalling [154], cell cycle checkpoints [155], Wnt signalling/development [156] and apoptosis [157]. The B'56α has also been shown to down regulate expression of the oncoprotein c-Myc, and is linked to cancer through numerous pathways [158].

(III) B" subunit

The B" PR72 subunit is only expressed in the heart and skeletal muscles, whereas, PR130 is found in all tissue [159]. The B" family of proteins have been found to regulate retinoblastoma protein (Rb) via phosphorylation at p107 and to up-regulate DNA damage response genes in the process [160].

(IV) B"' subunit

The B"' family is a calmodulin binding protein and interacts with PP2A to enhance calcium-dependent signalling [128]. Members of the B"' family of proteins include, SG2NA, striatin and mMOB1. The diverse nature of the B subunits make up of PP2A allows for the binding to an array of proteins and targets.

Name	Gene
A subunit	
PP2A catalytic subunit α	PPP2R1A
PP2A catalytic subunit β	PPP2R1B
B subunit	
PP2A regulatory B subunit α	PPP2R2A
PP2A regulatory B subunit β	PPP2R2B
PP2A regulatory B subunit γ	PPP2R2C
PP2A regulatory B subunit δ	PPP2R2D
PP2A regulatory B' subunit α	PPP2R5A
PP2A regulatory B' subunit β	PPP2R5B
PP2A regulatory B' subunit γ	PPP2R5C
PP2A regulatory B' subunit δ	PPP2R5D
PP2A regulatory B' subunit ε	PPP2R5E
PP2A regulatory B'' subunit α	PPP2R3A
PP2A regulatory B'' subunit β	PPP2R3B
PP2A regulatory B'' subunit γ	PPP2R3B
Striatin	STRN
SG2NA,	SG2NA
mMOB1	MOB4
C subunit	
PP2A catalytic subunit α	PPP2CA
PP2A catalytic subunit β	PPP2CB

Table 1.1 PP2A subunits

Components of the PP2A holoenzyme structure and their corresponding gene code identifications.

1.3.13 Post-translational Regulation of PP2A

PP2A can be regulated through several independent mechanisms. The most important modifications of PP2A occur at the post-translational level and involves (I) phosphorylation, (II) methylation and (III) nitrosylation of various residues.

(I) Phosphorylation

Phosphorylation of the catalytic subunit of PP2A at Tyr 307 reduces catalytic activity [161] and reduces binding of the B55 and PR61 (α, β and ε) regulatory subunits to the PP2Aa/PP2Ac dimer. In doing so the catalytic activity of PP2A to specific substrates is altered [162]. Interestingly, phosphorylation of PP2Ac at Tyr 307 also influences leucine methylation of the catalytic subunit [163].

(II) Methylation

The catalytic subunit of PP2A is methylated at the C-terminal Leu 309. This is catalysed by protein phosphatase methyltransferase (PPM1/LCMT) and is opposed by the phosphatase methylesterase (PME-1) [164, 165]. Reversible methylation of PP2A is absolutely critical as knocking-out PME-1 in mice results in premature death [166]. Furthermore, methylation of the C subunit is a dynamic process that plays a role in cellular response to acute stimuli [167]. PP2A methylation also controls binding of PP2A to PP2A phosphatase activator (PTPA). PTPA is an ATP-dependent isomerase that has been shown to activate PP2A when bound to the A/C subunit dimer [168].

(III) Nitrosylation

There is also evidence to support nitrosylation in regulating PP2A activity. In particular, nitrosylation of tyrosine residues prevents the inhibitory effects of phosphorylation of PP2A thus increasing its enzymatic activity [169]. The most obvious residue being for nitrosylation is Tyr 307, as a result of nitric oxide synthase (NOS2) activity [170].

1.3.14 Role of PP2A in Cellular Transformation and Cancer

1.3.14.1 Okadaic acid

The first implication that PP2A is involved in tumour suppression emerged from studies using okadaic acid. As previously stated, okadaic acid is a cytotoxin polyether first isolated from marine sponge [72]. It is a selective but not specific inhibitor of PP2A with okadaic acid been a widely known tumour promoter with resulting exposure inducing tumour formation in mouse skin [72, 171]. Studies have also shown that at low concentrations of okadaic acid, HEK cells are conferred with the ability to grow in an anchorage-independent fashion as well as with increased cell proliferation abilities [172].

1.3.14.2 SV40 Virus and Oncogenic Transformation

Further indications of the tumour suppression capabilities of PP2A come from observations that several tumour-promoting viruses such as polyomaviruses such as SV40 (Simian virus 40) inhibit PP2A activity [133]. Many other viral proteins also interact with subunits of PP2A, including the HIV antigen HIV-1 Vpr, the HCV antigen HCV NS5A and the E7 antigen of the HPV virus [173, 174]. SV40 is a DNA tumour virus with a genome of ~5 kb and is one of the best-studied members of the polyomavirus family [175]. Preliminary studies in murine cell models found that expression of SV40 virus in cells promoted oncogenic

transformation [176]. In NIH 3T3 cells, expression of LT (Large T antigen) from a retrovirus led to anchorage-independent growth in the absence of ST (Small T antigen), contrary to earlier studies [177]. This study showed that ST was only necessary for efficient transformation when LT was expressed from an SV40 virus at relatively low levels, while LT expression from a retrovirus lead to significantly higher levels of LT and no requirement for ST. As well as these findings, similar studies found that activated Ras can also transform murine cells in the context of LT expression [178]. Thus, in murine models at least, ST functions are not required but enhance transformation induced by limited LT. However, in humans cellular transformation is somewhat different. Expression of LT in human cells is sufficient to bypass cellular senescence, immortalisation requires the additional expression of the human telomerase catalytic subunit (hTERT) [179]. Human cells expressing LT remain morphologically normal, but expression of both SV40 oncoproteins induces morphological changes reminiscent of cell lines derived from tumours [180, 181]. However, to generate fully transformed human cells capable of tumour formation in nude mice, the expression of hTERT and oncogenic Ras is necessary in addition to LT and ST [180, 182]. However, in the context of absolute transformation, LT, hTERT, and oncogenic Ras expression is fully dependent upon the additional expression of ST, which is the fundamental difference between murine and human oncogenic cell transformation.

In the context of PP2A in cellular transformation, the ST domain of the SV40 virus, competitively binds to the active site of PP2A displacing B subunits from the A/C PP2A heterodimer is central [183]. In addition to SV40 small t antigens [184], adenovirus E4orf4 proteins [185] also bind to and inhibit PP2A activity aiding in cellular transformation. Studies have also shown specific suppression of the PP2A B56$^{\gamma}$ subunit is able to replace ST and induce anchorage-independent growth and tumour formation [126, 172]. Overexpression of PP2A B56$^{\gamma}$ reversed ST-induced transformation. In contrast, suppression of the PP2A

B55α subunit was unable to transform cells, and B55α overexpression did not inhibit transformation by ST. These findings suggest that PP2A complexes containing the B56γ subunit are specifically involved in transformation in this system.

1.3.14.3 PP2A Subunits and Cancer

The tumour suppressor role of PP2A was further confirmed by showing that loss of the Aβ subunit of PP2A transforms immortalised human cells [186]. PP2A alone is no longer considered a tumour suppressor, unless it contains B-subunits, which target key oncogenes or tumour suppressors [71]. Indeed, knockdown of B56α, B56γ, PR72/130 or PTPA within the PP2A complex activates oncogenic pathways regulated by c-Myc, Wnt, and PI3K (phosphoinositide 3-kinase)/Akt in a subunit specific manner [186].

1.3.14.4 PP2A Subunit Mutations

PPP2CA, the gene that encodes the Cα isoform of the catalytic subunit is decreased in majority of human androgen-independent prostate cancer cell lines and in cancerous lesions as compared with the adjacent normal/benign tumour tissues [187]. Somatic missense mutations in PPP2R1A, which encodes for Aβ isoform of PP2A, are found in high-grade endometrial and ovarian carcinomas [188]. Mutations of the Aβ isoform are also reported in colorectal cancers [189]. Interestingly, a recent study has found that the functional -241 (-/G) variant in the PPP2R1A promoter region is associated with a decrease risk HCC development [190]. Point mutation in PPP2R5C/B56γ in lung cancer was reported to impair p53-dependent tumour suppression ability of PP2A [191]. B55β silencing by promoter methylation has been reported in human colorectal cancer [192], while a SNP in B56ε is associated with onset, risk and survival in patients with soft tissue sarcoma [193].

1.3.14.5 Regulation of PP2A by miRNA

As well as mutation and subunit deletions, post transcriptional regulation of PP2A subunits also play a role in cancer. Recent studies indicated that miRNA-31 represses PPP2R2A/B55α in human and mouse lung cancer [194], while miRNA-222 targets the subunit in HCC, [195]. In the latter study, inhibition of miRNA-222 inhibited migration of HCC cells. Wong *et al.* also found that miRNA-22 inhibited B55α protein expression and activated p-AKT signalling in Hep3B cells [195]. In breast and cervical cancer, miR-520 has been found to regulate Cα expression [196, 197], while miR-200c regulates Aβ subunit expression in Oesophageal cancer [198]. For a more comprehensive list of miRNAs that target PP2A subunits see table 1.2. Despite the accumulating evidence to support genetic mutations and post-transcriptional/translational modifications of PP2A in cancers, they general occur at low frequency and are not sufficient to explain the significance of PP2A in all cancers. In recent years, focus has shifted towards endogenous inhibitor proteins of PP2A as having a more influential and prominent role to play in cancer.

PP2A subunit or regulator	miRNA identified	Cancer or disease involved
Cα (PPP2CA)	miR-520h	Lung Cancer [196]
		Cervical cancer [197]
Cα (PPP2CA)	miR-155	Systemic lupus erythematosus [199]
Aβ (PPP2R1B)	miR-200c	Oesophageal cancer[198]
B55α (PPP2R2A)	miR-222	Hepatocellular carcinoma [195]
B55α (PPP2R2A)	miR-222	Lung cancer [200]
B55α (PPP2R2A)	miR-31	Lung cancer [194]
B56α (PPP2R5A)	miR-1	Heart disease [201]
B56α (PPP2R5A)	miR-155	Infection (macrophage response to bacteria)[202]
B56α (PPP2R5A	mIR-136	Keratinocytes wound healing [203]
B56ε (PPP2R5E)	miR-19a	Acute lymphoblastic leukaemia [204, 205])
B56ε (PPP2R5E)	miR-23a	Gastric cancer [205]
B56γ (PPP2R5C)	miR-135	Lymphoma [206]
SET	miR-199b	Choriocarcinoma [207]
CIP2A	miR-375	Oral cancer [208]

Table 1.2: miRNAs that target the PP2A catalytic core subunits A and C, various regulatory B subunits and inhibitory PP2A proteins.

1.3.15 PP2A Endogenous Inhibitory Proteins

In addition to pharmacological inhibition, viral proteins and subunit deletions/alterations, endogenous cellular proteins are also able to inhibit or promote PP2A activity (Figure 1.8).

1.3.15.1 $I1^{PP2A}$

$I1^{PP2A}$ is a sphingosine or dimethylsphingosine (DMS) sensitive PP2A inhibitor [209], which is also known as acidic leucine-rich nuclear phosphoprotein 32 member A (ANP32A), putative HLA associated protein-1 (PHAP1) or pp32 [210]. Overexpression of $I1^{PP2A}$ has been linked with restoring drug-resistant cell lines to caspase-dependent apoptosis [210]. Another member of the ANP32 family, ANP32e (formerly cerebellar developmental-regulated protein 1, CPD1), is also a PP2A inhibitor and plays a role in neuronal development [211].

1.3.15.2 Alpha 4

Alpha 4 (α4) has been shown to inhibit PP2A by binding directly to the catalytic subunit of PP2A and displacing the PP2Aa scaffolding subunit [212, 213]. When existing A/C complexes are destabilised by heat shock, α4 can bind to and stabilise the released C subunit. However, rather than acting as a competitor to A/C binding, α4 binding to the catalytic subunit maintains PP2A holoenzyme activity under stress conditions [214]. This suggest that α4 binds and inhibits PP2A activity of either newly synthesised or released C subunits from active A/C complexes. This prevents PP2A from acting un-wieldy during cellular stress and aids in reconstitution of the holoenzyme.

1.3.15.3 SET protein

SET protein ($I2^{PP2A}$) was first identified as a myeloid leukaemia associated SET-CAN fusion gene and later shown to inhibit PP2A activity [215]. SET overexpression has been reported mainly in

haematological cancers such as chronic myelogenous leukaemia (CML), where it operates downstream of the oncogenic BCR-ABL kinase [216]. However, it is also found in solid tumours; Wilms tumours (Carlson et al., 1998) prostate cancer [217] and lung cancer [218]. As SET is a member of the INHAT (inhibitor of acetyltransferase) complex it attenuates gene transcription preventing acetylation of histones thereby altering chromatin structure [219, 220]. Juxtaposed to this, SET can also increase gene expression in a histone acetylation dependent and independent manner [221]. SET has been implicated in regulating many cellular processes such as cell cycle and cell growth [222] However, it still unknown whether specific cancer-related pathways are affected by dysregulation of SET or if its oncogenic effects are a direct result of PP2A inhibition.

SET can inhibit PP2A at relatively low expression without inhibiting other phosphatases [218]. Inhibition of PP2A requires SET to be phosphorylated on serine 9 and 24, by protein kinase C phosphorylation [215] or on serine 93 PI3Kγ [223]. More recent work indicates that phosphorylation of SET on serine 171 by PKD can reduce SET mediated inhibition of PP2A [224]. SET-mediated inhibition of PP2A is also inhibited by the sphingolipid signalling molecule ceramide, a well-known activator of PP2A [225, 226]. Ceramide activates PP2A through direct binding to SET protein. This produces a conformational change in SET, which disrupts the binding dynamics to PP2A resulting in SET's displacement [225]. Interestingly, fingolimod (FTY-720), which is a sphingosine analogue used to treat multiple sclerosis, reactivates PP2A through disruption of SET/PP2A binding by altering ceramide production [218]. Targeting SET protein has become an attractive option in the development of new anti-cancer drugs, and is one of the main targets in PAD (Protein phosphatase 2A activator drug) drug development.

1.3.15.4 Cancerous inhibitor of protein phosphatase 2A (CIP2A)

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an endogenous PP2A inhibitor found overexpressed in a variety of cancers [227-229]. CIP2A was first discovered through the identification of autoantigens against a 90-kDa protein in HCC and was originally named as p90 for its molecular weight. However, the role of CIP2A in cancer was not well defined until it was shown to inhibit PP2A-mediated dephosphorylation of c-Myc at serine-62 (Figure 1.6) [227]. An increase in the oncogene c-Myc has been reported, on average in 50% of human cancers [230]. Early studies of c-Myc found that deregulated expression of Myc was sufficient to drive tumourigenesis in a number of transgenic mouse tissues [231]. Myc plays a role in tumour initiation as well as DNA replication, metabolic reprogramming and tumour stemness [232]. Protein phosphatase 2A (PP2A) plays a prominent role in controlling accumulation of the proto-oncoprotein c-Myc, with the B56α subunit of PP2A selectively associating with the N terminus of c-Myc. resulting in a dramatic reduction in c-Myc levels [158]. Other studies have found that the B56δ subunit modulates c-Myc expression [233]. Thus, the identification of CIP2A as protein that prevents PP2A regulation of c-Myc is very important. CIP2A has been found to stabilise c-Myc by preventing its proteasomal degradation [227, 234]. Junttila *et al.* also showed that CIP2A promotes anchorage-independent cell growth and *in vivo* tumour formation all of which are hallmarks of c-Myc overexpression. The direct structural mechanism by which CIP2A inhibits PP2A remains unclear, as no crystal structure of PP2A as yet exists. However, using known CIP2A protein sequences, predictions of its structure and potential phosphorylation sites can be made using computational software (Figure 1.7).

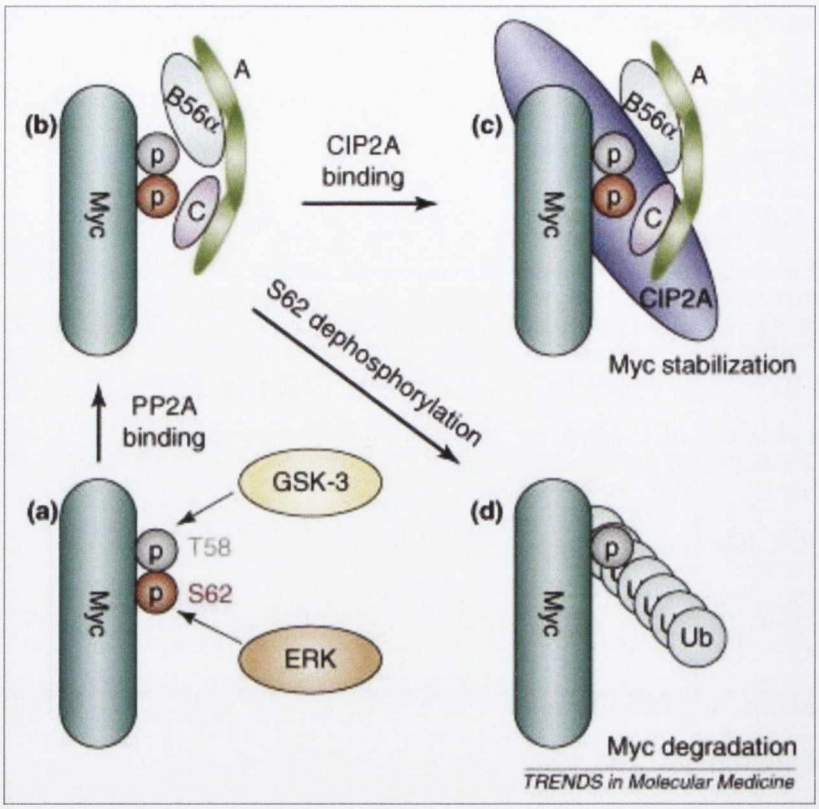


Figure 1.6: PP2A inhibitor CIP2A stabilising Myc protein and preventing ubiquitination.

(a) Myc is phosphorylated at threonine 58 (T58) and serine 62 (S62) by GSK-3 and ERK kinases, respectively. (b) This facilitates PP2A binding to the N-terminus of phosphorylated Myc via B56α. (c) CIP2A then directly interacts with Myc and inhibits PP2A-mediated S62 dephosphorylation, which stabilises Myc protein. (d) Without the presence of CIP2A, PP2A dephosphorylates Myc at S62 but not T58. Myc is then ubiquitinated on T58 and targeted for proteolytic degradation. Diagram taken from Westermarck *et al.* [235].

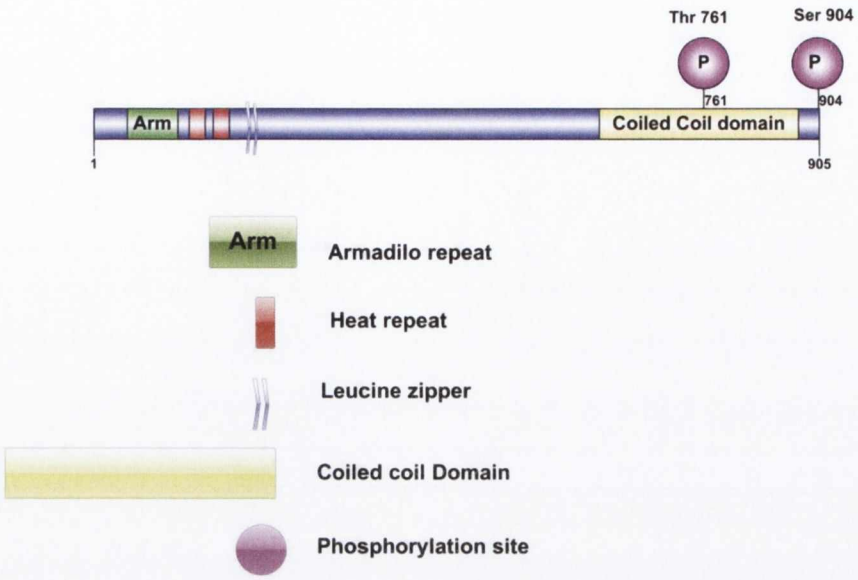


Figure 1.7: Predicted Structure of CIP2A

Predicted modular structure of CIP2A showing shown Armadillo repeats, heat repeats, leucine zipper and a coiled coil region (631-879). Armadillo repeats contain 42 amino acid motif composed of three α-helices. HEAT repeats form rod-like helical structures which are involved in intracellular transport; they are structurally related to armadillo repeats. Leucine zipper protein motifs are protein–protein interaction domain consisting of amphipathic α helices that dimerise in parallel, either as homodimers or heterodimers, to form a coiled-coil structure. Coiled coil domains are a motif in which 2-7 alpha-helices are coiled together. CIP2A FASTA sequence was analysed for predicted protein structure using bioinformatic software[236] [237]. Thr 761 and Ser 904 phosphorylation sites have been identified previously [238]. Image was created using IBS 1.0 software [239].

1.3.16 Role of CIP2A in the Cell

CIP2A is involved in centrosome separation and mitotic spindle dynamics within cell cycle progression [240]. CIP2A strongly interact with NEK2 during G2/M phase, thereby enhancing NEK2 kinase activity facilitating centrosome separation in a PP2A dependent manner. In support of this, depletion of CIP2A by siRNA interference delays the mitotic cell cycle without fluctuations in NEK2 expression level [240]. In a similar mechanism to c-MYC stabilisation, CIP2A associates with mTORC1 and acts as an allosteric inhibitor of mTORC1-associated PP2A, thereby enhancing mTORC1-dependent growth signalling and inhibiting autophagy within the cell [241]. CIP2A works in tandem with H-Ras to promote epithelial-mesenchymal transition (EMT) in cells through the up–regulation of EMT markers such as Vimentin and SNAIL protein [242]. This supports earlier data by Junttila *et al.* showing CIP2A mediated cellular transformation up-regulated Ras mediated cellular transformation [227]. Cooperation with Ras protein has also been recently noted in phosphoproteomics data, where CIP2A depletion down-regulates Ras signature pathways, mimicking that of Ras knockdown [243]. Since PP2A is crucial in transforming immortalised human cells in a Ras-dependent manner, these studies further emphasise the role of CIP2A as a cancerous inhibitor of PP2A.

1.3.17 CIP2A as a Therapeutic Target

Though recently discovered, CIP2A has attracted a lot of attention as a potential drug target. Along side SET, CIP2A is now considered a therapeutic target in the treatment of chronic myeloid leukaemia (CML) [244] and a potential target in challenging prostate cancer [245]. As CIP2A overexpression is associated with drug resistance in tumour cells it is directly and indirectly a target for many of the new chemotherapeutic agents. CIP2A has been show to mediate the effects of bortezomib on p-AKT and apoptosis in hepatocellular carcinoma cell lines [246]. CIP2A has also been show to mediate the effects of some Tyrosine Kinases inhibitors (TKi); Erlotinib derivatives have been shown to inhibit hepatocellular carcinoma by targeting CIP2A to reactivate protein phosphatase 2A [247]. mTOR targeting agents have also been shown to reduce CIP2A levels and aid in the chemosensitisation of other anti-cancer compounds [248].

1.3.18 CIP2A: Inflammation and Cancer

Cancers with a chronic inflammatory component such as gastric cancer, HCC and HPV-lung adenocarcinoma overexpress CIP2A. Chronic infection with *H. pylori* has been found to increase CIP2A in gastric cancer [249, 250], while the HPV E6 oncoprotein has been implicated in the up-regulation of CIP2A through IL-10 in lung adenocarcinoma [251]. CIP2A is also regulated post-transcriptionally by miR-375 in oral cancer cells. Transient transfection of miR-375 in oral cancer cells reduces the expression of CIP2A, resulting in decrease of Myc protein levels and leading to reduced proliferation, colony formation, migration, and invasion [208]. miR-375 itself is down regulated by bacterial infections such as *H. pylori* [252], while low expression of miR-375 has been reported in Hepatitis B viral induced hepatocellular carcinoma [253] Interestingly, *H. pylori,* IL-10 and miR-375 are external stimuli to tumour cells, with IL-10 being produced by anti-inflammatory macrophages within the microenvironment [254].

These observations facilitated a component of this study; investigating the influence of macrophage co-culture on CIP2A expression in a HCC cell line (Chapter 6).

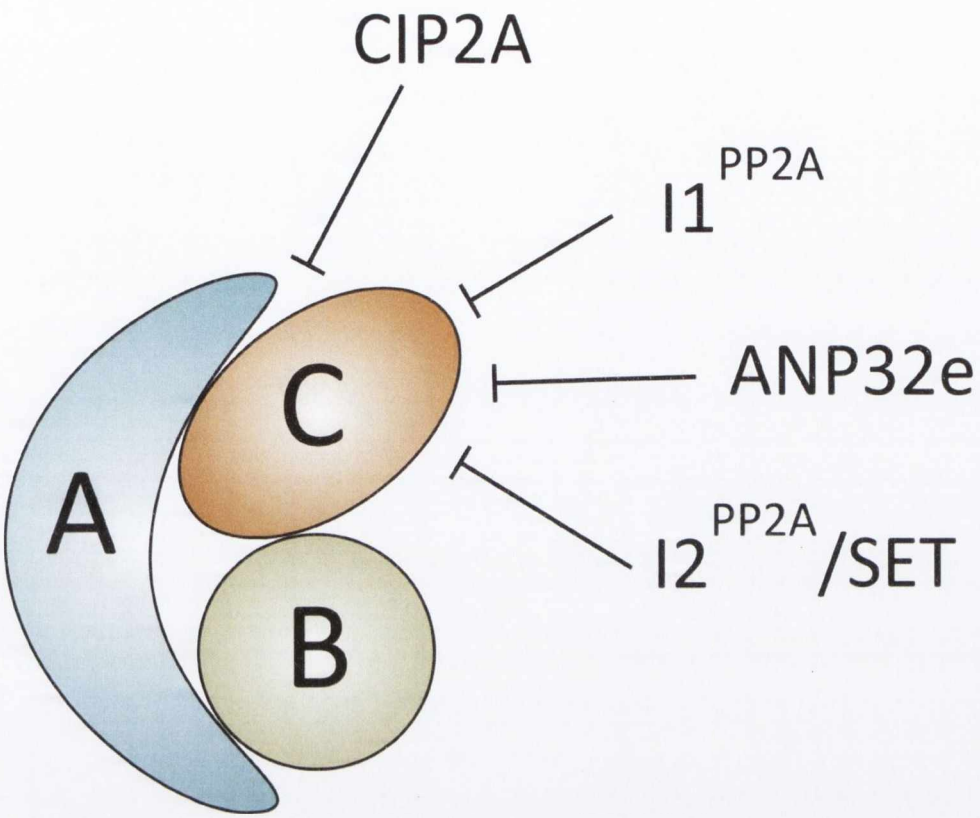


Figure 1.8 Inhibition of PP2A by endogenous protein inhibitors

Endogenous protein inhibitors of PP2A predominantly target the catalytic subunit (C) of PP2A; $I1^{PP2A}$, ANP32e and SET are found to inhibit PP2A through interactions with the C subunit. The precise binding of CIP2A to PP2A is unclear, however, CIP2A has been found immunoprecipitated with the scaffolding subunit (A) of PP2A [227].

1.4 Matrix Metalloproteinases and the Regulation of the Extracellular Matrix and Tumour Microenvironment

1.4.1 Physiological Function of the Extracellular Matrix

Communication between cells is mediated through cell-cell and matrix-cell interactions. The main function of the extracellular matrix (ECM) is to provide support and anchorage to cells through interactions with cells of similar function [255]. These interactions regulate morphological changes, differentiation, apoptosis, tissue repair and cell invasion. The ECM is composed of a variety of components of which collagens, proteoglycans, glycoproteins, elastin, fibronectins and laminins provide structural support to cells and surrounding tissues. Furthermore, the ECM can act as a reservoir for a variety of growth factors, proteinases and cytokines [256]. Importantly, the ECM forms the external framework for normal and tumour cells alike, with the term "remodelling" being used to describe the proteolytic degradation of the ECM by matrix metalloproteinases and subsequent release of cytokines and growth factors from this structural protein matrix.

1.4.2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases are a family of zinc-dependent enzymes, which belong to a larger family called the metzincin-superfamily [257]. MMPs are capable of degrading practically all ECM components along with facilitating the release of a host of non-ECM molecules that include growth factors and their receptors, cytokines, chemokines, adhesion receptors and cell surface proteoglycans as well as enzymes involved in angiogenesis, cellular invasion and migration [258]. To date, 28 members of the MMP/TIMP family (24 MMP and 4 TIMP) have been identified in man. MMPs were originally classified based on a combination of their substrate specificity and cellular localisation as

collagenases, gelatinases, stromelysins and the membrane-type MMPs (MT-MMPs. However, many of the family members have multiple and frequently overlapping substrates. With the advancement in gene classification and protein structure, MMP classification has moved towards a new classification based on domain structure.

1.4.3 Structure of MMPs

MMPs are extracellular proteins, but recent studies have indicated that MMP-1, MMP-2 and MMP-11 are also found intracellular and may act on intracellular proteins [259]. Structurally, MMPs are classified into four groups; archetypal MMPs, matrilysins, gelatinases and furin-activatable MMPs [258]. Archetypal MMPs are further classified based on their substrate specificities into collagenases, stromelysins and other MMPs. Furin-activated MMPs can be subdivided even further into secreted, type 1 transmembrane, GPI anchored and type 2 transmembrane MMPs (Figure 1.9). Structurally, MMPs typically consist of a pro-peptide of about 80 amino acids, a catalytic metalloproteinase domain of about 180 amino acids, a linker peptide of variable lengths (also called the 'hinge region') and a hemopexin (Hpx) domain of about 200 amino acids. Exceptions to this are MMP-7 (matrilysin-1), MMP-26 (matrilysin-2) and MMP-23; they lack the linker peptide and the Hpx domain and MMP-23, which have a unique cysteine-rich domain and an immunoglobulin-like domain after the metalloproteinase domain. Two gelatinases, gelatinase A (MMP-2) and gelatinase B (MMP-9), have three repeats of a fibronectin type II motif in the metalloproteinase domain. The zinc binding motif HEXXHXXGXXH in the catalytic domain, and the "cysteine switch" motif PRCGXPD in the propeptide are common structural signatures, where three histidines in the zinc binding motif coordinate and the cysteine in the propeptide coordinates with the catalytic zinc ion. This Cys-Zn^{2+} coordination keeps proMMPs inactive by preventing a water molecule essential for catalysis from binding to the zinc ion.

The catalytic domain consists of a 5-stranded β-pleated sheet, three α-helices and connecting loops. It contains two zinc ions, one catalytic and one structural. Usually three calcium ions are also found within the region, which are thought to stabilise the structure [260]. The catalytic domain also contains a conserved methionine, forming a "Met-turn", eight residues after the zinc binding motif, which forms a base to support the structure around the catalytic zinc [261, 262]. The reason for the "Met-turn" conservation within the family remains unclear. However, it has been postulated that it may be essential for the structural integrity of the active site through stabilisation of zinc binding [262]. The main determinant of substrate specificity within the MMP family is the S_1 pocket of the active site. The S_1 pocket is not uniform in size or shape across the MMP family. This pocket is hydrophobic in nature, but variable in depth among MMPs. The MMP-9 and MMP-2 pockets are described as a tunnel leading towards solvent, whereas the pocket for stromelysin is described as tunnel leading out to solvent [263].

The propeptide domain within MMPs consists of three α chains and connecting loops. The proteinase susceptible "bait region" is located between helix 1 and helix 2, but the structure has not been resolved due to the flexible nature of the region, except for that of proMMP-2, which is stabilised by a disulphide bond [264, 265]. The "cysteine switch" lies in the substrate binding pocket but the orientation of this polypeptide segment is opposite from that of a peptide substrate. The SH group of the cysteine interacts with the catalytic zinc ion. Upon activation the interaction of Cys–Zn^{2+} is disrupted, which allows a water molecule to bind to the zinc atom. In the case of proMMP-1, the pro-domain interacts with the hemopexin domain rendering it inactive compared to active MMP-1 [266].

Substrate specificities of MMPs have been studied either by identifying the cleavage sites of protein substrates or by a series of synthetic peptide substrates [267]. MMPs possess the ability to cleave peptide

bonds in from of a hydrophobic residue (Leu, Ile, Met, Phe or Tyr), which fits into the S_1 pocket [268]. In addition to interactions at the active site, some substrates also interact with non-catalytic domains of the MMPs, in particular large molecules of the ECM such as collagens, gelatin and laminins [269]. This is as a result of the three type-II fibronectin binding sites within the catalytic domain of MMP-2 and MMP-9 [269, 270].

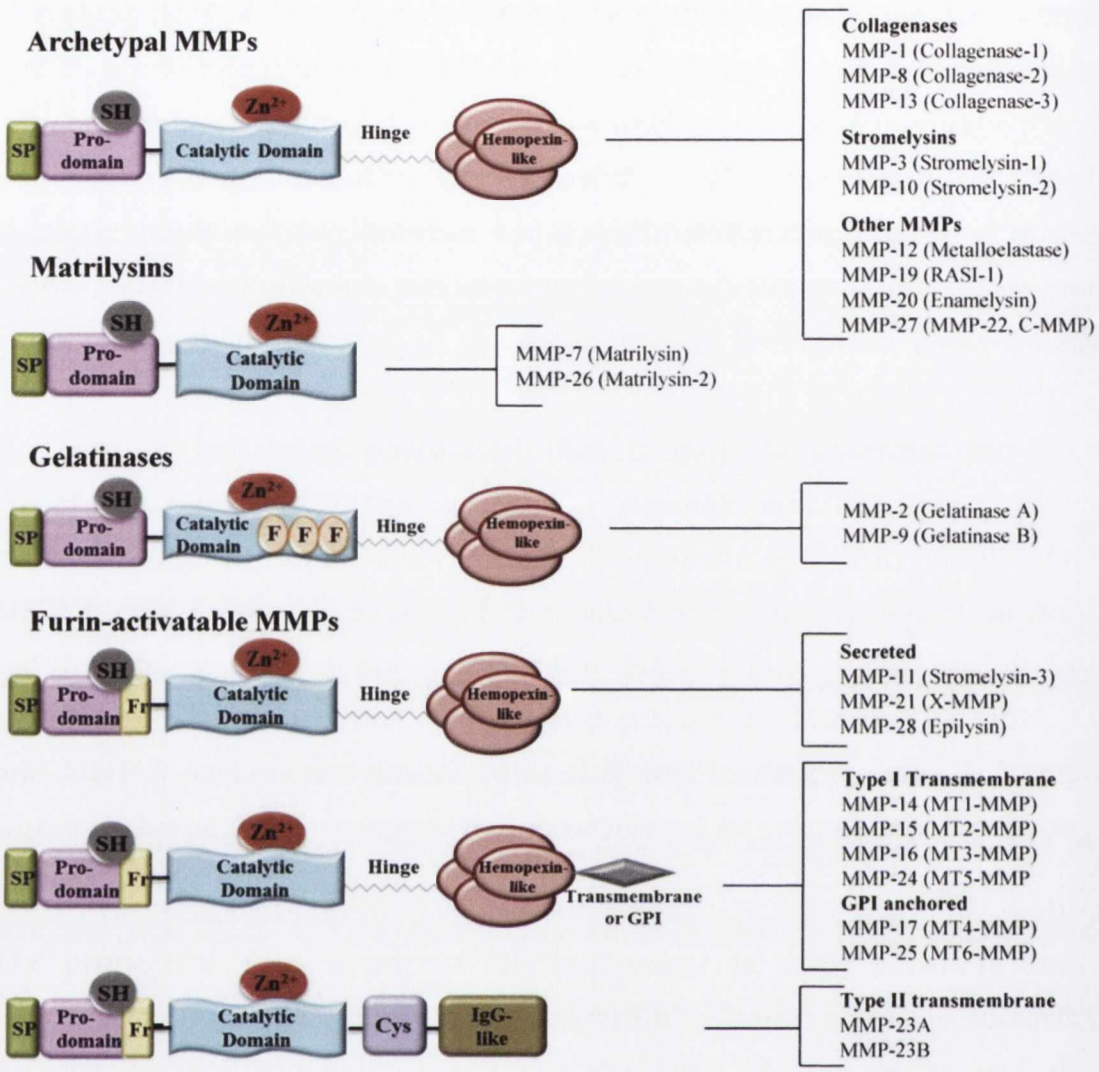


Figure 1.9 Domain structures of the major MMP classes.

The major domains consist of the signal peptide (SP; predomain), propeptide domain (Pro-domain), a catalytic domain with a zinc (Zn^{2+}) binding motif, a cysteine (Cys) or hinge region, a haemopexin domain and in some cases a transmembrane or glycosylphosphatidylinositol (GPI) domain or a IgG-like domain. Taken from Gong *et al.* [271].

1.4.4 Regulation of MMPs

The MMPs are capable of widespread destruction resulting in undesired ECM remodelling, so it is not surprising that they are tightly regulated to avoid unnecessary damage to tissue. MMP regulation occurs at three levels: transcriptional, proenzyme activation and the interaction of active MMPs with tissue inhibitors of metalloproteinases (TIMPs) [272].

1.4.4.1 Transcription Regulation

At the transcriptional level, MMP expression is regulated through gene specific promoter sequences. MMPs can be classified into three basic groups based on their promoter sequences [273]. Group one is characterised by a TATA box and an activator protein-1 (AP-1) binding site in the proximal region of the promoter. Examples of MMPs within this grouping are MMP-1, 3, 7, 9, 10, 12, 13, 19 and 26 [273, 274]. The second group include MMP-8, 11 and 21; this group contains only the TATA box but not the proximal AP-1 binding sites. The third and last group of MMP promoters lack both TATA boxes and AP-1 binding sites. Members of this family include MMP-2, 14 and 28, which are mainly expressed constitutively within the cell.

The AP-1 binding sites in conjunction with the PEA3 binding sites are responsible for the expression of MMPs in response to growth hormones and cytokines [274]. Growth hormones and cytokines activate MAPK-signalling cascades resulting in trans-activation of the MMP promoter. In particular p38-MAPK can increase c-Jun and c-Fos (components of the AP-1 complex) expression resulting in transcriptional activation of genes containing AP-1 transcription factor binding sites (Figure 1.10).

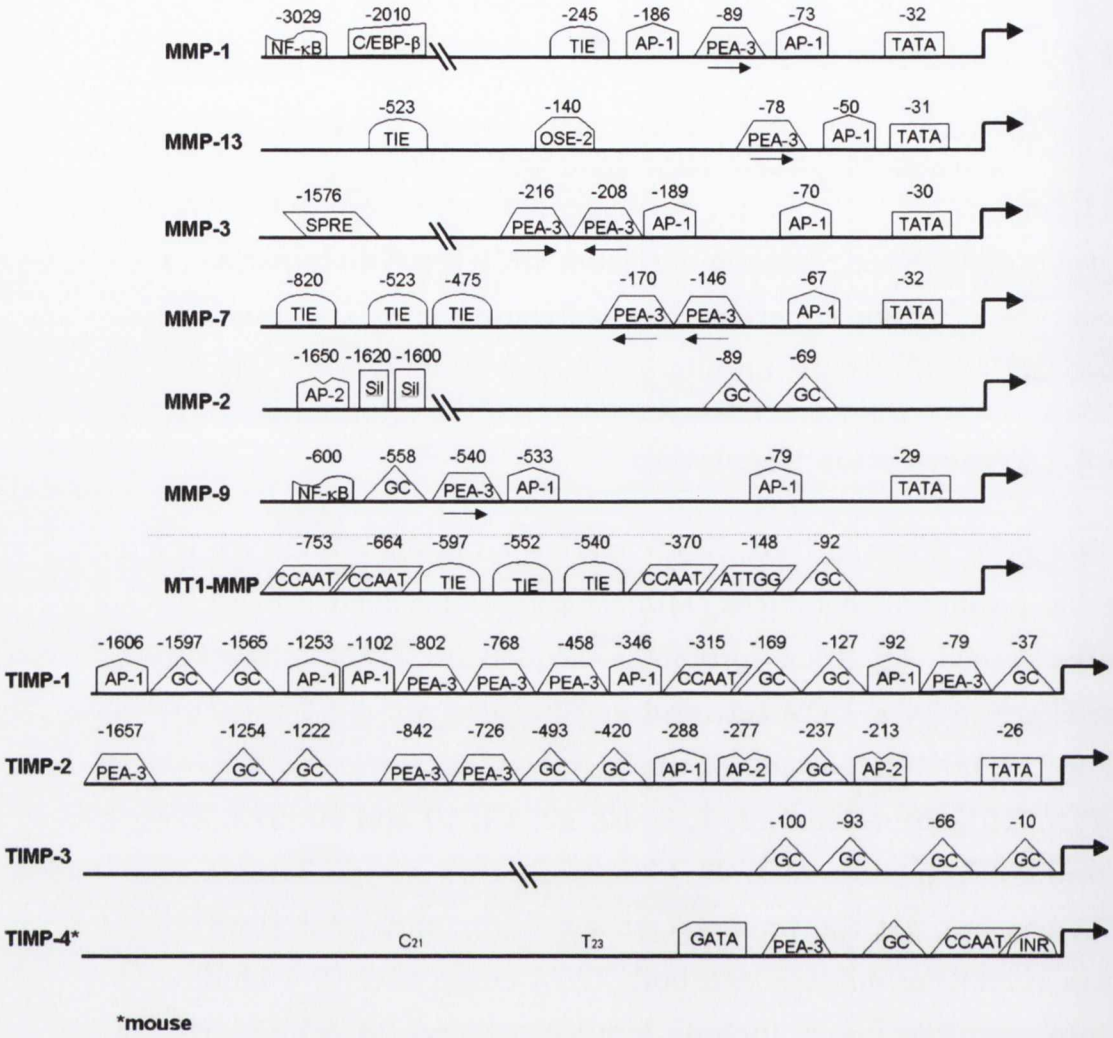


Figure 1.10 Transcription factor-binding elements located within the proximal promoters of select MMPs and TIMPs.

Abbreviations: C/EBP-β, CCAAT enhancer-binding protein; TIE, TGF-β inhibitory element; AP-1, activator protein-1; PEA-3, polyoma enhancer A binding protein-3; OSE-2, osteoblastic cis-acting element; TATA, TATA box; AP-2, activator protein-2; Sil, silencer; GC, Sp-1 binding site; NF-κB, NF-κB binding site; CCAAT, CCAAT box; GATA, GATA binding site; INR, initiator-like sequence. Image taken from Deschamps *et al.* [273].

1.4.4.2 Zymogen Activation

Once synthesised, MMPs are secreted by cells as inactive zymogens (proenzymes), which become activated by removal of the NH_2-terminal propeptide (signal peptide) generating active MMPs [256]. This removal is usually achieved through proteolytic or oxidative cleavage. However, some proMMPs (MMP-14, 15, 16) contain a furin-like enzyme recognition site between the propeptide and the catalytic domain, and are activated by furin, which is a serine protease [275]. Once the propeptide is removed, the Zn^{2+} becomes available for the binding of the substrate, resulting in a conformational change.

MMPs can also be activated by other MMPs. MMP-3 activates pro-MMP-1, 7, 8, 9 and 13, whereas MMP-2 activates pro-MMP1, 9 and 13 [272]. The activation of MMP-2 is somewhat different as it occurs at the cell surface and requires MMP-14 (MT1-MMP) and tissue inhibitor of MMP-2 (TIMP-2) [276].

1.4.4.2 Endogenous Inhibitors

A third form of regulation of MMP activity is through their interaction with endogenous inhibitors. Proteins such as alpha 2-macroglobulin, tissue factor pathway inhibitor-2, β-amyloid precursor protein, RECK and chlorotoxin are all reported to inhibit MMP activity [268, 277]. However, the best studied inhibitors and probably the ones with the most biological relevance are the tissue inhibitors of matrix metalloproteinases. TIMPs inhibit MMPs in a reversible manner, with four different types of TIMPs being identified in humans (TIMP-1, 2, 3, 4) [278].

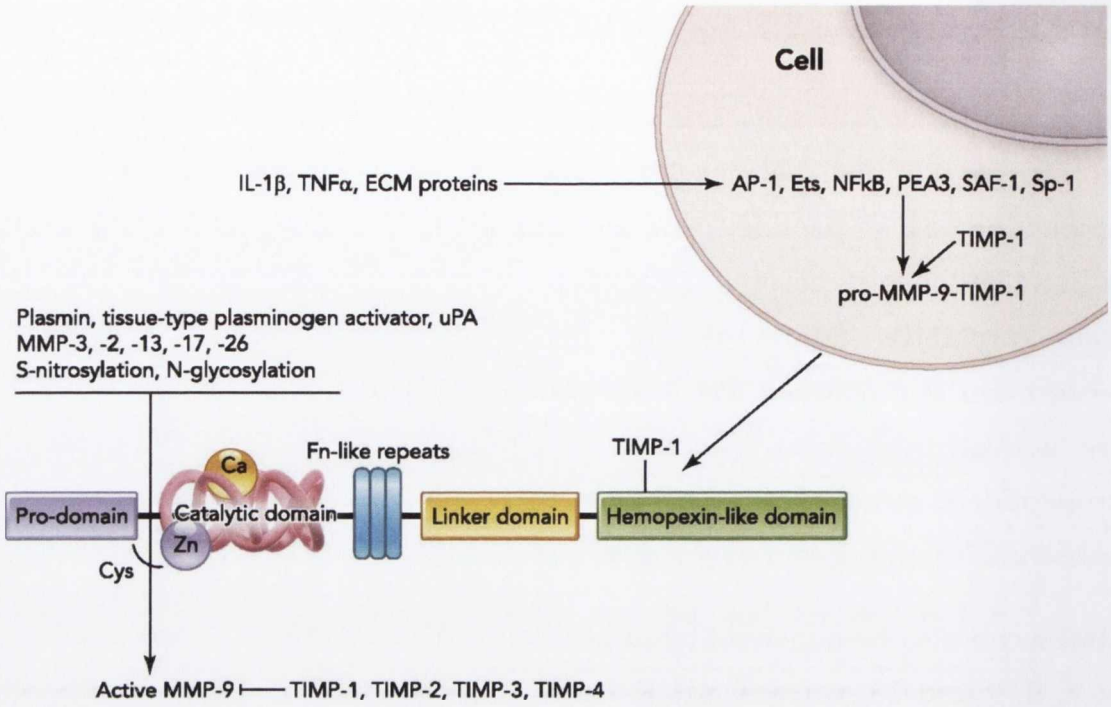


Figure 1.11 A schematic representation of the structure of MMP-9 and regulation by transcription factors and endogenous TIMP activity. Image taken from Yabluchanskiy *et al.* [279].

1.4.5 Tissue Inhibitors of Matrix Metalloproteinase (TIMPs)

1.4.5.1 Discovery, Classification and Structure

The first TIMP was described in 1975 as a protein in culture medium of human fibroblasts and in human serum, which was able to inhibit collagenase activity [280]. All mammalian TIMPs have two distinct domains, an N-terminal domain of about 125 amino acid residues and a C-terminal domain of about 65 residues; the conformation of each domain is stabilised by three disulphide bonds [281]. The four human TIMPs are about 40% identical in sequence to each other; the most similar pair being TIMP-2 and TIMP-4, which are 50% identical in sequence, whereas TIMP-1 is only 37 to 41% identical to the other TIMPs [278]. The N-terminal domain folds wedge-like as an independent unit and slots into the active site cleft of the MMP in a similar way to the substrate [282, 283]. Several cell types including fibroblasts, osteoblasts, endothelial cells and tumours, produce TIMPs.

1.4.5.2 TIMP-1

TIMP-1 is an extensively glycosylated protein with a molecular weight of 28 kDa. Human TIMP-1 is secreted by a variety of cell types and has wide tissue distribution [284]. TIMP-1 inhibits multiple MMPs with the exception of MT1-MMP, but is most effective against MMP-9. In addition, TIMP-1 binds to the C-terminal haemopexin domain of proMMP-9 slowing its activation [285]. The secreted protein has 184 amino acids and six intramolecular disulphide bonds. TIMP-1 has been shown in several studies to have many diverse biological functions including roles in gonadal steroidogenesis, ovulation, tissue remodelling during tumour progression, growth factor activity, including erythroid potentiating activity (EPA) and inhibition of angiogenesis *in vitro* [285].

1.4.5.3 Regulation of TIMP-1

Expression of TIMP-1 is regulated at the level of transcription by various growth factors, cytokines and hormones. Phorbol esters and IL-1β stimulate both MMP and TIMP-1 expression [286], whereas MMP expression is decreased and TIMP-1 expression increased by TGF-β and retinoic acid [285]. IL-6, EGF, oncostatin M and glucocorticoids also stimulate TIMP-1 expression. TNF-α has also been shown to stimulate TIMP-1 at low concentrations, while inhibiting expression at high levels [287]. The TIMP-1 promoter contains several transcription factor-binding sites including AP-1, AP-2, Spl and PEA3, but does not contain a TATA box (Hammani *et al.*, 1996; Deschamps *et al.*, 2006. The AP-1 site at -92 bp upstream of the initiation site is essential for basal activity [288].

1.4.5.3 Role of TIMP-1 in Cancer

TIMP-1 is a multi-functional protein that in addition to inhibiting MMP can directly regulate apoptosis, inflammation and cell proliferation [289]. With regards in cancer, TIMP-1 expression can be increased or decreased depending on the malignancy. In HCC, high protein levels of TIMP-1 have been reported [290] and are an essential promoter of formation of pre-metastatic niche in HCC [291]. High levels of TIMP-1 are also linked to development of drug resistance in breast cancer cell lines [292]. In melanoma, TIMP-1 assembles into a supramolecular complex consisting of CD63 and β1-integrins, which activates PI3K signalling conferring drug resistance [293]. In addition, TIMP-1 can suppress both intrinsic and extrinsic cell death pathways via an MMP-independent mechanism in lymphocytes and breast epithelial cells [294, 295]. Overexpression of TIMP-1 in cells leads to the up-regulation of oncogenic miR-210 in a CD63/PI3K/AKT/HIF-1-dependent pathway in lung adenocarcinoma cells [296]. These new functions of TIMP-1 further demonstrate the importance of understanding its regulation.

1.4.6 The Tumour Microenvironment and Tumour Inflammation

Tumours do not make up the vast majority of the ECM or tumour microenvironment (TME), with the ECM being influenced by a multifaceted of different cell types including neoplastic cells, fibroblasts, endothelial cells and immune cells all interacting with one another continuously (Figure 1.11). Virchow, who hypothesised that the origin of cancer was at sites of chronic inflammation, first described the presence of inflammatory cells such as macrophages, neutrophils, mast cells and T cells in tumours back in 1863. This was in part based on his observation that certain irritants, which caused tissue injury and inflammation enhance cell proliferation [297]. Together, the TME provides the tumour with essential growth factors and optimum conditions necessary for survival

1.4.6.1 T cells

Within the tumour microenvironment, T cells play a dual role in tumour progression. T-cells are a heterogeneous mix originating from different lineages. The $CD4^+$ T cells, best known as helper cells are subdivided into, type 1 $CD4^+$ T cells (T_H1) and type 2 $CD4^+$ T cells (T_H2). Other T cell subtypes include $CD8^+$ T cells and cytotoxic T lymphocytes (CTLs). The latter kill tumour cells following recognition of antigens expressed on the surface of tumour cells. The T_H1 cells are describes as anti-tumourigenic as they secrete INF-γ and IL-1β which directly kill tumour cells within the TME [298]. T_H1 also aid $CD8^+$ T cells in tumour rejection. This is counter-regulated by T_H2 and $CD4^+$ T regulatory cells which block the activation of $CD8^+$ T cells [299]. T_H2 cells also inhibit apoptosis and enhance tumour growth through secretion of anti-inflammatory cytokines such as IL-10 [300]. A third group of T cells; the regulatory T cells regulate the immune response of other immune cells. It is still not exactly known how this regulation is controlled; previous research showed two possible mechanisms, either via direct

cell-cell contact between the regulatory T cells and the other immune cell, or via the secretion of anti-inflammatory mediators, like IL-10 [301]. Given the critical importance of T cells in tumour biology, they represent a major therapeutic target for development of cancer immunotherapies.

1.4.6.2 Fibroblasts

Besides immune cells, the tumour microenvironment also contains fibroblasts. Fibroblasts are embedded in the extracellular matrix (ECM) of tissues, where they synthesise many components of the ECM. Moreover they also regulate inflammation, epithelial differentiation and are involved in wound healing [302]. Besides synthesis, they also regulate the reduction of the ECM by secreting enzymes that regulate this degradation such as MMPs. Furthermore, the fibroblasts also secrete growth factors influencing their neighbouring epithelial cells including VEGF, TGF-β, platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2) which are frequently deregulated in cancer [303, 304]. Cancer associated fibroblasts (CAFs) are commonly identified by their expression of α-smooth-muscle actin [305]. Interestingly, as well as providing scaffolding support to the TME, fibroblasts are a significant source of immune-modulatory cytokines such as INF-γ, IL-6 and TNF-α which can influence the mobilisation of immune cells [306]. In this regard, it has also been suggested that normal fibroblasts help to prevent tumour progression and formation through the alteration in the concentration of immunoregulatory soluble factors.

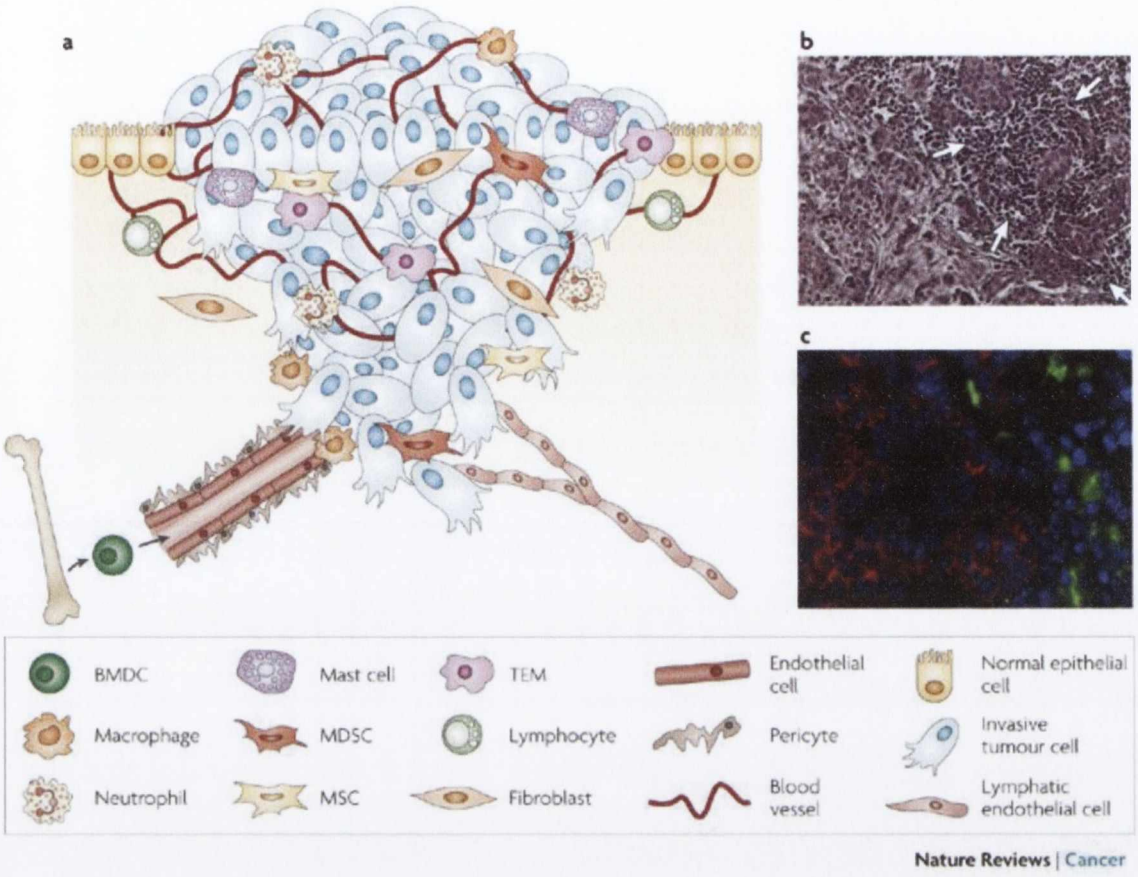


Figure 1.11: Schematic representation of the tumour microenvironment

(a) Cancer cells in primary tumours are surrounded by a complex microenvironment comprising of numerous cells. (b) Invasive human breast cancer cells stained with haematoxylin and eosin in which infiltration of leukocytes is evident. (c) Macrophages (green) at the invasion edge of pancreatic islet cancer cells (red). Image taken from Joyce *et al.* [307].

1.4.6.3 Macrophages

Macrophages constitute a heterogeneous population of cells that are grouped into Mϕ (unpolarised), M1 (or classically activated pro-inflammatory) and M2 (or alternatively activated anti-inflammatory) macrophages [308]. M1 macrophages are pro-inflammatory macrophages that provide immediate defence against foreign pathogens and co-ordinate leukocyte infiltration [309]. M2 macrophages are anti-inflammatory macrophages that play a role in anti-inflammatory signalling, as well as being responsible for wound healing and tissue remodelling [310].

Within the tumour, "tumour associated macrophages" (TAMs) represent up to 50% of the tumour mass [311]. Their presence predicts poor prognosis in up to 80% of all tumours [312, 313], and chemo responsiveness of patients with HCC [314]. It is now generally accepted that TAMs have an M2 phenotype and pro-tumourigenic as they promote tumour cell survival, proliferation, and dissemination [315-317]. As tumours are seen as "foreign" to the immune system, it is an interesting and contentious issue as to how macrophages assist tumour growth/spread.

Although the tumour microenvironment is thought of as an immunosuppressed environment [318], tumours can "re-educate" or program macrophages to aid in their progression. Central to this is the release of macrophage-derived factors such as MMPs, growth factors (VEGF), tumour-favourable cytokines and chemokines (IL-8, IL-10) [319]. The mechanism by which tumours repolarise macrophages is unclear. However, many believe it is through a combination of T_H-2 derived cytokine immune-editing [320] and tumour associated factors such as microsomes or tumour derived cytokines such as IL-10 [321, 322]. A recent study has also found that tumour-derived lactic acid can polarise macrophages to M2-like TAMs fostering the idea that tumours

posses the ability to re-educate macrophages by creating an acidic/hypoxic microenvironment [317].

Human macrophages produce several MMPs including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-12 that can participate in remodelling of the extracellular matrix during inflammation. Indeed, within the tumour, TAMs are a major source of MMP-9 [323]. In a mouse of pancreatic cancer MMP-9 secreted from tumour-infiltrating inflammatory cells liberates VEGF-A from its matrix reservoir facilitating tumour angiogenesis and ECM remodelling [324]. The authors postulate that TAMs may also promote formation of the metastatic niche by providing high levels of MMP-9, IL-8 and VEGF to the tumour.

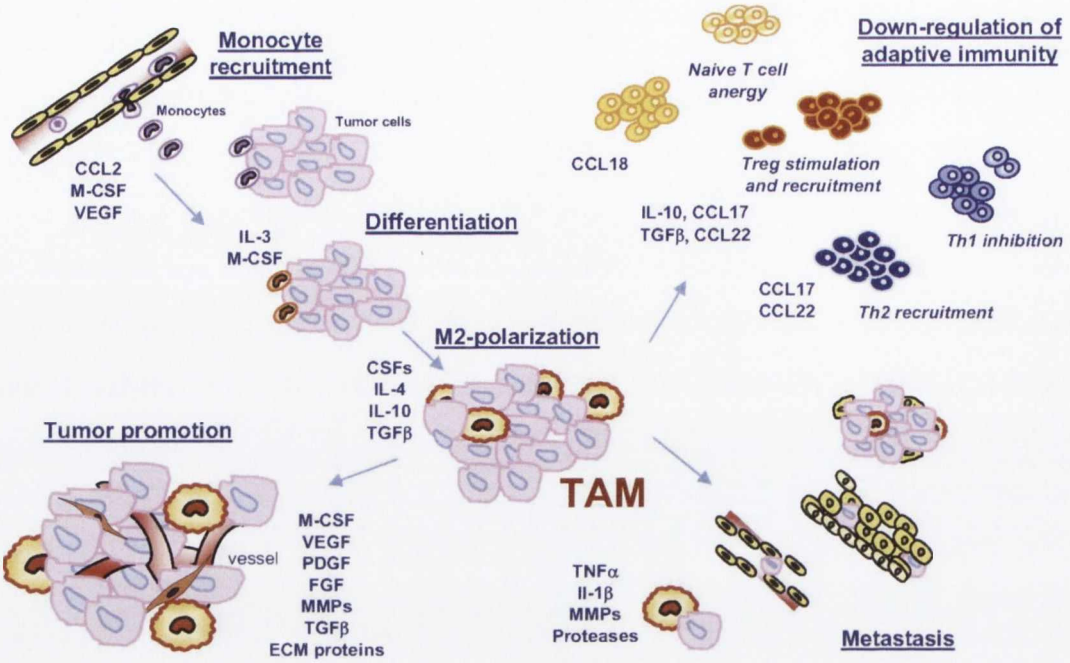


Figure 1.12: Overview of tumour associated macrophage (TAM) polarisation and role in formation of the tumour microenvironment.

The main factors involved in monocyte recruitment are the chemokine CCL2, M-CSF, and VEGF. Tumour macrophages resemble M2-polarised cells and play a pivotal role in tumour growth and progression. TAM actively work for the tumour: they produce several molecules that sustain malignant cell survival, modify neoplastic ECM proteins, promote the development of a newly formed vessel, and assist tumour cells in their progression. TAM affect adaptive immune responses significantly by recruiting and stimulating T-regs and recruiting T_H2 lymphocytes, which in turn inhibit T_H1 cells, and by inducing anergy of naïve T cells. Image adapted from Solinas *et al.* [325].

1.4.7 PP2A Inhibition and Implications for the Tumour Microenvironment

PP2A regulates numerous internal signalling pathways that are implicated in regulating the ECM and tumour microenvironment (TME) of which MAPK and ERK signalling are key. However, the mechanism by which PP2A modulates the tumour microenvironment has not been studied in detail.

1.4.7.1 PP2A and Nuclear Factor Kappa B Signalling

The Nuclear Factor Kappa B (NF-κB) regulates numerous MMPs including MMP-1 and MMP-9 [326], while also regulating TNF-α, IL1β and numerous other soluble tumour soluble factors. PP2A has been found to regulate the NF-κB pathway by preventing the release of the IκKB, an inhibitor protein of NF-κB from being phosphorylated [327]. Inhibition of PP2A thus promotes NF-κB pathway activation, which could influence the tumour microenvironment by up-regulation of gene expression involved in inflammation such as MMPs, cytokines and growth factors.

1.4.7.2 PP2A regulation of MAPK and ERK Signalling

The most likely signalling pathways involved in PP2A mediated alteration of the tumour microenvironment are the mitogen activated protein kinase (MAPK) pathways. PP2A is a known regulator of MAPK signalling, with PP2A inhibition being central to their activation [328]. The MAPKs are stratified into the extracellular-regulated kinases (ERK), the stress activated or p38 protein kinases and the c-Jun N-terminal kinases (JNK). These pathways regulate MMPs, cytokines and growth factor expression in many cells type. Indeed hyper-phosphorylation of MAPK signalling is linked to their over expression in a variety of tumours [329, 330].

PP2A regulates p38 signalling by association and dephosphorylation of the upstream activator of p38, MKK3 [331]. PP2A seems to regulate not only JNK activity but also the upstream kinases MKK4 and MEKK-1, which in turn regulate JNK [328]. *In vitro* studies indicate that PP2A can dephosphorylate and inactivate both MEK1, 2 and ERK1, 2 proteins in cells [332]. Similarly, in drosophila S2 cells, PP2A-containing B56 specifically inhibited ERK1, 2 phosphorylation [129]. These data are supported by inhibitor data showing okadaic acid to induce MEK1, 2 and ERK1, 2 phosphorylation and activity *in vitro* [333]. Thus, through a deregulation of these intrinsic pathways, loss of PP2A potentially promotes not only favourable intracellular conditions for oncogenesis but also extracellular ones too.

1.4.7.3 PP2A regulation of MMPs and Cytokines in Cancer

MMPs expression is coupled to ERK, MEK and p38 MAP kinases dependent signalling [334-337]. The role of PP2A in the regulation of the MMPs is unclear. However, few studies have linked PP2A to MMP or cytokine regulation. The first evidence of potential involvement of protein phosphatases in regulating the ECM came from studies using pharmacological inhibitors of PP1 and PP2A, okadaic acid (OA). OA has been shown to increase MMP-3 (stromelysin-1) expression in murine keratinocytes [338]. This has been confirmed and extended by others to include collagenase, interstitial collagenase (rat homologue of collagenase-3/MMP-13), MMP-13 and MMP-9 in fibroblasts and chondrocytes [339-342]. Interestingly, these MMPs have a TATA box, proximal AP-1 and PEA3 binding sites in their promoters [343]. However, Westermarck *et al.* has also shown OA to increase TIMP-1 mRNA in HT-1080 cells, which doesn't have TATA box, but instead possesses AP-1 and PEA3 binding sites in it's promoter [288, 342].

Okadaic acid also been shown to increase soluble IL-8 expression [344-346] as well as IL-6 production from alveolar epithelial cells [346]. As well as this, okadaic acid increases expression and secretion of vascular

endothelial growth factor gene, which is key in angiogenesis [347]. Together these data highlight the important role of PP2A in modulating expression of key factors associated with tumour cell migration and invasion [339-341].

1.4.7.3 PP2A and Hypoxia

Another factor, which is critical in influencing the TME and ECM, is the transcription factor HIF-1α [348]. Hypoxia decreases PP2A activity in glioblastoma multiforme cells [349]. Interestingly in this study, brain biopsies with high HIF-1α protein expression correlated with reduced protein expression of PP2Aa, PP2Ac and PP2Ab (B55) subunits. However, this study also shows that hypoxia increases PP2A activity in glioblastoma expression of PP2Aa, PP2Ac and PP2Ab (B55) subunits. However, this study also shows that hypoxia increases PP2A activity in glioblastoma. PP2A activity is increased under hypoxia in hypoxia-activated Smad3-specific dephosphorylation [350]. Paradoxically, okadaic acid up-regulates HIF-1α protein expression in HUVEC cells [351]. It is plausible that hypoxia induces changes to the PP2A composition by switching regulatory subunits thus, targeting PP2A specificity within tumours. Further work is needed to investigate the role PP2A plays in hypoxia and MMP regulation within the TME with understanding the PP2A enzymatic activity equilibrium a key factor in this.

1.5 Aims of this study

The overall scope of this thesis is to investigate the functional consequences resulting from PP2A inhibition and its impact on the tumour microenvironment in a cell line model of hepatocellular carcinoma. In particular, characterisation of the functional and molecular implications of pharmacological inhibition of PP2A by okadaic acid using a phosphoproteomics approach is studied. Altered signalling pathways and common phosphorylation motifs will be assessed using Bioinformatic software and phosphorylated proteins will be validated. Delineated upstream regulator pathways are assessed resulting from okadaic acid induced PP2A inhibition.

Based on emerging research linking protein phosphatase modulation to the regulation of the extracellular matrix, the effects of PP1, PP2A and PP2B inhibition on members of the MMP/TIMP family and others is examined in hepatic stellate cells and hepatic epithelial carcinoma cells. Key regulatory factors of PP1 and PP2A activity such as the catalytic and scaffolding subunit will be investigated along with the implications of CIP2A expression in relation to MMP-9 abundance. Regulatory cis-elements on the MMP-9 promoter are investigated by mutational analysis and the implications on okadaic acid-mediated regulation of MMP-9. The functional consequences of inhibition of PP2A by okadaic acid and CIP2A overexpression are considered in hepatic epithelial carcinoma cells.

Based on the ever-growing body of conflicting data on the role TIMP-1 has in cancer, we investigate the signal transduction and transcriptional regulation of tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) by PP2A. In particular, the work focuses on the effect that okadaic acid and CIP2A overexpression has on TIMP-1 in three hepatic cell lines, as cell line differences may account for some of the discrepancies within the literature. The roles of NF-κB, PI3K, MEK, JNK signalling in okadaic acid-mediated TIMP-1 expression is also

investigated in conjunction with loss of PP2A function. I also aim to investigate regulation of TIMP-1 promoter using wild type and mutant promoter constructs with the goal of identifying potential regulatory sites responsible for okadaic and CIP2A regulation of TIMP-1 expression.

Bidirectional communication between cells and their microenvironment is critical for both normal tissue homeostasis, and for tumour growth. As macrophages constitute a large proportion of the tumour mass, the fourth study considers the role of tumour-associated macrophages (TAMs) in altering PP2A activity in hepatocellular carcinoma. It considers the effects of "smouldering inflammation" resulting from macrophage co-culture CIP2A expression and functional consequences of tumour microenvironment. The positive feed back onto anti-inflammatory macrophages resulting from alterations in PP2A activity is also investigated.

Chapter 2.

Materials

2.1 Consumables

Product	Manufacturer
3MM ® chromatography paper	Sarstedt
6-well plate, sterile	Sarstedt
12-well plate, sterile	Sarstedt
24-well plate, sterile	Sarstedt
48-well plate, sterile	Sarstedt
96-well plate, sterile	Sarstedt
Cell scraper, sterile	Sarstedt
Cryogenic vials, sterile	Corning
Hybond-P, Hydrophoic polyinylidene difluoride (PVDF) membrane	GE Healthcare
Petri dish, sterile	Sarstedt
White 96-well plates	Greiner Bio-One
MicroAmp ® Optica 8-Cap Strip/Caps	Applied Biosciences
Corning 6 well transwell plate (0.4 μM pore)	Corning
Corning 6 well transwell plate (8.0 μM pore)	Corning
Corning 24 well transwell plate (8.0 μM pore)	Corning
Serological pipettes, sterile	Corning
Syringes and needles, sterile	Becton, Dickinson and Company
BioPionte Pipette tips, sterile	BioPointe Scientific
T75-cell culture flask, Nunclon Δ Surface filter cap, sterile	Nunc

Table 2.1 Consumables

2.2 Equipment

Equipment	Company, Model
MX3000p, Real Time PCR machine	Applied Biosystem
Analytical balance	Mettler, AE240
Autoclave	Dixon
Fluorescence microscope	EVOS
Automated pipettes	Gilson, Inc. (2 µl, 10µl, 100µl, 200µl, 1000 µl, 5000 µl, Pipetman Ultra 8-channel (20-300 µl))
Centrifuge	Hettich Zentrfugen, EBA 12R/mikro 22R
Digital imaging	Fusion Fx imaging system, Vilber Lourmat
Inverted microscope	VWR, VistaVision™
Freezer (-80°C)	Thermofischer Scientific , Revco Valure Plus
Gel documentation system	Syngene, GeneGenius
Gel electrophoresis system	Bio-Rad, Mini-Protean
Incubator (37°C, 5% CO2, 95% rh))	HERAcell 240i
Laminar flow hood	Mason Technology, BioBan 48
Luminometer	Thermofischer Scientific, Fluoroskan AscentFL
Microplate reader	BioTek EL 808
Microplate washer	BioTek ELx405
Micro-volume UV-Vis	Thermofisher Scientific, Nanodrop ND 8000
Spectrophotometer	
Neubauer haemocytometer, improved	BRAND GMBH + CO KG, Blaubrand ®
pH meter	Mettler-Toledo Inc., MP320
Thermocycler	MJ Research Inc, PTC-100

Table 2.2 A list of equipment and supplier

2.3 Prokaryotic cells

In the experimental process of this study the bacterial strain TOP 10 *E. coli*, which were a kind gift or Dr. Deirdre Ni Eidhin, was used, The genotype of the TOP 10 E/coli strain is F-*mrc*A Δ (*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15n Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gla*U *gal*K *rps*L (Str^{R}) *end*A1 *nuo*G λ-.

2.4 Eukaryotic cells

2.4.1 Hep3B cells

Hep3B cells are human epithelial cells derived hepatoma cells that produce the hepatitis B virus surface antigen that are derived from an 8 year old male [352]. These cells grow adherently in culture. Hep3B cells were a kind gift from Dr. Steven Gray, who purchased them from the American Type Culture Collection.

2.4.2 LX-2 cells

LX-2 cells are hepatic stellate cells, which were prepared through spontaneous immortalization in low serum conditions and grow adherent [353]. Prof. Scott L. Friedman (Mount Sinai School of Medicine, New York) kindly donated these cells.

2.4.3 Huh-7 cells

HuH-7 is a well-differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumour in a 57-year-old Japanese male [354]. These cells grow adherently in culture. Huh-7 cells were a kind gift from Dr. Steven Gray, who purchased them from the American Type Culture Collection.

2.4.3 THP-1 cells

THP-1 cells are a human leukaemia monocytic cell line isolated from a 1-year old infant male, which have been extensively used to study

monocyte/macrophage functions [355]. THP-1 cells can be activated to macrophages with PMA stimulation and further differentiated with T_H1 or T_H2 cytokines. THP-1 cells were a kind gift from Dr. Steven Gray, who purchased them from the American Type Culture Collection.

Chapter 3

Okadaic acid Triggered Alteration in The Phosphoproteome of Hep3B Cells: PP2A Priming Cells for Aggression

3.1 Introduction

A deregulation in phosphatase activity is a signature of many malignancies, including hepatocellular carcinoma (HCC) [356-358]. This deregulation allows for uncontrolled proliferation of tumour cells impacting on protein synthesis, metabolism and cell migration [3]. Recent developments in cancer therapeutics have focussed on targeting cell surface proteins with humanised monoclonal antibodies or intracellular enzymes with small molecule inhibitors such as kinase inhibitors. The rapid expansion of proteomic technologies has resulted in considerable activity in the intracellular protein discovery pipeline. However, the resulting advancements have not aided in HCC drug development with metastasis and drug resistance the leading cause of death in HCC. Exploring the intracellular mechanisms resulting from reduced phosphatase activity and the association of this with HCC metastasis could help to develop novel reagents and drugs for the treatment of liver cancer.

In recent years, the advancement in proteomics has permitted us to detect the dynamic changes and post-translational modifications in proteins in cancer cells, and using systems biology has led to a greater understanding of the hierarchical control of oncogenic pathways [359, 360]. While the emphasis has been on these kinase dependent pathways and their phosphoprotein substrates, less is known about the regulation of these pathways by phosphatases, in particular the role of serine/threonine protein phosphatases 2A (PP2A) in facilitating both cell proliferation and migration. The majority of serine/threonine dephosphorylation reactions are catalysed by a handful of phosphatases, giving rise to the fallacy that these protein phosphatases are promiscuous and unregulated enzymes. This idea could not be more farfetched. PP2A—the most abundant of the serine-threonine phosphatases—is, in fact, a tightly controlled enzyme with a highly diverse array of regulators including post-translational modifications, subunit depletions and recently discovered inhibitory binding proteins

[128, 227]. Understanding the protein networks that PP2A regulates will lead to a greater understanding of the cell proliferation that tumour cells undergo following PP2A inhibition. For many years, inhibition of PP2A with okadaic acid (OA), a serine/threonine phosphatase inhibitor was known to promote tumourigenesis [361]. Inhibition of PP2A prevents the dephosphorylation of signalling proteins such as AKT, GSK-3, c-Myc, MAPK and cyclin-dependent kinase (CDK) to name but a few [362-365]. As to the diversity of its regulatory role, PP2A can be seen as a master controller in tumour kinase pathway networks.

1. Therefore, the aims of this study were to use an *in vitro* and computational experimental approach to identify potential proteins, pathways and upstream regulators involved in cellular proliferation, cell migration and angiogenesis following PP2A inhibition using a hepatic epithelial carcinoma cell line, Hep3B.
2. Apply a targeted phosphoproteomic approach combined with a selective enrichment of phosphorylated phosphoproteins following PP2A inhibition. Mass spectrometry analysis and bioinformatics were used to identify phosphorylated proteins.
3. Validate the identification of selective proteins by Western blotting and used bioinformatics to identify potential pathways altered and to predict phosphorylation motifs and their associated kinases following PP2A inhibition
4. Identify a potential regulator of tumour cell oncogenesis resulting from PP2A inhibition.

3.2 Methods

3.2.1 Materials

Rabbit anti-ROCK2, anti-HYOU1, anti-eif3H (D9C1), anti-VASP (9A2) and anti-moesin (Q480) were purchased from Cell Signalling Technology (Massachusetts, USA). Mouse anti-c-Myc was purchased from Santa Cruz (California, USA). Secondary goat anti-rabbit and sheep anti-mouse HRP conjugated antibodies were obtained from Dako Denmark A/S (Denmark). Okadaic acid (OA) was obtained from Calbiochem (Germany). FTY-720 was obtained from Sigma-Aldrich (Ireland). All other chemicals were obtained from Sigma-Aldrich (Ireland) unless otherwise stated.

3.2.2 Cell culture

Human epithelial hepatocellular carcinoma (Hep3B) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and 4 mM-glutamine, at 37°C in an humidified atmosphere containing 5% CO_2. Hep3B experiments were performed under serum free conditions. Human umbilical vein endothelial cells (HUVECs) were cultured in HAMs F-12K Medium supplemented with 0.1 mg/ml heparin; 0.03-0.05 mg/ml endothelial growth media (EGM-2), antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and an adjusted final concentration of 10% (v/v) foetal bovine serum.

3.2.3 Scratch Assay

Cells were grown to confluence in complete DMEM culture media (Fisher Scientific, Pittsburgh, PA, USA) in 6 well plates and a scratch 1 mm wide was made using a sterile pipette tip along the centre of the dish. Cells were extensively washed in sterile phosphate buffered saline (PBS) before replacing the media with serum free DMEM. Cells were exposed to OA (40 nM), FTY-720 (2.5 μM), FTY-720+OA or media alone for 24 h. A demarcated area of the wound was photographed on a Cell Imaging System (EVOS® FL) and subsequent images captured at 0 and 24 h at 4× magnification.

3.2.4 Cell proliferation Assay

Hep3B were seeded in 96-well plates (5 x 10^3 cells/well) and exposed to media, OA (40 nM) and the PP2A activator FTY-720 (2.5 μM), and DMSO (0.1% v/v) for 0, 3, 6, 12, 24 and 48 h. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; (5 mg/mL) was added at each time point and the cells incubated for a further 2 h. At the end of the incubation period, the culture medium was removed, and the purple formazan deposits were dissolved in DMSO. Formazan production was quantified by spectrophotometry at a wavelength of 540 nm with a reference wavelength of 650 nm (BioTek, EL 808, Bedfordshire, UK). Samples were analysed in triplicate.

3.2.5 Tube Formation Assay

HUVECs were detached by trypsinisation and after neutralization of trypsin, were counted and resuspend in EGM-2 containing 1% FCS at 4×10^5 cells/mL. HUVECs were seeded at 2×10^4 cells per well in Matrigel-precoated 96-well plate (BD Biosciences, catalogue no. 354605). HUVECs were then exposed to OA (40 nM), FTY-720 (2.5 μM), FTY-720+OA and media for 6 h before visualisation on EVOS® FL Cell Imaging System. Each experiment was performed in triplicate. Tube formation was evaluated using ImageJ software [366].

3.2.6 Cell Lysis and Phosphoprotein Enrichment

Although PP2A is serine/threonine phosphatase, it also has tyrosine dephosphorylation capabilities [367]. As no antibody would recognise all serine or threonine phosphorylation sites independent of the surrounding amino acid sequence, a whole phosphorylated enrichment approach was used. Cell lysates enriched for phosphoproteins were obtained using a TALON phosphoprotein enrichment kit (Clontech, Mountain View, CA) in accordance with the manufacturer's instructions. Cells were grown in a T75 flask to 70% confluency before being serum depleted and exposed to either OA (40 nM) or media for 24 h. Briefly, cells were washed three times with 20 ml of sterile PBS, before being strapped from the flask using a rubber policeman (Sarstedt, UK). Cells were then centrifuged at 500-x g for 5 min in a pre-weighed centrifuged tube. The supernatant was decanted and any residual liquid aspirated. The cell pellets were then weighed again, before being placed in an -80^{0}C freezer for 1 hour to disrupt the cell membrane. The cell pellets were then resuspend in 25 μl/mg of cells of extraction buffer (Buffer A) and pipetted up and down approximately 20 times. The cell pellets were then incubated at 4^{0}C for 10 min with mixing by inverting the tube approximately every minute. Cell extracts were then centrifuged at 10,000-x g for 20 min at 4^{0}C. During this time the phosphocolumns were assembled for phosphoprotein extraction.

This is important as the column resin needs to settle out of suspension and the columns equilibrated with Buffer A solution until the final pH eluent read pH 6.0. After centrifugation, the supernatant was transferred to a clean tube and the pellet was disposed off. The supernatant was then added to the phosphocolumns and gently agitated on a platform shaker for 30 min at 4^{0}C to allow the phosphorylated proteins to bind to the columns. After agitation, the columns were allowed to settle out of suspension. Columns were then washed with 1 ml of the elution buffer (Buffer B) and the fraction collected on ice. This was repeated 14 times with 1 ml of Buffer B. Samples were kept on ice and stored at -20^{0}C prior to determination of protein concentration by Bradford Assay (Sigma Aldrich, Ireland). For SDS-PAGE, MS/MS analysis and Western blot, fraction three was used.

3.2.7 SDS-Page and In-gel digestion

Enriched phosphoproteins were supplemented with Laemmli buffer (250 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 0.1% phenol red, 50% glycerol, 5% Ficoll 400 and 5% *lithium dodecyl sulphate;* final concentrations) and subjected to gel electrophoresis using Thermo Scientific™ Precise™ Tris-Glycine 10% gels (Fisher Scientific, Ireland). Following staining with EZBlue™ Gel Staining Reagent (Sigma-Aldrich, Ireland) for 3 h and destaining (60:30:10% (v/v/v) dH_2O, CH_3OH, CH_3COOH) for 1 h, the bands of interest were excised and sent to the University of St Andrews, Fyfe, United Kingdom, for mass spectrometry. Dr. Catherine Botting performed the mass spectrometry. The proteins were digested in-gel with trypsin (Promega) after reduction and alkylation, on an Investigator Progest digestion robot (Genomic Solutions Ltd., UK) using standard protocols (Shevchenko et al., 1996). Briefly, a volume of 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 sufficient to cover the gel pieces was added, and the proteins were reduced for 1 h at 56 °C. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM

iodoacetamide in 100 mM NH_4HCO_3. After 45 min incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50-100 µL of 100 mM NH_4HCO_3 for 10 min, dehydrated by addition of acetonitrile, rehydrated in 100 mM NH_4HCO_3, and dehydrated again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were dried in a vacuum centrifuge. The gel pieces were rehydrated in a digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/µL of trypsin (Boehringer Mannheim, sequencing grade) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 5-10 µL of the same buffer, but without trypsin, to keep the gel pieces wet during enzymatic cleavage (37 °C, overnight). Peptides were extracted by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile (20 min for each change) at room temperature and dried down.

3.2.8 nLC-ESI-MS/MS

The resultant peptides were separated on an UltiMate nanoLC (LC Packings, Amsterdam) equipped with a PepMap C18 trap and column, with a 60 min gradient of increasing acetonitrile concentration, containing 0.1 % formic acid. The eluent was injected into a Q-Star XL tandem mass spectrometer (Applied Biosystems, CA) and analysed in Information Dependent Acquisition (IDA) mode, 1 sec of MS followed by 2 x 3 sec MS/MS of the 2 most intense peaks seen by MS. These masses were then excluded from analysis for the next 60 seconds.

3.2.9 Database searches

MS/MS data for doubly and triply charged precursor ions were converted to centroid data, without smoothing, using the Analyst QS1.1 mascot.dll data import filter with default settings. Swiss-Prot and NCBI databases were interrogated to allow for greater sequence identification. *Homosapien* species restriction was applied. The data were searched

with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, and one missed cleavage. Three or more matching peptides and a significant probability score ($P < 0.05$; MOWSE score > 50) were required for a secure identity assignment. All MASCOT search results were inspected manually to ensure correct matches.

3.2.9.1 PhosphoSitePlus™

All phosphorylation sites of okadaic acid stimulated phosphorylated proteins were identified using the PhosphoSitePlus Plus database [368]. For each hit from the PhosphoSite Plus, the occurrence of at least one serine/threonine phosphorylation sites was carefully validated using the SMART (Simple Modular Architecture Research Tool) domain identification program. PhosphoSitePlus was also used to identify common phosphorylation motifs in the data set (Supplementary data).

3.2.9.2 GPS 2.0 Kinase-Specific Phosphorylation Prediction and iGPS (GPS Algorithm With the Interaction Filter)

For prediction of site-specific kinase-substrate relations, phosphoproteins were prepared in the FASTA format. We used iGPS 1.0 to predict the site-specific kinase-substrate relations for all phosphoproteins identified following PP2A inhibition [369]. During the prediction, high threshold and the Exp./String interactions were chosen.

3.2.9.3 Phosphorylation Motif Analysis

Motif-X was used for the identification of phosphorylation motifs present in the phosphoproteins identified in Hep3B cells following PP2A inhibition [370]. All okadaic acid stimulated phosphorylated proteins were converted to FASTA sequence for phosphorylation site prediction. A phosphopeptide with a length of 15 amino acids with 7 residues upstream and 7 residues downstream from the phosphorylation site was selected, with serine, threonine and tyrosine phosphorylation sites all being covered. If the phosphorylation site was located in the N-terminus or C-terminus of the protein sequence, the phosphopeptide was complemented with the necessary number of "*"s replacing of amino acids. Motif-x parameter settings of "pre-aligned", central S, T or Y, with =15, occurrence = 20 and significance = 0.000001 were adopted. IPI Human proteome in Motif-x was set as the background.

3.2.9.4 Gene Ontology Data Mining

The PANTHER Classification System (Pathway version 10.0; www.pantherdb.org) was used to identify protein families or groups of evolutionarily related proteins from all of the phosphoproteins identified by mass spectrometry. DAVID (http://david.abcc.ncifcrf.gov/) was used to convert protein IDs to Ensembl format for gene ontology. Molecular function, biological process and pathway analysis ontology was assigned to the phosphoproteins identified in Hep3B cell line with and without OA. To view significantly enriched "Pathway Commons" following PP2A inhibition, Gene Ontology enrichment analysis was also carried out in WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) [371] using the Fisher's exact test with an adjusted *P* value cut-off of 0.05 enriched Gene Ontology terms were further analysed using the Pathway Commons algorithm to generate a parsimonious list of enriched terms [372]. Pathways identified considered biological processes and pathways that were comprised more than 10 genes.

3.2.10 Immunoblotting

Confirmation of phosphoprotein expression, elution and c-Myc expression was determined using immunoblotting. Protein samples (1 μg) were boiled for 1 min in Laemmli buffer supplemented with 2-β-mercaptoethanol (1% v/v). The samples were subjected to SDS-PAGE (10% gel) and semi-dry transferred onto PVDF membrane (Amersham, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS-T (Tris-base 10 mM, NaCl 100 mM, HCl 1 M, and Tween-20 0.1% (v/v) containing 5% (w/v) Marvel) and probed with 1° antibodies directed against ROCK2, eif3H, HYOU1, VASP, moesin (Cell Signalling Technology, Massachusetts, USA; 1:1000 dilution) and c-Myc (Santa Cruz, California, USA) for 24 h at 4°C. Following extensive washing, membranes were incubated with a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP 1:2500 dilution). Signals were visualised by chemiluminescence detection (30% H_2O_2, 250 mM luminol in DMS0, 90 mM 4-iodophenylboronic acid in DMSO and 10 mM Tris-HCl) [373] and the signal recorded using a Fusion Fx imaging system (Vilber Lourmat, France).

3.2.11 Ingenuity Pathways Analysis (IPA)

IPA software (Ingenuity Systems, Redwood City, CA) was used as a bioinformatics tool to help better understand the experimental data in relation to published research by identifying relationships, mechanisms, functions, and pathways of relevance through easy dynamic pathway modelling. The differentially expressed phosphoprotein *UniProtKB/Swiss-Prot* IDs and corresponding Benjamini–Hochberg adjusted probability values were uploaded into Ingenuity Pathways Analysis (IPA) software, version 7.5 to identify the networks, diseases and disorders associated with the differentially expressed proteins. The probe sets were mapped to the corresponding IPA gene objects. These "focus" genes were combined with Ingenuity's proprietary data and knowledge base to construct a molecular network. We also used an IPA tool called "Canonical Pathways Analysis" to identify the most statistically significantly over-represented pathways from the IPA pathway library in the data set (by Fischer's exact test). The IPA software, using literature-based pathway databases to connect focus genes and other genes in the knowledge base, generated graphical network models of key pathways. The 'IPA Path Designer' mode was used to generate final network images.

3.2.12 Statistical Analysis

Cell proliferation and invasion data were compared to untreated control. Data were analysed by or 2-way ANOVA with *post hoc* analysis (Dunnett). Data are expressed as mean ± S.E.M., and a value of $P<0.05$ is taken to indicate statistical significance.

3.3 Results

3.3.1 Effect of PP2A inhibition and activation on Hep3B cell proliferation and migration

Cells exposed to OA increased cell proliferation after 12, 24 and 48 h compared to untreated control (Figure 3.1; $P<0.05$). FTY-720 did not alter cell proliferation at the earlier time points, however, it was significantly decreased at the 48 h time point compared to untreated control and OA ($P<0.05$). The effects of OA (40 nM) and FTY-720 (2.5 μM) exposure on cell migration and proliferation were assessed in Hep3B cells by scratch assay (Figure 3.2A). OA increased Hep3B cell migration by 43.7 ± 4.3% compared to untreated control ($P<0.05$), while FTY-720 had no effect compared to the untreated control (Figure 3.2B). FTY-720+OA reduced cell invasion compared to OA alone by 18.7 ± 4.9%. FTY-720 alone did not alter wound closure compared to untreated control ($P<0.05$).

3.3.2 Effect of PP2A inhibition and activation on HUVEC tube formation

As well as an increase in cell migration and proliferation, we were interested to see if alterations in PP2A activity altered the ability of HUVECs to form tubules. Exposure of HUVEC cells to OA resulted in an increase in tubule formation, which is indicative of an increase in angiogenesis. Activation of PP2A by FTY-720 inhibited HUVEC tube formation, while FTY-720+OA reduced tube formation compared to OA alone (Figure 3).

3.3.3 Isolation of phosphorylated proteins and Identification of pSer/Thr and pTyr containing proteins by nanoLC-MS/MS

Fraction 3 from the enrichment process was found to have the greatest protein concentration. Phosphorylated and non-phosphorylated proteins from OA exposed (40 nM) and untreated fraction 3 samples were resolved by SDS-PAGE and stained with EZBlue™ (Figure 3.3). Phosphorylated protein bands were excised and subjected to spectrometry analysis as described in section 3.2.8. A total of 281 proteins were identified by the evaluation of the nanoLC-MS/MS data (Supplemental Table 1, data available on request) under basal conditions. A further 180 (Table 3.1) proteins were identified following exposure to OA (40 nM). 7 proteins were present in the untreated fraction that was not detected in the OA treated fraction (Supplemental Table 1, data available on request) giving a total of 461 phosphorylated proteins identified by LC-MS/MS. To further support these results, each protein sequence was manually determined using FASTA identification software:

http://genome.nci.nih.gov/tools/reformat.html;
http://www.ensembl.org/index.html

3.3.4 Categorisation of the identified proteins

PANTHER Gene Ontology and DAVID Functional Annotation Tool were used to categorise all the identified proteins based on their biological function. They were categorised into 12 processes (Figure 5) with cellular process (GO:0009987), metabolic process (GO:0008152) and cellular organisation (GO:0016043) the major ontologies of the newly identified phosphorylated proteins. DAVID protein ontology identified proteins based on their protein type; acetylated proteins, phosphoproteins (P=1.04E-28), cytoplasmic proteins, nucleotide-binding proteins, ubiquitin conjugation proteins and ATP-binding

proteins were all identified as being the most significant (Figure 6; P<0.05). 80% of the proteins identified were described as phosphoproteins. Pathways Common identified statistically over-represented pathways, of which the majority are involved in cancer cell proliferation and are current drug targets for cancer therapy (Table 3.2). Major pathways identified using WebGestalt software include gene expression (P=9.61E-17), plasma membrane oestrogen signalling (P=5.9E-16), PAR-1 mediated thrombin signalling (P=1.85E-15), EGF signalling (P=1.89E-15) and mTOR signalling (P=1.85E-15).

3.3.4 Identification of predominate phosphorylation sites and *in silico* kinase regulation

Utilising a combination of, GPS 3.0, PhosphoSitePlus database and iGPS, 112 proteins (62%) were identified as having experimentally proven phosphorylation sites (Table 3.3). Each protein was examined further and known phosphorylation sites (Ser/Thr and Tyr phosphorylation sites) and corresponding peptide sequence were mapped (data available on request). In addition, each protein was interrogated by manual examination of published literature using PubMed.

Following identification of okadaic acid (OA) induced phosphorylated proteins; the relevant phosphorylation motifs within the dataset were deduced. 6 serine, 2 threonine and 2 tyrosine phosphorylation motifs were enriched (Table 3.4; Table 3.5; Table 3.6) and (Figure 3.7). Many of the motifs identified are putative motifs to our data set, however, statistical graphic representation of the serine phosphorylation motifs identified **S**E as the dominant phosphorylation motif (Figure 3.8; P<0.000001). A DNA dependent ATM kinase substrate serine motif **GQ**S**QS* was also identified. The other four serine motifs identified were found to be putative motifs with no known kinase substrates. A putative threonine CDK-like motif *TK/*T*K** was also identified. Motif-X identified two putative Src kinase tyrosine motifs *YGQH** and

Y**G. Following motif identification, each phosphorylation site was then mapped for its relevant kinase. 182 serine/threonine kinases and 63 tyrosine kinases were predicted, giving a total kinome of 245 being altered following inhibition of PP2A by OA (Supplemental table 2, data available on request).

3.3.5 Validation of newly phosphorylated proteins by Western blot

To test for false positives and for validation of the mass spectrometry data, 5 proteins were chosen as a result of their roles in cell migration, proliferation and protein synthesis/translation (moesin, eif3H, VASP, ROCK2 and HYOU1). VASP, eif3H, ROCK2 and HYOU1 were all detected in the OA enriched phosphoprotein fraction while moesin was detected in both untreated and OA exposed fractions (Figure 3.8). As an internal control, corresponding whole cell fractions (WCL) were also probed. Moesin, eif3H, VASP, ROCK2 and HYOU1 were detected in WCL from untreated and OA-exposed samples.

3.3.7 *In silico* protein network analysis of identified PP2A inhibitory phosphoproteome

Gene IDs of all 180 proteins was mapped onto Ingenuity software, of which 178 could be mapped using Ingenuity's database. Using Ingenuity pathway analysis, the top canonical pathways, upstream regulators and biological processes were identified in our network. The top 3 canonical pathways identified were RhoA signalling, signalling by Rho Family GTPases and EIF2 signalling (Table 3.9). 14-3-3-mediated signalling, DNA methylation and transcriptional repression signalling, mTOR signalling and IL-8 signalling were also identified (Supplemental Table 3, data available on request). The top biological processes identified were RNA post-translational modification (*P*=8.67E-07), cellular growth and proliferation (2.93E-06), cell death and survival (P=3.48E-06), protein synthesis (*P*=4.45E-0.6) and cell morphology (P=6.78E-05) (Table 10). Ingenuity pathway analysis was also used to create a network of protein-protein interactions within the newly identified phosphorylated protein data set (Figure 2.9). Within this network, ELAV like RNA binding protein 1 (ELAVL1) was the most connected node with 18 connections (edges). The next most connected node was heat shock 70 kDa protein 4 (HSPA4) with 5 connections. The top canonical pathways identified were Myc (P=4.59E-11), sirolimus (mTOR signalling; P=1.65E-07) insulin receptor signalling (P=2.19E-06), 1,2-dithiol-3-thione (P=6.48E-0.6) and methylprednisolone (P=2.35E-0.5; Table 11). For both Myc and sirolimus, growing out the nodes from a central core, proteins such as lactate dehydrogenase A (LDHA), arginase 1 (ARG-1), stathmin 1 and nucleophosmin (Figure 3.10) were commonalities. Myc was identified as the top up-stream regulator resulting from PP2A inhibition (Figure 3.9A). The diversity of proteins identified is intriguing as they range from enzymes (DNMT1, FASN, ARG1, GLUD1), cytoskeleton proteins (MSN, CTNNA1, NCL), kinases (ROCK2), transcription factors (RUVBL1, RUVBL2, RBBP7, YBX1) and chaperone proteins (CSTB, HSPA14) to proteins associated with

translation (EIF4G1; Figure 3.9A). As Myc was identified as the major upstream regulator *in silico*, we then investigated whether inhibition of PP2A by OA altered c-Myc expression *in vitro*. OA increased c-Myc protein expression in Hep3B cells (Figure 3.9B). This translation from an *in silico* to *in vitro* experimental approach shows the applicability of the *in silico* experiments.

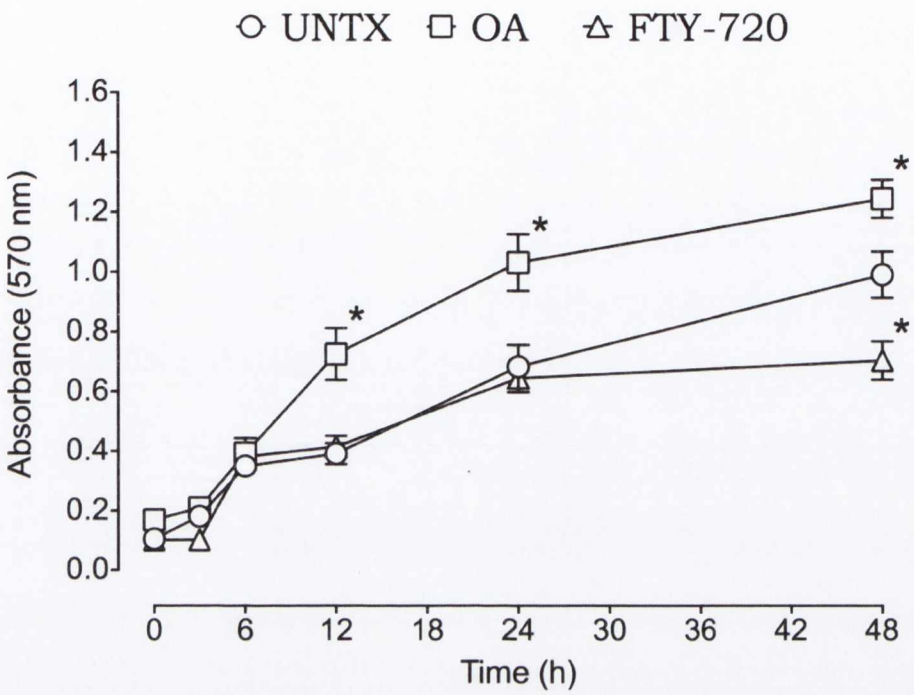


Figure 3.1: Temporal Effect of OA and FTY-720 on Hep3B proliferation Hep3B cells were treated with OA (40 nM) and FTY-720 (2.5 μM) over a 48 h time period and cell proliferation determined using MTT assay. Data were assessed using 2-way ANOVA with *post hoc* analysis (Bonferroni) and are expressed as mean ± S.E.M. * indicates $P < 0.05$ (n=5) compared to UNTX

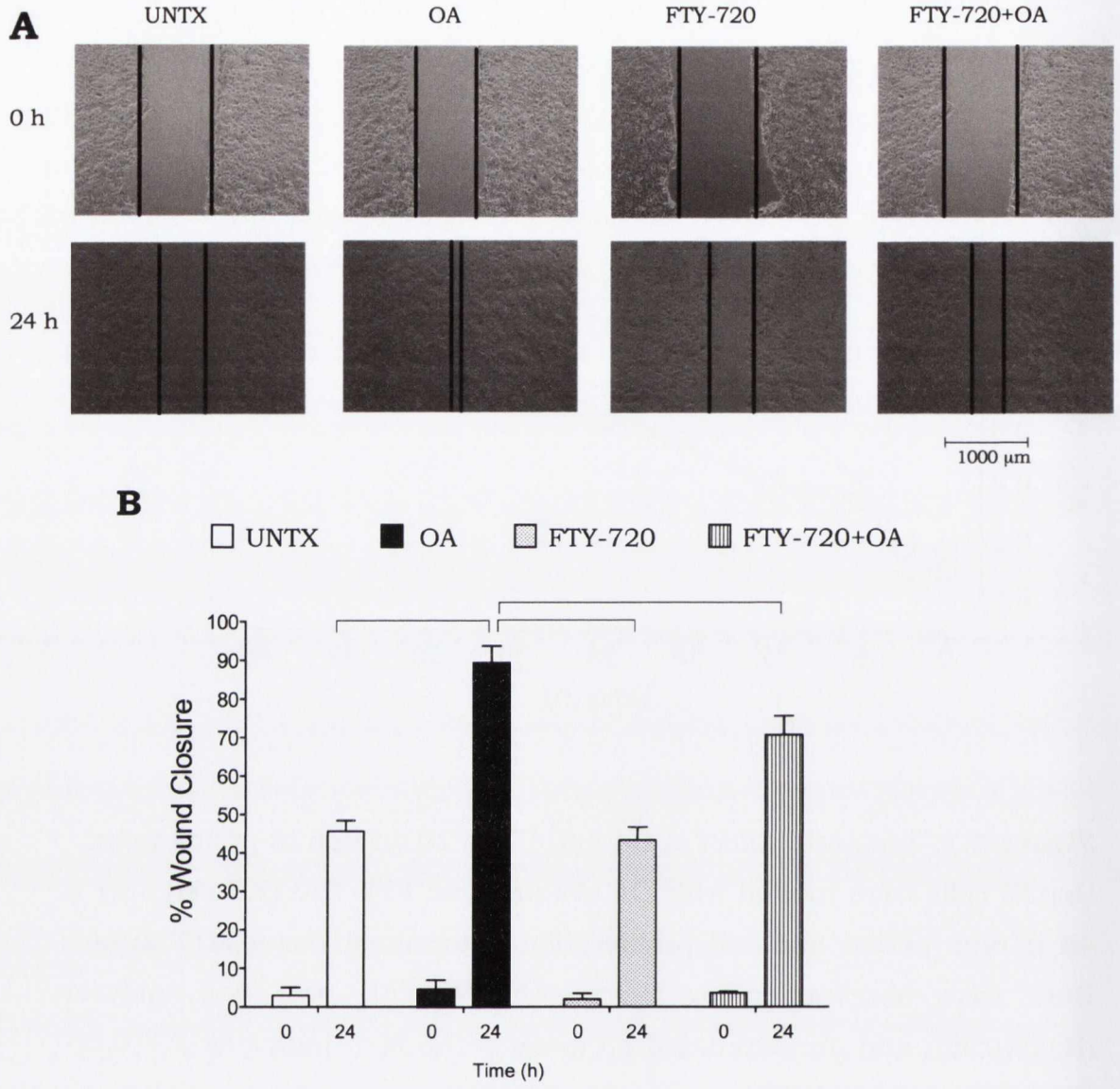


Figure 3.2: Increase in Hep3B cell migration following PP2A inhibition

(A) Scratch assay following Hep3B cell exposure to DMEM, OA (40 nM), FTY-720 (2.5 µM) and FTY-720+OA. (B) Time course of Hep3B cell proliferation following exposure to OA and FTY-720. Images were captured on EVOS imaging system and analysed using ImageJ. Data were analysed using 2-way ANOVA with *post hoc* analysis (Bonferroni) and are expressed as mean ± S.E.M. Horizontal bars represent significance at $P<0.05$ (n=5).

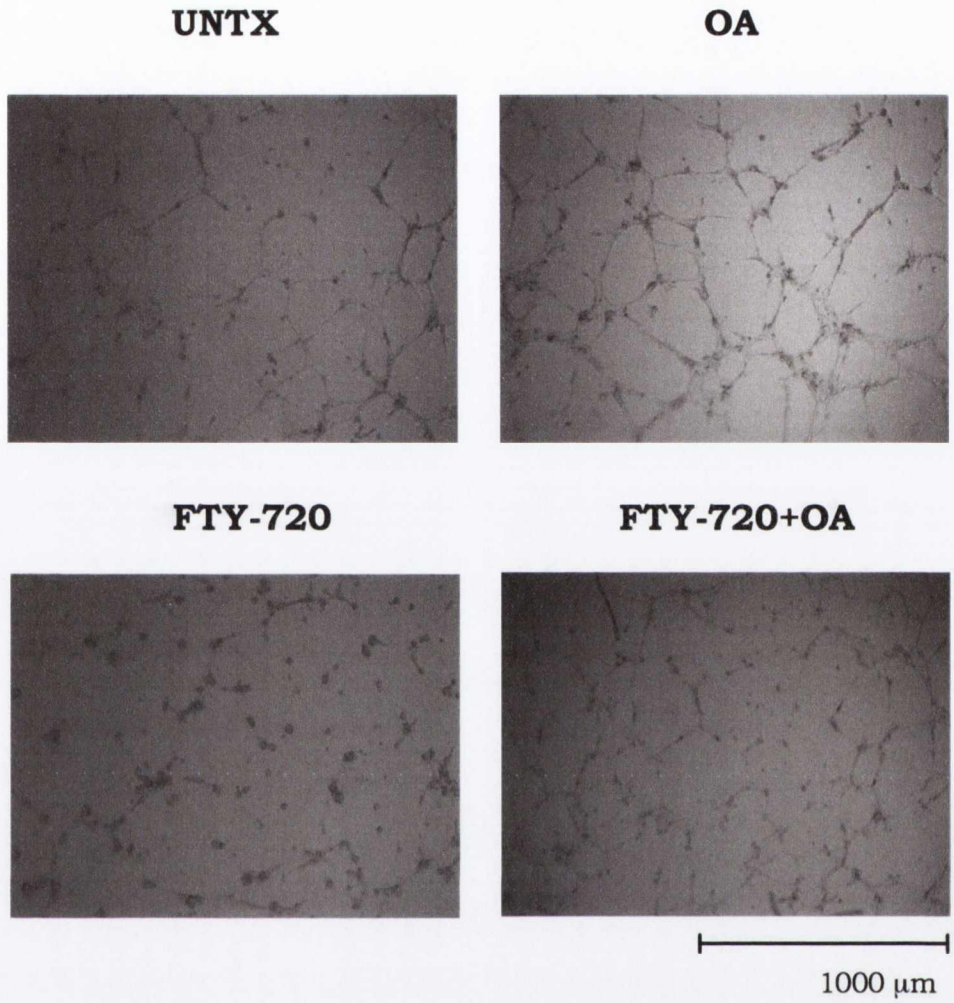


Figure 3.3: The effect of OA and FTY-720 alone and in combination on HUVEC tube formation

Representative images of HUVEC tube formation following PP2A inhibition and activation by pharmacological drugs. HUVEC cells were seed on a matrigel-coated surface and exposed to OA (40 nM), FTY-720 (2.5 μM), OA+FTY-720 or media (UNTX) for 6h. Images were captured on EVOS imaging system (n=3).

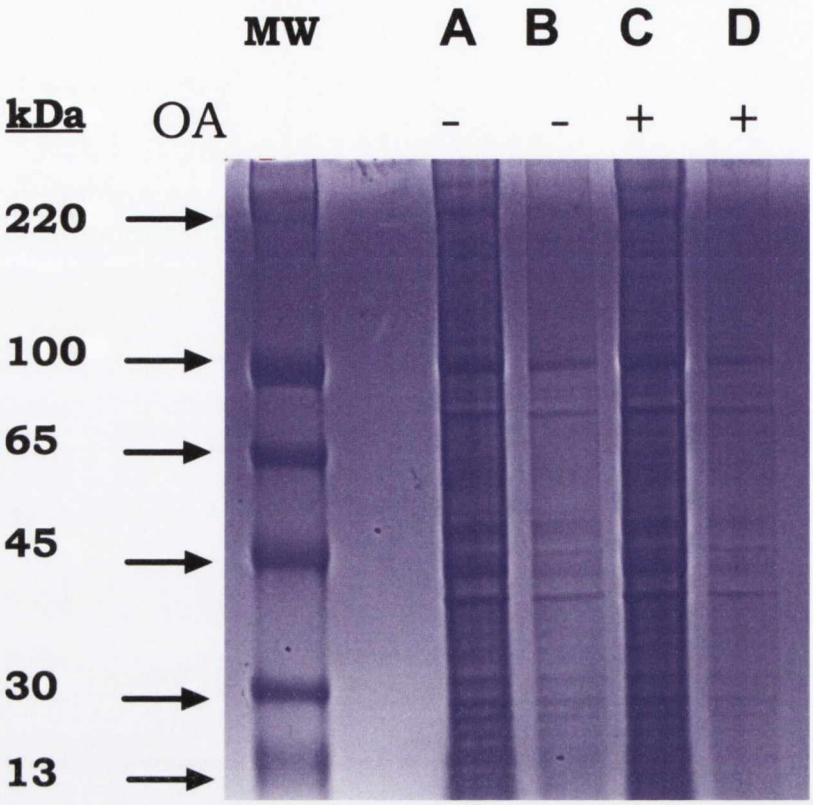


Figure 3.4: SDS-PAGE of un-phosphorylated and phosphorylated proteins following exposure to OA.

Enriched proteins isolated from Hep3B cells were subjected to electrophoresis to separate the complex protein mix and stained with coomassie blue. MW is the molecular weight ladder. (A) Untreated non-phosphorylated fraction, (B) untreated phosphoprotein fraction, (C) OA (40 nM) non-phosphorylated fraction, (D) OA (40 nM) phosphorylated fraction. Gel slices were carefully excised and sent for protein identification by LC/MS/MS.

Table 3.1: Phosphorylated proteins identified following okadaic acid stimulation.

Phosphorylated proteins identified in Hep3B cells following exposure to OA (40 nM) by mass spectrometry. Proteins are listed by their gene ID, Ensemble ID and descriptions as defined by the HUGO Gene Nomenclature Committee [374].

Gene ID	Ensembl ID		Description
DNMT1	ENSG00000130816	DNMT1	DNA (cytosine-5-)-methyltransferase 1
NPM1	ENSG00000181163	NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)
PRDX4	ENSG00000123131	PRDX4	peroxiredoxin 4
FLG2	ENSG00000143520	FLG2	filaggrin family member 2
ARG1	ENSG00000118520	ARG1	arginase 1 [
GANAB	ENSG00000089597	GANAB	glucosidase, alpha; neutral AB
VCP	ENSG00000165280	VCP	valosin containing protein
HSPA4	ENSG00000170606	HSPA4	heat shock 70kDa protein 4
ERC2	ENSG00000187672	ERC2	ELKS/RAB6-interacting/CAST family member 2
NOP2	ENSG00000111641	NOP2	NOP2 nucleolar protein
USP39	ENSG00000168883	USP39	ubiquitin specific peptidase 39
SEPT11	ENSG00000138758	SEPT11	septin 11
PABPN1	ENSG00000100836	PABPN1	poly(A) binding protein, nuclear 1

CORO1C	ENSG00000110880	CORO1C	coronin, actin binding protein, 1C
NCL	ENSG00000115053	NCL	nucleolin
LBR	ENSG00000143815	LBR	lamin B receptor
ETF1	ENSG00000120705	ETF1	eukaryotic translation termination factor 1
RBM8A	ENSG00000265241	RBM8A	RNA binding motif protein 8A
CNN3	ENSG00000117519	CNN3	calponin 3, acidic
HNRNPA3	ENSG00000170144	HNRNPA3	heterogeneous nuclear ribonucleoprotein A3
PDAP1	ENSG00000106244	PDAP1	PDGFA associated protein 1
CHMP4B	ENSG00000101421	CHMP4B	charged multivesicular body protein 4B
PSMD13	ENSG00000185627	PSMD13	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13
SPRR2D	ENSG00000163216	SPRR2D	small proline-rich protein 2D
PLCB3	ENSG00000149782	PLCB3	phospholipase C, beta 3 (phosphatidylinositol-specific)
MSN	ENSG00000147065	MSN	moesin
XRN2	ENSG00000088930	XRN2	5'-3' exoribonuclease 2
USP7	ENSG00000187555	USP7	ubiquitin specific peptidase 7 (herpes virus-associated)
UHRF1	ENSG00000276043	UHRF1	ubiquitin-like with PHD and ring finger domains 1
ZC3H15	ENSG00000065548	ZC3H15	zinc finger CCCH-type containing 15

NT5C2	ENSG00000076685	NT5C2	5'-nucleotidase, cytosolic II
KDM2A	ENSG00000173120	KDM2A	lysine (K)-specific demethylase 2A
PSMD11	ENSG00000108671	PSMD11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11
CLPX	ENSG00000166855	CLPX	caseinolytic mitochondrial matrix peptidase chaperone subunit
MCMBP	ENSG00000197771	MCMBP	minichromosome maintenance complex binding protein
PRPF31	ENSG00000105618	PRPF31	pre-mRNA processing factor 31
PGAM5	ENSG00000247077	PGAM5	phosphoglycerate mutase family member 5
RAN	ENSG00000132341	RAN	RAN, member RAS oncogene family
LDHA	ENSG00000134333	LDHA	lactate dehydrogenase A
PURB	ENSG00000146676	PURB	purine-rich element binding protein B
RAB1B	ENSG00000174903	RAB1B	RAB1B, member RAS oncogene family
MYL12B	ENSG00000118680	MYL12B	myosin, light chain 12B, regulatory
NAGK	ENSG00000124357	NAGK	N-acetylglucosamine kinase
PPFIA1	ENSG00000131626	PPFIA1	protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1
KRT84	ENSG00000161849	KRT84	keratin 84
KRT76	ENSG00000185069	KRT76	keratin 76
RPS27A	ENSG00000143947	RPS27A	ribosomal protein S27a
ACACA	ENSG00000278540	ACACA	acetyl-CoA carboxylase alpha

HYOU1	ENSG00000149428	HYOU1	hypoxia up-regulated 1
CST5	ENSG00000170367	CST5	cystatin D
XP32	ENSG00000198854	C1ORF68	chromosome 1 open reading frame 68
HIST1H2BJ	ENSG00000124635	HIST1H2BJ	histone cluster 1, H2bj
SMARCC1	ENSG00000173473	SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1
SF3B2	ENSG00000087365	SF3B2	splicing factor 3b, subunit 2, 145kDa
BAIAP2L1	ENSG00000006453	BAIAP2L1	BAI1-associated protein 2-like 1
NELFE	ENSG00000204356	NELFE	negative elongation factor complex member E
NCOA5	ENSG00000124160	NCOA5	nuclear receptor coactivator 5
FKBP4	ENSG00000004478	FKBP4	FK506 binding protein 4, 59kDa
RUVBL1	ENSG00000175792	RUVBL1	RuvB-like AAA ATPase 1
KPNA2	ENSG00000182481	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
RTKN	ENSG00000114993	RTKN	rhotekin
DDX47	ENSG00000213782	DDX47	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47
PEG10	ENSG00000242265	PEG10	paternally expressed 10
FAM98A	ENSG00000119812	FAM98A	family with sequence similarity 98, member A
CCDC47	ENSG00000108588	CCDC47	coiled-coil domain containing 47
AMY1A	ENSG00000174876	AMY1B	amylase, alpha 1B (salivary) [Source:HGNC Symbol;Acc:HGNC:475]

AMY1A	ENSG00000237763	AMY1A	amylase, alpha 1A (salivary) [Source:HGNC Symbol;Acc:HGNC:474]
AMY1A	ENSG00000187733	AMY1C	amylase, alpha 1C (salivary) [Source:HGNC Symbol;Acc:HGNC:476]
HMGB3	ENSG00000029993	HMGB3	high mobility group box 3
CCDC124	ENSG00000007080	CCDC124	coiled-coil domain containing 124
PDCD10	ENSG00000114209	PDCD10	programmed cell death 10
ALAD	ENSG00000148218	ALAD	aminolevulinate dehydratase
PSMD7	ENSG00000103035	PSMD7	proteasome (prosome, macropain) 26S subunit, non-ATPase, 7
CHMP3	ENSG00000115561	CHMP3	charged multivesicular body protein 3
AKT1S1	ENSG00000204673	AKT1S1	AKT1 substrate 1 (proline-rich)
SNRNP200	ENSG00000144028	SNRNP200	small nuclear ribonucleoprotein 200kDa (U5)
DSG1	ENSG00000134760	DSG1	desmoglein 1
HYOU1	ENSG00000149428	HYOU1	hypoxia up-regulated 1
TCERG1	ENSG00000113649	TCERG1	transcription elongation regulator 1
SPRR3	ENSG00000163209	SPRR3	small proline-rich protein 3
SMC4	ENSG00000113810	SMC4	structural maintenance of chromosomes 4
EPRS	ENSG00000136628	EPRS	glutamyl-prolyl-tRNA synthetase
CTNNA1	ENSG00000044115	CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa
TUBB6	ENSG00000176014	TUBB6	tubulin, beta 6 class V
TUBA1C	ENSG00000167553	TUBA1C	tubulin, alpha 1c

DDX3X	ENSG00000215301	DDX3X	DEAD (Asp-Glu-Ala-Asp) box helicase 3, X-linked
EIF4A3	ENSG00000141543	EIF4A3	eukaryotic translation initiation factor 4A3
LAD1	ENSG00000159166	LAD1	ladinin 1
VASP	ENSG00000125753	VASP	vasodilator-stimulated phosphoprotein
NFIB	ENSG00000147862	NFIB	nuclear factor I/B
TFG	ENSG00000114354	TFG	TRK-fused gene
PRPF31	ENSG00000105618	PRPF31	pre-mRNA processing factor 31 [Source:HGNC Symbol;Acc:HGNC:15446]
PRPF31	ENSG00000105618	PRPF31	pre-mRNA processing factor 31 [Source:HGNC Symbol;Acc:HGNC:15446]
HSPA14	ENSG00000187522	HSPA14	heat shock 70kDa protein 14
XRCC5	ENSG00000079246	XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)
KATNAL2	ENSG00000167216	KATNAL2	katanin p60 subunit A-like 2
RPL24	ENSG00000114391	RPL24	ribosomal protein L24
MARCKSL1	ENSG00000175130	MARCKSL1	MARCKS-like 1
EIF3H	ENSG00000147677	EIF3H	eukaryotic translation initiation factor 3, subunit H
PCBP1	ENSG00000169564	PCBP1	poly(rC) binding protein 1
STRAP	ENSG00000023734	STRAP	serine/threonine kinase receptor associated protein
RPL14	ENSG00000188846	RPL14	ribosomal protein L14
RPLP0	ENSG00000089157	RPLP0	ribosomal protein, large, P0

YWHAG	ENSG00000170027	YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma
STMN1	ENSG00000117632	STMN1	stathmin 1
LGALS7	ENSG00000178934	LGALS7B	lectin, galactoside-binding, soluble, 7B
LGALS7	ENSG00000205076	LGALS7	lectin, galactoside-binding, soluble,
FASN	ENSG00000169710	FASN	fatty acid synthase
ASCC3	ENSG00000112249	ASCC3	activating signal cointegrator 1 complex subunit 3
HRNR	ENSG00000197915	HRNR	hornerin
CCAR1	ENSG00000060339	CCAR1	cell division cycle and apoptosis regulator 1
CYFIP1	ENSG00000273749	CYFIP1	cytoplasmic FMR1 interacting protein 1
ARHGEF2	ENSG00000116584	ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2
PDCD11	ENSG00000148843	PDCD11	programmed cell death 11
CSTB	ENSG00000160213	CSTB	cystatin B (stefin B)
MCM5	ENSG00000100297	MCM5	minichromosome maintenance complex component 5
CALD1	ENSG00000122786	CALD1	caldesmon 1
ATXN2L	ENSG00000168488	ATXN2L	ataxin 2-like
PARN	ENSG00000140694	PARN	poly(A)-specific ribonuclease
SMTN	ENSG00000183963	SMTN	smoothelin
PAICS	ENSG00000128050	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole

			succinocarboxamide synthetase
SPATS2L	ENSG00000196141	SPATS2L	spermatogenesis associated, serine-rich 2-like
NELFE	ENSG00000204356	NELFE	negative elongation factor complex member E
CD2BP2	ENSG00000169217	CD2BP2	CD2 (cytoplasmic tail) binding protein 2
C22ORF28	N/A	N/A	N/A
KIAA0930	ENSG00000100364	KIAA0930	KIAA0930
PRPF31	ENSG00000105618	PRPF31	pre-mRNA processing factor 31
GTF3C5	ENSG00000148308	GTF3C5	general transcription factor IIIC, polypeptide 5, 63kDa
RBBP7	ENSG00000102054	RBBP7	retinoblastoma binding protein 7
PRKAA1	ENSG00000132356	PRKAA1	protein kinase, AMP-activated, alpha 1 catalytic subunit
DPYSL2	ENSG00000092964	DPYSL2	dihydropyrimidinase-like 2
ASNS	ENSG00000070669	ASNS	asparagine synthetase (glutamine-hydrolyzing)
ZNF207	ENSG00000010244	ZNF207	zinc finger protein 207
PSMA3	ENSG00000100567	PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3
TCEA1	ENSG00000187735	TCEA1	transcription elongation factor A (SII), 1
TALDO1	ENSG00000177156	TALDO1	transaldolase 1
CBX5	ENSG00000094916	CBX5	chromobox homolog 5
DSC1	ENSG00000134765	DSC1	desmocollin 1
KIAA1967	ENSG00000158941	CCAR2	cell cycle and apoptosis regulator 2

LIMA1	ENSG00000050405	LIMA1	LIM domain and actin binding 1
SART3	ENSG00000075856	SART3	squamous cell carcinoma antigen recognized by T cells 3
ARCN1	ENSG00000095139	ARCN1	archain 1
PACSIN3	ENSG00000165912	PACSIN3	protein kinase C and casein kinase substrate in neurons 3
PAK2	ENSG00000180370	PAK2	p21 protein (Cdc42/Rac)-activated kinase 2
NELFE	ENSG00000204356	NELFE	negative elongation factor complex member E
FRMD8	ENSG00000126391	FRMD8	FERM domain containing 8
LRPAP1	ENSG00000163956	LRPAP1	low density lipoprotein receptor-related protein associated protein 1
BCKDK	ENSG00000103507	BCKDK	branched chain ketoacid dehydrogenase kinase
PRPF31	ENSG00000105618	PRPF31	pre-mRNA processing factor 31
CCDC50	ENSG00000152492	CCDC50	coiled-coil domain containing 50
ELAVL1	ENSG00000066044	ELAVL1	ELAV like RNA binding protein 1
PTMS	ENSG00000159335	PTMS	parathymosin
FAM50A	ENSG00000071859	FAM50A	family with sequence similarity 50, member A
PDHB	ENSG00000168291	PDHB	pyruvate dehydrogenase (lipoamide) beta
LGALS7	ENSG00000178934	LGALS7B	lectin, galactoside-binding, soluble, 7B
LGALS7	ENSG00000205076	LGALS7	lectin, galactoside-binding, soluble, 7
PKP1	ENSG00000081277	PKP1	plakophilin 1
SUPT5H	ENSG00000196235	SUPT5H	suppressor of Ty 5 homolog (S. cerevisiae)
ARHGAP35	ENSG00000160007	ARHGAP35	Rho GTPase activating protein 35
FARP1	ENSG00000152767	FARP1	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-

			derived)
AP3B1	ENSG00000132842	AP3B1	adaptor-related protein complex 3, beta 1 subunit
SMARCA1	ENSG00000102038	SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
DDB1	ENSG00000167986	DDB1	damage-specific DNA binding protein 1, 127kDa
TJP2	ENSG00000119139	TJP2	tight junction protein 2
NRD1	ENSG00000078618	NRD1	nardilysin (N-arginine dibasic convertase)
CCT8	ENSG00000156261	CCT8	chaperonin containing TCP1, subunit 8 (theta)
GLUD1	ENSG00000148672	GLUD1	glutamate dehydrogenase 1
RUVBL2	ENSG00000183207	RUVBL2	RuvB-like AAA ATPase 2
PDCD4	ENSG00000150593	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
YBX1	ENSG00000065978	YBX1	Y box binding protein 1
VPS4A	ENSG00000132612	VPS4A	vacuolar protein sorting 4 homolog A (S. cerevisiae)
ABI1	ENSG00000136754	ABI1	abl-interactor 1 [
ARFGAP2	ENSG00000149182	ARFGAP2	ADP-ribosylation factor GTPase activating protein 2
BZW2	ENSG00000136261	BZW2	basic leucine zipper and W2 domains 2
BZW1	ENSG00000082153	BZW1	basic leucine zipper and W2 domains 1
AAMP	ENSG00000127837	AAMP	angio-associated, migratory cell protein
POLR2E	ENSG00000099817	POLR2E	polymerase (RNA) II (DNA directed) polypeptide E, 25kDa

TMOD3	ENSG00000138594	TMOD3	tropomodulin 3 (ubiquitous)
EIF4G1	ENSG00000114867	EIF4G1	eukaryotic translation initiation factor 4 gamma, 1
TGM3	ENSG00000125780	TGM3	transglutaminase 3
CHD4	ENSG00000111642	CHD4	chromodomain helicase DNA binding protein 4
ROCK2	ENSG00000134318	ROCK2	Rho-associated, coiled-coil containing protein kinase 2
SPRR1A	ENSG00000169474	SPRR1A	small proline-rich protein 1A
POGZ	ENSG00000143442	POGZ	pogo transposable element with ZNF domain
SF3B3	ENSG00000189091	SF3B3	splicing factor 3b, subunit 3, 130kDa
SUMO3	ENSG00000184900	SUMO3	small ubiquitin-like modifier 3
SEPT10	ENSG00000186522	SEPT10	septin 10
HNRNPF	ENSG00000169813	HNRNPF	heterogeneous nuclear ribonucleoprotein F
PTPN11	ENSG00000179295	PTPN11	protein tyrosine phosphatase, non-receptor type 11
AKT2	ENSG00000105221	AKT2	v-akt murine thymoma viral oncogene homolog 2
DUSP9	ENSG00000130829	DUSP9	dual specificity phosphatase 9
PSMD14	ENSG00000115233	PSMD14	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14

Table 3.1: Phosphorylated proteins identified following okadaic acid stimulation

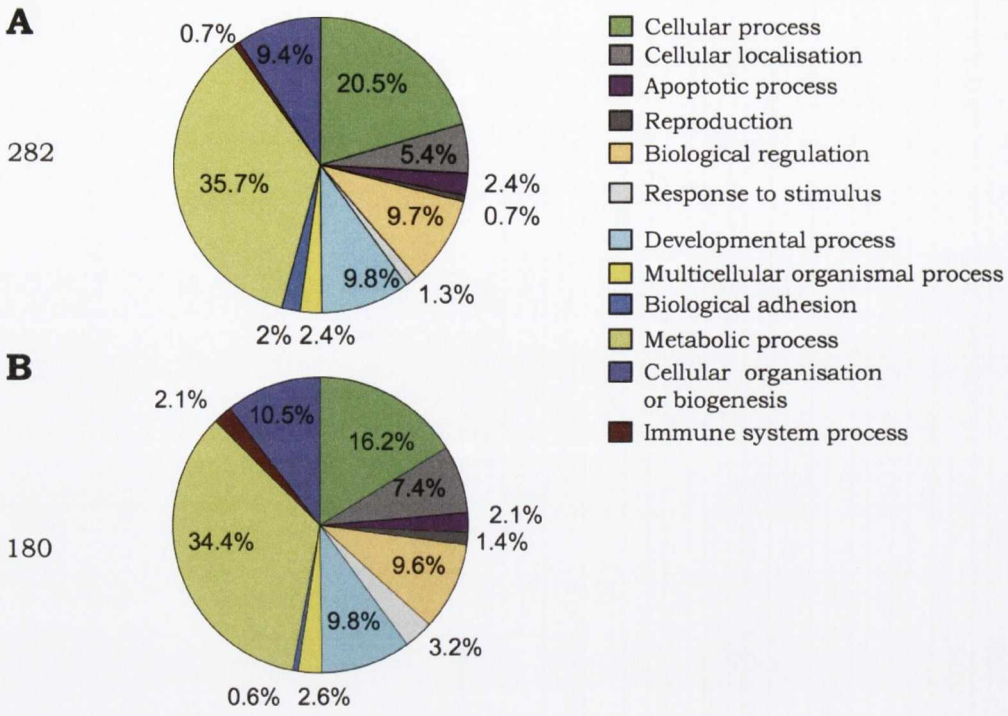


Figure 3.5: Gene ontology of Phosphoproteins identified

Following MASCOT peptide identification, newly identified proteins were subjected to PANTER gene ontology for functional identification. (A) Phosphoproteins identified Hep3B cells under basal conditions and their biological functions. (B) Phosphoproteins identified in OA exposed Hep3B cells and their biological function.

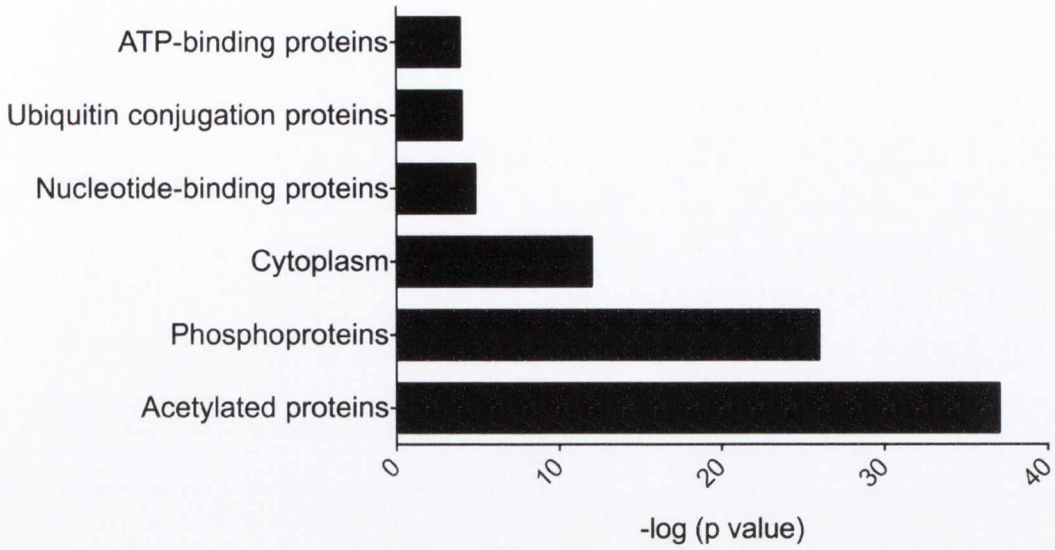


Figure 3.6: Protein ontology of newly identified proteins as determined using DAVID following exposure to okadaic acid

List of newly identified proteins following exposure to okadaic acid ($P<0.005$) as identified using DAVID protein ontology analysis.

Pathway Name	p-Value
Gene Expression	P=9.61E-17
Plasma membrane oestrogen receptor signalling	P=5.19E-16
Thrombin/protease-activated receptor (PAR) pathway	P=1.85E-15
PAR1-mediated thrombin signalling events	P=1.85E-15
Endothelins	P=1.85E-15
GMCSF-mediated signalling events	P=1.89E-15
IGF1 pathway	P=1.89E-15
EGF receptor (ErbB1) signalling pathway	P=1.89E-15
mTOR signalling pathway	P=1.89E-15
Arf6 signalling events	P=1.89E-15

Table 3.2: Pathways identified by WebGestalt.

List of pathways associated with the phosphoproteins identified following exposure to OA in Hep3B cells that are statistically over-represented using WebGestalt software. Criteria: at least 10 genes represented in the pathway and adjusted P value ≤0.05 (hypergeometric *t* test with P value adjusted by multiple test adjustment, Benjamini and Hochberg [375]).

Table 3.3: List of phosphoproteins identified with phosphorylation sites

List of proteins identified with phosphorylation sites predicted using GPS 3.0. Phosphoproteins were stratified based on their phosphorylation specificity for serine/threonine, tyrosine, or serine/threonine/tyrosine. 112 proteins were identified as having experimentally proven phosphorylation sites.

Proteins with identified phosphorylation sites			**Serine/Threonine/ Tyrosine**	**Serine/Threonine**	**Tyrosine**	
ABI1	HNRNPF	RPL24	ABI1	AMPKa1	AP3B1	RBBP7
ACACA	HRNR	RPS27A	ACACA	CCT8	ARG1	SART3
AKT1S1	HSPA4	RTKN	AKT1S1	EIF4G1	CBX5	SMARCA1
AKT2	HYOU1	RUVBL1	AKT2	FKBP4	CCAR1	TALDO1
AMPKa1	KPNA2	RUVBL2	ARHGEF2	LBR	CCDC50	TCERG1
AP3B1	KRT3	SART3	CALD1	LRPAP1	CD2BP2	TGM3
ARG1	LBR	SMARCA1	CHD4	PDCD10	CNN3	TJP2
ARHGEF2	LIMA1	SMARCC1	DDB1	RPL14	CSTB	VPS24
CALD1	LRPAP1	STMN1	DPYSL2	RPL24	CTNNA1	VPS4A

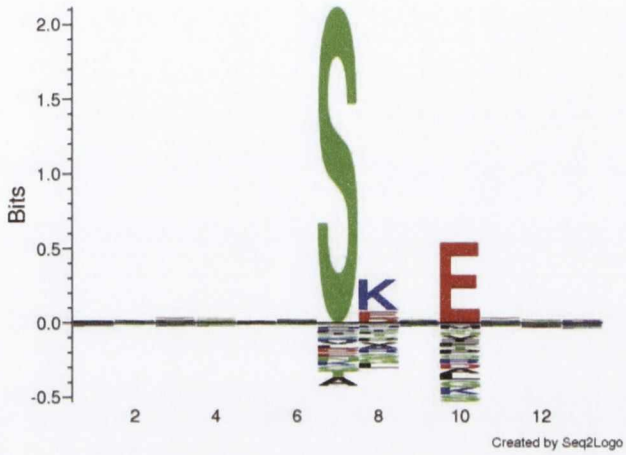
CBX5	MCM5	STRAP	EIF3S3	RTKN	CYFIP1	EIF3H
CCAR1	MSN	SUPT5H	EPRS	RUVBL2	DKFZ	
CCDC50	MYL12B	TALDO1	FASN	STMN1	DNMT1	
CCT8	NCL	TCEA1	GLUD1	STRAP	DSG1	
CD2BP2	NFIB	TCERG1	HSPA4	TCEA1	DUSP9	
CHD4	NOP2	TFG	HYOU1	TMOD3	EIF4A3	
CNN3	NPM1	TGM3	KPNA2	TUBB8	ETF1	
CSTB	NRD1	TJP2	LIMA1	USP39	FAM50A	
CTNNA1	PACSIN3	TMOD3	MSN		FLG2	
CYFIP1	PAICS	TUBB6	NCL		FRMD8	
DDB1	PAK2	TUBB8	NOP2		GRLF1	
DKFZp686M2119	PARN	USP39	NPM1		HNRNPF	
DNMT1	PDAP1	VASP	PACSIN3		HRNR	
DPYSL2	PDCD10	VCP	PAK2		KRT3	
DSG1	PDCD4	VPS24	PLCB3		MCM5	
DUSP9	PEG10	VPS4A	PSMA3		MYL12B	
EIF3S3	PKP1	XRCC5	ROCK2		NFIB	
EIF4A3	PLCB3	YBX1	RPS27A		NRD1	
EIF4G1	PPFIA1	YWHAG	RUVBL1		PAICS	

EPRS	PRDX4	SMARCC1	PARN
ETF1	PRPF31	SUPT5H	PDAP1
FAM50A	PSMA3	TFG	PDCD4
FASN	PTPN11	TUBB6	PEG10
FKBP4	RAB1B	VASP	PKP1
FLG2	RBBP7	VCP	PPFIA1
FRMD8	ROCK2	XRCC5	PRDX4
GLUD1	RPL14	YBX1	PRPF31
GRLF1	EIF3H	YWHAG	PTPN11
		EIF3H	RAB1B

	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
1	.....S..E...	9.91	451	5027	72620	1094911	1.35
2	....Q.SG.GQ..	49.37	21	4576	44	1022291	106.62
3	GQH...S.Q....	47.17	26	4555	48	1022247	121.56
4	..GQ..S...QS.	48.83	23	4529	58	1022199	89.5
5	.....SK.....	6.14	274	4506	46081	1022141	1.35
6	..G...S.....H	17.59	40	4232	1996	976060	4.62

Table 3.4: Serine phosphorylation Motifs identified by Motif-x software.

Serine phosphorylation motifs identified following exposure to okadaic acid (40 nM). Protein sequences were inputted in FASTA sequences and searched for (S) as the central amino acid. Significance was taken as P=0.00001 with International Protein Index database used as a reference for clustering.



Single-letter code	Full name
A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
Q	Glutamine
E	Glutamic acid
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

Figure 3.7: Amino acid frequency in serine phosphorylation motifs.

Amino acid distribution surrounding phosphorylated serine residues as predicted using Motif-X. Enrichment of serine amino acids at the central position is shown and the frequency of amino acid occurrence calculated and given P-values plotted as a P-weighted Kullback-Leibler graph with Hobohm clustering [376].

	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
1	.....T.K....	7.09	426	6241	36237	687043	1.29
2	.....TK.....	6.02	345	5815	29717	650806	1.3

Table 3.5: Threonine Phosphorylation Motifs identified by Motif-X.

Threonine phosphorylation motifs identified following exposure to okadaic acid (40 nM). Protein sequences were inputted in FASTA sequences and with central amino acid character threonine (T) searched. Significance was taken as P=0.00001 with International Protein Index database used as a reference for clustering.

	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
1	.....YGQH...	36.28	25	3232	43	322611	58.03
2	.....Y....G.	7.08	292	3207	21515	322568	1.37

Table 3.6: Tyrosine Phosphorylation Motifs identified by Motif-X

Tyrosine phosphorylation motifs identified following exposure to okadaic acid (40 nM). Protein sequences were inputted in FASTA sequences and with central amino acid character tyrosine (Y) searched. Significance was taken as P=0.00001 with International Protein Index database used as a reference for clustering.

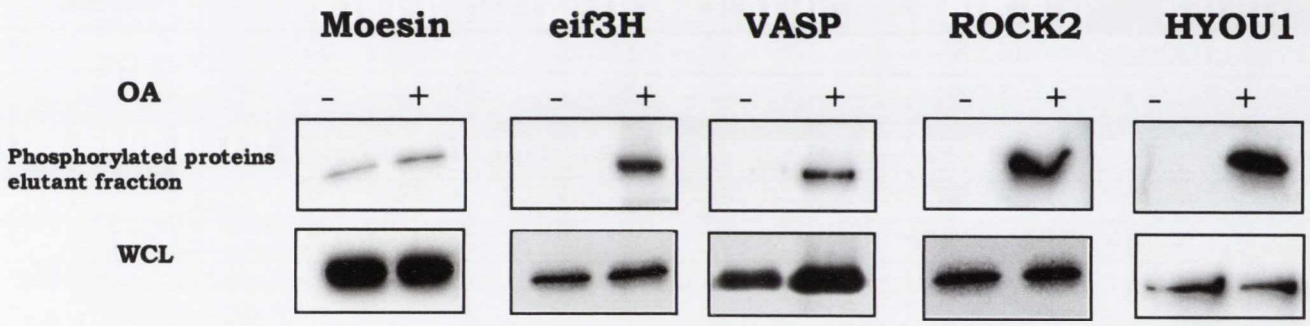


Figure 3.8: Conformation of MS/MS protein identification by western blot

Serum starved Hep3B cells was exposed to media or OA (40 nM) for 24 h. Phosphorylated and non-phosphorylated proteins were isolated as described above in "Materials and Methods". Phospho-cell lysates and non-phosphorylated proteins (1 μg) were Western blotted (WB) and probed against anti-moesin, anti-eif3H, anti-VASP, andti-ROCK2 and anti-HYOU1.

Top Canonical Pathways	-log(p-value)	Ratio	Overlaps with dataset	Molecules
RhoA Signalling	4.01E00	5.74E-02	7/122 (6%)	SEPT11, MYL12B, SEPT10, ARHGAP35, MSN, RTKN, ROCK2
Signalling by Rho Family GTPases	3.66E00	3.85E-02	9/234 (4%)	PAK2, CYFIP1, SEPT11, ARHGEF2, MYL12B, SEPT10, MSN, ROCK2, STMN1
EIF2 Signalling	3.65E00	4.32E-02	8/185 (4%)	RPL24, EIF3H, RPS27A, EIF4A3, RPL14, AKT2, RPLP0, EIF4G1
Protein Ubiquitination Pathway	3.39E00	3.53E-02	9/255 (4%)	HSPA4, USP39, PSMD7, PSMD14, PSMD13, PSMA3, USP7, HSPA14, PSMD11
14-3-3-mediated Signalling	3.25E00	5.13E-02	6/117 (5%)	AKT1S1, YWHAG, TUBB6, PLCB3, TUBA1C, AKT2
DNA Methylation and Transcriptional Repression Signalling	3.18E00	1.5E-01	3/20 (15%)	CHD4, DNMT1, RBBP7
mTOR Signalling	2.88E00	3.72E-02	7/188 (4%)	AKT1S1, PRKAA1, EIF3H, RPS27A, EIF4A3, AKT2, EIF4G1
Axonal Guidance Signalling	2.82E00	2.54E-02	11/433 (3%)	PAK2, PTPN11, TUBB6, MYL12B, VASP, PLCB3, PSMD14, DPYSL2, TUBA1C, ROCK2, AKT2

Table 3.7: List of top canonical pathways identified using Ingenuity Pathway Analysis.

Top 8 pathways were identified from the phosphorylated proteins identified following PP2A inhibition. Validated proteins ROCK2, HYOU1, VASP, EIF3H and MSN were all identified in the top pathway.

Top Upstream Regulators	
Upstream Regulator	***P*-value**
Myc	4.59E-11
Sirolimus	1.65E-07
INSR	2.19E-06
1,2-dithiol-3-thione	6.48E-0.6
Methylprednisolone	2.35E-0.5

Table 3.8: List of top 5 upstream regulators identified by Ingenuity Pathway analysis.

Top Biological Processes		
Name	**Number of Proteins**	***P*-value**
RNA post-translational modification	15	8.67E-07
Cellular growth and proliferation	71	2.93E-06
Cell death and survival	71	3.48E-06
Protein synthesis	29	4.45E-0.6
Cell morphology	49	6.78E-05

Table 3.9: List of top 5 biological processes identified by Ingenuity Pathway analysis.

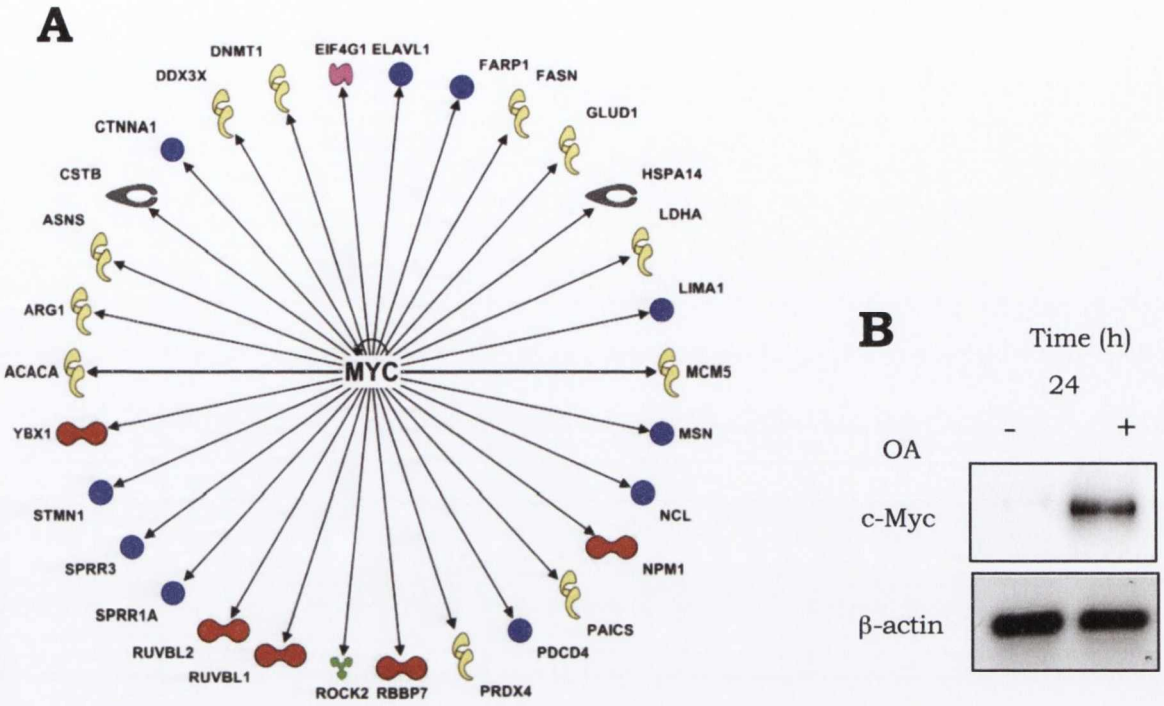


Figure 3.9: Myc protein interaction network

(A) Ingenuity pathway analysis identified network of proteins that were all regulated by Myc. The network is displayed graphically as nodes and Myc identified as the central upstream regulator. (B) Expression of c-Myc protein in Hep3B cells following exposure to OA (40 nM; n=4) determined by Western Blotting.

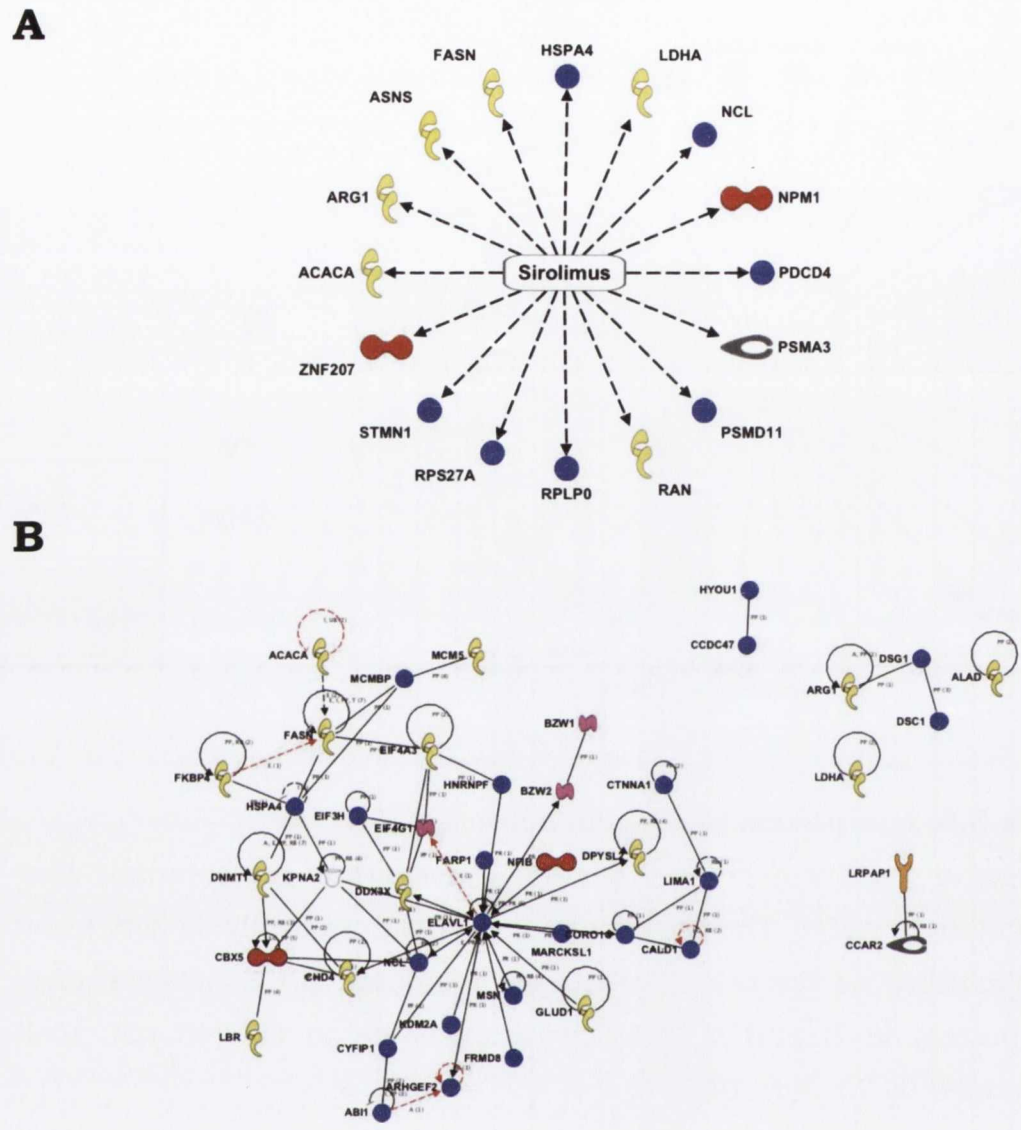


Figure 3.10: (A) Sirolimus protein interaction network and (B) IPA protein-protein interaction network with ELAV1 as the central node

3.4 Discussion

The unrestricted growth of transformed cells is caused by the cumulative dysregulation of multiple cellular pathways involved in normal growth control. Kinases form an intrinsic part of the oncogenic process, turning on and off important cell homeostasis proteins through modification of proteins by phosphorylation. To counter-balance this, relatively few protein phosphatases catalyse the dephosphorylation processes, with just two serine/threonine phosphatase family members, PP2A and PP1, controlling 90% of all serine/threonine dephosphorylation events [377]. This present study highlights the diversity of phosphoproteins phosphorylated following exposure to the serine/threonine phosphatases inhibitor okadaic acid at concentrations selective for PP2A inhibition [361].

The identification of proteins undergoing both serine/threonine and tyrosine phosphorylation in response to PP2A inhibition is essential for understanding the molecular mechanisms and relevant signalling pathways that are deregulated in cancers with reduced PP2A activity. Our study highlights the functional consequences of reduced PP2A activity; an increase in cell proliferation, migration and the increase ability of endothelial cells to form tubules. Previous studies have found okadaic acid to increase cell proliferation at sub-lethal dosages in gastric and colon epithelial cell lines [378]. The increase in cell proliferation resulting from OA exposure was due to the increased expression of cyclin B1 and phosphorylation of threonine 308 on AKT. Regarding okadaic acid and angiogenesis, we found low concentration OA increases HUVEC tube formation, while FTY-720 decreased tube formation. This is in keeping with previous work that found low concentration of FTY-720 decreases HUVEC tube formation and also cell invasion in hepatocellular carcinoma [379]. Using a novel phosphoprotein purification method and state of the art mass spectrometry we investigated the molecular mechanisms that underline

these processes. 282 phosphorylated proteins were identified, while 180 newly phosphorylated proteins were identified in response to okadaic acid in a cell line used to study HCC, Hep3B. This is in keeping with recent studies using okadaic acid as a tool to detect phosphoproteins [380]. Using the TALON phosphoprotein enrichment method however, had its limitations. Many transient low phosphorylation proteins were not detected such as those associated with MAPK, ERK and JNK signalling. This perhaps is due to the sensitivity of the enrichment process and maybe overcome using a TiO_2 phosphopeptide enrichment method or using an iTRAQ labelling method. However, due to the cost limitations, this could not be achieved with this present study. From this, we sought to characterise the proteins identified using bioinformatics, and elucidate if an overall regulator of phosphorylated proteins could be identified.

Gene ontology highlighted the diversity of the phosphorylated proteins identified by mass spectrometry, with metabolic process, cellular processes and cellular organisation the most common ontologies featured. Further data mining using DAVID protein function ontology found acetylated proteins, phosphoproteins and cytoplasmic proteins, nucleotide-binding proteins and ATP-binding proteins to be the most abundant protein type. It is not surprising that metabolic processes make up 34.4% of the phosphoproteins identified following OA exposure considering the importance of phosphorylation events in the regulation of cellular metabolism [381, 382] and cellular processes [383]. The identification of acetylation as the most statistically significant protein type (98 proteins were classified as being acetylated), however, this was not expected. The increase in phosphorylation following okadaic acid exposure may exert pleiotropic effects on protein abundance, which may alter the acetylation state of many of these proteins. Studies of histone post-translational modification cross-talk have identified alterations of secondary *cis* (intramolecular) modifications, such as the phosphorylation of S10 on histone H3 increasing the acetylation on the

Lys-14 residue [384]. This study found phosphorylation and acetylation to be synergistically linked, with EGF stimulation of C3H 10 T1/2 cells resulting in a rapid and sequential phosphorylation and acetylation of various histone associated proteins [384, 385]. Indeed, previous studies have implicated protein phosphatases in the regulation of acetylation. PP1 dependent phosphorylation of CREB at S133 promotes the recruitment of co-activator histone acetylase (HAT) paralogs CBP and P300, which in turn stimulate acetylation of promoter-bound histones [386]. Studies have also found that PP2A interacts with histone deacetylase proteins such as HDAC3 and HDAC6 in the regulation of STAT3 signalling [387, 388]. The authors postulate whether okadaic acid exposure and subsequent loss of PP2A activity is somewhat involved the regulation of protein acetylation and other post-translational modifications. Supporting this, okadaic acid exposure resulted in the phosphorylation of DNMT1 (DNA methyltransferase 1). Previous studies have indicated the loss off the catalytic subunit of PP2A increases DNMT1 activity and subsequent up-regulation of genes associated with methylation [389]. Our data indicates that PP2A inhibition may result in not only the phosphorylation of proteins but also in their acetylation and methylation.

Pathways associated with gene expression and receptor kinase signalling following OA exposure included oestrogen receptor, PAR pathway, GM-CSF, IGF and EGF signalling. Many of these pathways are current targets for pharmacological intervention in a variety of tumours, especially solid form tumours such as HCC and Breast cancer [390-393].

The mass spectrometry data were validated in a select number of proteins in keeping with our overall aims of identifying proteins involved in cell proliferation and migration following loss of PP2A activity. Eif3H, VASP, ROCK2, Moesin and HYOU1 were all confirmed to be present in our phosphoprotein-enriched sample following OA exposure compared to untreated samples. Moesin (MSN), belongs to a family of proteins

called ERM (ezrin/radixin/moesin) proteins that are involved in coupling actin filaments to the plasma membrane [394]. Moesin functions as a cytoskeletal linker, working in an elaborate regulatory network of many cellular processes including cell migration and metastasis. Moesin has been found phosphorylated at T588 by numerous protein kinases, primarily PKC and ROCK [395, 396]. Rho-ROCK phosphorylation of moesin has also been found to play a central role in Rho- and Rac-induced reorganization of the cytoskeleton [397]. Moesin has been shown to be critical for melanoma cell invasion in the lung in 3D culture models, controlling not only cell polarity but also adhesion dependent activation of Rho and subsequent myosin II contractility [398]. As well as having a role in tumour cell invasion, moesin has also been implicated in EMT. In a human mammary cell line MCF-10A, moesin was found to be essential for EMT, relocalising from cell–cell adhesions to filopodia of the cell [399]. Moreover, this study suggested that ROCK is responsible for the increase of phosphorylated moesin during EMT. As PP2A inhibition has been found to stimulate ROCK kinase activity, exposure to okadaic may induce EMT in Hep3B cells through the activation of ROCK and subsequent phosphorylation of moesin. Interestingly, moesin was detected in both untreated and OA exposed samples. This could be due to limitation of the enrichment process, which is unlikely, as other proteins were not detected in untreated enriched samples. More likely, it exists in a phosphorylated state under basal/unstimulated conditions. This situation arises from the fact that antibodies against unphosphorylated epitopes were used for the Western blot analysis. Although this it may be considered a limitation of our approach, using epitope-independent phosphoprotein enrichment more than compensates for this.

Eif3H is a member of the eukaryotic translation initiation factor family (eIF), which is involved in the initiation phase of eukaryotic translation [400]. We also identified phosphorylated eif4G1 and eif4A3 using mass spectrometry following exposure to OA. Previous studies have indicated

a role for PP2A in the dephosphorylation of another member of the eIF family, eif4E [401, 402]. Our study extends this to show that inhibition of PP2A by okadaic acid results in phosphorylation of other members of the eukaryotic translation initiation factor family. Taken together, these data support PP2A negatively regulating eIF phosphorylation and eIF complex assembly through dephosphorylation mechanisms, highlighting a potential novel mechanism by which PP2A exerts its tumour-suppressive function.

With regards to the focal adhesion protein VASP, a link protein between signal transduction pathways and the microfilament system, OA exposure resulted in its phosphorylation in Hep3B cells. This is consistent with high concentrations of OA increasing VASP phosphorylation in microglial cells, which reduces retraction of membrane ruffles and chemotaxis [403]. OA also increases VASP phosphorylation in platelets [404] in a PP2A specific manner; PP1 was found to have no role. The effect was so pronounced in platelets that the authors commented that VASP phosphorylation was a very sensitive marker of OA action in human platelets. However, neither of these studies report OA mediated increase in total VASP protein expression, which was found in this study.

ROCK2 is a key regulator of the actin cytoskeleton and cell polarity and is involved in regulation of smooth muscle contraction, actin cytoskeleton organization, stress fibre and focal adhesion formation, neurite retraction, cell adhesion and motility via phosphorylation of various oncogenic proteins [405, 406]. In endothelial cells, the PP2A regulatory subunit Bα controls cell contractility through a RhoA dependent mechanisms [407]. The identification of phosphorylated ROCK2 and the identification of RhoA and Rho family GTPase signalling as the top canonical pathway altered by OA, would support PP2A dependent dephosphorylation of RhoA/ROCK signalling being critical in the biology of solid tumours [405, 408].

HYOU1 is a heat shock protein that serves as a molecular chaperone of the endoplasmic reticulum during environmental stress, particularly during oxygen deprivation [409]. In the present study, we found HYOU1 to be phosphorylated following okadaic acid-PP2A inhibition, leading us to speculate that HYOU1 is involved in OA induced cell proliferation and migration in Hep3B cells.

Given the large number of phosphorylation sites that are known today, it is difficult to identify phosphorylation motifs by a simple manual inspection of the sequences surrounding the phosphorylated residues. As well as this, prediction of potential phosphorylation sites is complex with amino acid conformation being crucial. Although there are a number of programs available for identifying sequence patterns from sequence data, most of them are not specifically designed to identify phosphorylation motifs *per se*. Motif-x is a web-based program that uses an iterative statistical approach to identify over-represented patterns from any sequence dataset and has recently been used for predicting phosphorylation motifs [370]. We used PhosphoSitePlus and GPS 3.0 to predict and verify phosphorylation sites of our identified phosphorylated proteins. As we were more interested in serine/threonine phosphorylation events, a greater emphasis was put towards its analysis. Using iGPS and PhosphoSitePlus, we were able to identify 62% of our proteins as haven experimentally proven phosphorylation sites; this differs from DAVID gene ontology, which predicted 80% phosphoproteins. Motif-x identified six putative serine phosphorylation motifs following bioinformatics analysis of the phosphorylated protein's sequences. Of these six serine motifs, only two had known kinase substrates, **S**E (pSX[E/pS*/pT*], Casein Kinase II substrate motif) and **GQ**S**QS* (pSX[E/pS*/pT*] DNA dependent Protein kinase substrate motif, ATM kinase substrate motif, Casein Kinase II substrate motif) [410]. The Casein Kinase II (CK2) motifs are well established for being CK2 substrates [411]. A closer look at the motif **S**E reveals that this motif displays an acidic nature, which

make it more likely to be phosphorylated by acidic kinases such as CK2 [412]. Protein kinase CK2 phosphorylates and up-regulates Akt/PKB, and is a key player in promoting cell proliferation and anti-apoptosis events [413]. Following a loss of PP2A activity, over representation of a CK2 motif suggests activation and subsequent phosphorylation of CK2 substrate sites promoting a PP2A/OA mediated increase in cell proliferation and migration. Though a CK2 phosphorylation motif was enriched, iGPS kinase prediction software identified other kinase signature phosphorylation sites following OA exposure. Many of these kinases fall in family categories such as PKA, PKB (AKT), PKC, CDK, ERK, MAPK, JNK, EGFR, Eph, FGFR and PDGF. PP2A regulates many of these pathways, underpinning its potential as a "master regulator of kinase activity" [129, 414, 415].

Although bioinformatics and systems biology was important for identifying potential kinase networks activated following PP2A inhibition, we also found some discrepancies in the databases and between the software packages used. For example, while the glycolytic enzyme lactate dehydrogenase A (LDH-A) was found to be phosphorylated following mass spectrometry neither, PhosphoSitePlus, scan-x or GPS 3.0 were able to identify its phosphorylation sites. This is in contrast to a recent study showing tyrosine phosphorylation of LDH-A on residue T10 by FGFR1 kinase [416]. An increase in LDH-A phosphorylation results in an increase in lactic acid production which is a by-product of glycolysis and abnormal cell proliferation in tumours [3]. Our findings suggest that inhibition on PP2A promotes LDH-A phosphorylation and an increase in lactic acid production.

Following on from the bioinformatics results and phosphorylation site predictions we use Ingenuity Pathway Analysis to construct interactive networks of our phosphorylated proteins in order to identify top canonical pathways and upstream regulators that control our phosphorylated proteins. RhoA signalling, signalling by Rho Family GTPase, EIF2 signalling, 14-3-3 mediated signalling, DNA methylation

signalling and mTOR signalling were all identified as top canonical pathways altered by PP2A inhibition. Rho GTPases are activated by various stimuli including GPCR activation, RTK activation and ion channel depolarisation [417, 418]. They control multiple cellular processes, including actin and microtubule dynamics, gene expression, cell cycle, proliferation and cell polarity through their ability to bind to numerous down stream effectors, which leads to diverse signalling pathway activation [419, 420]. It was therefore not surprising that Rho signalling was the top canonical pathway identified following OA exposure and subsequent PP2A inhibition. Previous studies have shown that okadaic acid influences cell migration and proliferation through phosphorylation and translocation of myosin phosphatase target subunit 1 and influences myosin phosphorylation, stress fibre assembly and cell migration in HepG2 cells and is dependent on Rho signalling [421]. Regulatory subunits of PP2A have been found to positively influence Ras-MAPK signalling events [422] and more recently PP2A has been linked to Ras/Myc mediated upregulation of cyclin E during G2 and thus the continuation of stable G1 through the inhibition of quiescent G0 [131].

EIF2 signalling was a surprising canonical pathway to be altered following inhibition of PP2A by OA. As postulated previously, PP2A negatively regulates eIF phosphorylation and eIF complex assembly through dephosphorylation mechanisms thus resulting in EIF2 signalling's prominent position. Other phosphorylated proteins associated with this pathway include the 60S ribosomal protein L family of proteins RPL0, RPL14, RPL24 and the ribosomal protein S27a, RP327A. Genes in the RPL family provide instructions for making some of the approximately 80 different ribosomal proteins and are essential in EIF2 mediated signalling and the translational of new proteins from mRNA. The phosphorylation of these proteins is indicative of cell proliferation, where new protein synthesis resulting from an increase in translational is needed to sustain cell growth [423-425].

The identification of ELAVL1 as central to the phosphorylated protein interaction node network is noteworthy. ELAVL1 is a member of the ELAVL family of RNA-binding proteins that contain several RNA recognition motifs, and selectively bind AU-rich elements (AREs) found in the 3' untranslated regions of mRNAs, stabilises mRNA and promotes gene expression [426]. AREs signal degradation of mRNAs as a means to regulate gene expression, thus by binding AREs, the ELAVL family of proteins play a role in stabilizing ARE-containing mRNAs. Phosphorylation of ELAVL1 at S158 and S221 by PKC influences its subcellular distribution by facilitating its export to the cytosol [427]. Here it can influence gene expression by aiding in oncogene mRNA stability. Previous studies have shown that PP2A catalytic subunit knockdown and exposure to OA leads to increased mRNA stability and gene expression [346, 428]. Thus, okadaic acid inhibited PP2A may play a role in ERAVL1 mediated posttranscriptional gene regulatory mechanisms.

Both the oncogene Myc and the mTOR inhibitor sirolimus (rapamycin) were identified as the top upstream regulators following Hep3B exposure to okadaic acid. In the case of Myc, exposure to OA resulted in the up-regulation of c-Myc at the protein level in Hep3B cells and was detected by Western blot. Previous studies have shown that OA induces c-Myc protein expression by promoting phosphorylation at S62 thus stabilising c-Myc and preventing it from proteasomal degradation [364]. Similarly, the cancerous inhibitor of PP2A (CIP2A), interacts directly with the oncogenic transcription factor c-Myc by inhibiting PP2A activity toward c-Myc at S62, and thereby prevents c-Myc proteolytic degradation [227]. Our data suggest that okadaic acid exposure and subsequent loss of PP2A activity prevents c-Myc degradation in Hep3B cells. c-Myc also plays a role in the up-regulation of LDH-A and positively regulates aerobic glycolysis and promotes tumour progression [429]. Further work is needed to fully show whether PP2A, and more importantly CIP2A, plays a role in this phenomenon.

In conclusion, our data indicate okadaic acid mediated inhibition of PP2A and subsequent increase in cell proliferation and migration is potentially a complex and intricate process, in which a multitude of factors influence the phenotype change (Figure 3.11). Through the use of mass spectrometry and bioinformatics, we were able to construct potential mechanisms through which the newly identified proteins were phosphorylated and elucidate their role in PP2A mediated cell proliferation. This study highlights PP2A's role is not limited to dephosphorylation events, but also encompasses other posttranslational and even posttranscriptional modifications. Furthermore, OA-mediated PP2A inhibition potentially regulates both tyrosine and serine/threonine kinase pathways, the majority of which are targets for pharmaceutical pipeline drugs, tough formulating a more detailed network of interactions requires a more refined phosphoproteomic approach. However, it is the potential role of PP2A in the metabolic reprogramming of tumour cells that garners the greatest fascination, linking PP2A and c-Myc to Otto Warburg's hypothesis of change in cancer metabolism to glycolysis.

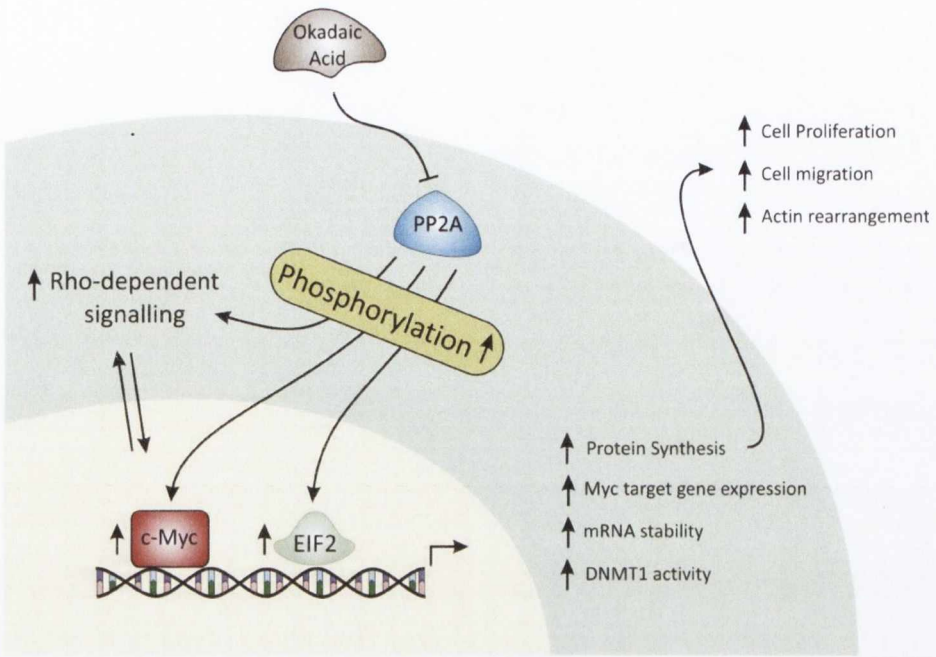


Figure 3.11: Overview of OA regulation of cell proliferation results

Okadaic acid inhibits PP2A, promoting the increase in the phosphorylation of proteins resulting in increased protein synthesis, Myc targeted gene expression and mRNA stability. Okadaic acid activates Rho-dependent signalling pathways, which mediate the increase in cell proliferation and migration.

Supplemental data:

All supplemental data are maintained on excel spread sheets. Tables are too large for thesis and are available upon request.

Chapter 4:

Protein Phosphatase 2A (PP2A): Key to Altering Markers of Extracellular Matrix Remodelling in Hep3B Cells and Functional Consequences for Tumour Invasion

4.1 Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer in the world [430]. Despite great advances in its diagnosis and treatment, the prognosis is dismal. Aetiologically, more than 80% of patients who develop HCC have pre-existing liver cirrhosis due to chronic inflammation as a consequence of hepatitis, alcohol abuse or non-alcoholic fatty liver disease [431].

Within a tumour, numerous inflammatory mediators, including cytokines, chemokines, growth and transcription factors, and enzymes such as the matrix metalloproteinase (MMP) and their associated tissue inhibitors (TIMP) cooperate to create a microenvironment conducive to tumour growth and invasion [432-435]. A recognised hallmark of invasion is the proteolytic breakdown of the extracellular matrix (ECM) through deregulation of the matrix metalloproteinase system [436, 437]. While many studies implicate MMP-9 [438-440], MMP-2, MMP-13, TIMP-1 and TIMP-3 are also involved [441-444]. Interestingly, their effects are not limited to degradation of the ECM. For example, MMP-9 modulates the innate immune response to tumours, facilitating both proliferation and regression of tumour cells [445, 446], while MMP-14 and TIMP-1 are implicated in development of chemoresistance in glioblastoma and breast cancer cell lines [292, 447]. Other members of the superfamily of metalloproteinases ADAMs (a disintegrin and metalloproteinase), have been implicated in the release or "shedding" of various receptor ligands involved in cancer and inflammatory processes; ADAM17 catalyses the release of soluble TNF-α, while ADAM10 facilitates the release of particular EGFR ligands[448, 449]

The "seed and soil" hypothesis with regard to the formation of the metastatic niche is intrinsically linked to MMPs. In particular, MMP-2 and MMP-9 facilitate disseminated primary tumour cell invasion, and release soluble pro-metastatic factors like VEGF-A, transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and interleukin 8 [IL-

8; 450].

A key molecular driver underpinning ECM degradation and release of pro-metastatic factors is over-activation of ERK, MEK and p38 MAP kinases dependent signalling pathways [334-336] or a reduction in activity of counter-regulatory phosphatases [451]. Within the latter family, serine/threonine PP form a major subfamily, which includes phosphoprotein phosphatase type I (PP1) and type 2A (PP2A). Together, these catalyse approximately 90% of all serine/threonine dephosphorylation events in cells. As stated previously, protein phosphatases are regulated by endogenous expression of the cancerous inhibitor of PP2A (CIP2A; alias p90, KIAA1524) and SET/I2^{PP2A}. In HCC and breast cancer CIP2A and SET are overexpressed promoting cell proliferation and conferment of drug resistance [452-456].

While our understanding of how kinases regulate cell motility, ECM degradation and subsequent migration are well established less is known on the role of protein phosphatase. In particularly little is known on how protein phosphatases control the MMP system. Previous work from our group has shown knockdown of the catalytic subunit of PP2A by siRNA did not mimic the MMP-9 response to OA in murine fibroblasts [339]. This may be explained by non-selective inhibition of PP2A as OA can also inhibit PP1 or PP2B as well as PP4 and PP6, with OA recently being found to have similar selectivity for PP4 and PP6 as PP2A [74]. The increase too, could be a consequence of indirect phosphorylation of signalling molecules such as Cdc25, histone H1, PKA, PKB, PKC, and IκB kinases [457], which are implicated in MMP regulation [147, 458, 459]. Nevertheless, the role of other phosphoprotein phosphatases (PP2B and PP1) and CIP2A in regulation of the MMP system and other modulators of ECM remodelling remains to be established in HCC.

Therefore, the aims of the present study were to:

1) Investigate if pharmacological inhibition of PP1 (tautomycetin), PP2A (okadaic acid) and PP2B (cyclosporin A) alters expression of the MMP system and other markers of ECM remodelling.
2) Establish protein phosphatase subunit involvement in MMP-9 regulation and the requirement for NF-κB and/or AP-1 binding motifs.
3) Ascertain if over-expression of CIP2A modulates MMP-9 abundance.
4) Examine if PP2A inhibition alters cell invasion in Hep3B cells.

4.2 Methods

4.2.1 Materials

Okadaic acid, tautomycetin and cyclosporin A were purchased from Calbiochem (Germany). All other chemicals were obtained from Sigma-Aldrich (Ireland). Mouse anti-PP2A (C subunit, clone 1D6) and goat anti-mouse HRP-conjugated secondary antibodies were obtained from Millipore (Ireland) and Sigma-Aldrich (Ireland), respectively. Mouse anti-CIP2A and HRP-conjugated β-actin antibodies were acquired from Santa Cruz Biotechnology (USA). LX-2 hepatic stellate cells were a gift from Prof Scott L. Friedman, Mount Sinai School of Medicine, USA, while Hep3B were a gift from Dr Steven Gray, Trinity College Dublin, Ireland. siRNA was obtained from IDT (Integrated DNA Technologies, Belgium), while DNA primers were purchased from Metabion (Germany). Details of the MMP-9 wild type reporter construct (pGL3-MMP9$_{1285}$-Luc) and its NF-κB (pGL3-MMP9/NF-κB$_{(-600)}$-Luc), and AP-1 mutated counterparts (pGL3-MMP9/AP-1$_{(-79)}$-Luc and pGL3-MMP9/AP-1$_{(-533)}$-Luc) have been previously published by our group [339]. The CIP2A expression plasmid, pcDNA3.1_CIP2aflag_WT, was kindly provided by Prof J Westermarck, University of Turku, Finland.

4.2.2 Cell Culture

Human epithelial hepatocellular carcinoma (Hep3B) and human hepatic stellate (LX-2) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) and 4 mM glutamine, at 37°C in an humidified atmosphere containing 5% CO_2. All experiments were performed under serum free conditions. siRNA knockdown and DNA transfection were performed under antibiotic free conditions.

4.2.3 Cell Viability

Hep3B and LX-2 cells seeded in 48-well plates (3 x 10^4 cells per well) were exposed to okadaic acid (OA), tautomycetin (TMC), cyclosporin A (CSA; 1-100 nM), media, doxorubicin hydrochloride (5 μM, positive control), or dimethylsulphoxide (DMSO; 0.1% v/v, solvent control) for 24 h. After 22 h, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) was added, and the cells incubated for a further 2 h. At the end of the incubation period, the culture medium was removed, and the purple formazan deposits dissolved in DMSO. Formazan production was quantified by spectrophotometry at a wavelength of 540 nm with a reference wavelength of 650 nm (BioTek, EL 808, Bedfordshire, UK).

4.2.4 Semi-Quantitative Real-Time PCR

Cells were grown in 24-well plates (4 x 10^4 cells per well) and total RNA isolated using TRI Reagent™ (Sigma Aldrich). Following treatment with DNase I (Sigma Aldrich), RNA was reverse transcribed using random hexamers and ReverseAid reverse transcriptase (Thermofisher, Dublin, Ireland). mRNA expression was analysed by semi-quantitative PCR using target specific primers (Table 4.1; Metabion) and Agilent Brilliant III SYBR green on a Mx3000P qPCR system (Agilent Technology). Gene expression was quantified using the comparative Ct method [2-ΔΔCt; 460]. For each primer pair, a no template control and no RT control was included. Authenticity of the primer sequences was verified by nucleotide search (Primer-BLAST; NCBI), while the size and specificity of the amplicons were checked by gel electrophoresis with UV trans-illumination (Fusion Fx imaging system, Vilber Lourmat, Marne-la-Vallée Cedex, France).

Primer	NM ID	Forward Sequence	Reverse Sequence
MMP-1	NM_001145938.1	AGAAAGAAGACAAAGGCAAGTTGA	TTCCCAGTCACTTTCAGCCC
MMP-2	NM_004530.5	CGCATCTGGGGCTTTAAACAT	CCATTAGCGCCTCCATCGTA
MMP-3	NM_002422.3	AAAGACAGGCACTTTTGGCG	CTTCATATGCGGCATCCACG
MMP-9	NM_004994.2	CATCCGGCACCTCTATGGTC	CATCGTCCACCGGACTCAAA
MMP-13	NM_002427.3	GGGTCTTCCAAAAGAAGTTAAG	ATATCTCCAGACCTGGTTTC
TIMP-1	NM_003254.2	CATCCGGTTCGTCTACACCC	TCTGCAGTTTGCAGGGGATG
TIMP-2	NM_003255.4	CAGCTTTGCTTTATCCGGGC	ATGCTTAGCTGGCGTCACAT
TIMP-3	NM_000362.4	GAAGAGAGTACCGGCATCGG	CCATTCTCCCCCTGCCAAAT
TIMP-4	NM_003256.3	CCTTGGTGCAGAGGGAAAGT	GTCCAGAGGCACTCGTTAGG
TGFB1	NM_000660.5	CCCACAACGAAATCTATGAC	TGTATTTCTGGTACAGCTCC
IL1B1	NM_000576.2	GCAACAAGTGGTGTTCTC	CAGATTCTTTTCCTTGAGGC
IL-8	NM_000584.3	CCACCGGAAGGAACCATCTC	TTCCTTGGGGTCCAGACAGA
ADAM-10	NM_001110.3	GACCACAGACTTCTCCGGAAT	TGAAGGTGCTCCAACCCAAG
ADAM-15	NM_001261466.1	ACCCCAGTGGCAGTTATGTG	CTTGGAGTCCCTGCCTTTGA
ADAM-17	NM_003183.4	TTGTGTGGTTTGGCCCTTCT	CTGCTTTTGCACCACAGGTC

JUN	NM_002228.3	GCATGAGGAAACGCATCGCTGCCTCCAAGT	GCGACCAAGTCCTTCCCACTCGTGCACACT
FOS	NM_005252.3	TGCCTCTCCTCATCAATGACCCTG	ATAGGTCCATGTCTGGCACGG
NFκB1	NM_003998.3	AAGAGGAGGTTTCGCCACCG	AAGGTATGGGCCATCTGCTGTT

Table 4.1 Details of primers and their sequences used in mRNA expression profiling.

4.2.5 Gelatin Zymography

MMP-9 and MMP-2 activity was measured by gelatin zymography as previously described [461]. In brief, conditioned medium denatured in a non-reducing Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 0.1% phenol red, 50% glycerol, 5% ficoll 400 and 5% lithium dodecyl sulphate; final concentrations) was loaded onto 8% SDS-polyacrylamide gels co-polymerised with gelatin (0.1% w/v). Following electrophoresis, gels were washed in 2.5% Triton X-100 and incubated for 24 h at 37°C in development buffer (50 mMTris-HCl, 10 mM $CaCl_2$, 50 mM NaCl, pH 7.6). Gels were stained with 0.25% (w/v) Coomassie blue R-250 in a glacial acetic acid: methanol: distilled H_2O mixture (1:3:6). Following destaining, gels were imaged, and lytic activity quantified by densitometry using a Fusion Fx imaging system. To test for non-specific proteases activity, identical gels were incubated in either the standard incubation buffer or one supplemented with EDTA (20 mM) to inhibit gelatinase activity.

4.2.6 siRNA Knockdown of Protein Phosphatases

Hep3B cells were seeded in 24-well plates (4 x 10^4 cells per well) 24 h prior to transfection with siRNA targeting PPP2CA (10 nM; catalytic subunit of PP2A), PPP2R1A (10 nM; scaffolding subunit of PP2A), PPP1CA (10 nM; catalytic subunit of PP1; Integrated DNA Technology, USA) or non-target siRNA (Fisher, USA) using HIPerFect as per the manufacturer's instructions (Qiagen, UK); a mock transfection was also included as a control. Knockdown of PPP2CA, PP2R1A and PPP1CA was confirmed by qPCR. The culture medium was collected 48 h later, and MMP-9 abundance and activity were determined using ELISA and zymography.

Primer	NM ID	Forward Sequence	Reverse Sequence
PPP2CA	NM_002715.2	GCCTCTGCGAGAAGGCTAAA	GAATGGTGATGCGTTCACGG
PPP2R1A	NM_014225.5	AAAGGGACGGAGCCAAGATG	GGGTAGTGAAGGTTCCCAGC
PPP1CA	NM_001244974.1	CAGGGTCCTGACACCCCATT	AGGTAAAAGAGACGCCACGG
CIP2A (KIAA1524)	NM_020890.2	TGGACCCACAAATCACCTCG	ATTACCTCCAAGTGCCGCAA
GAPDH	NM_002046.5	CTCTGCTCCTCCTGTTCGAC	GCGCCCAATACGACCAAATC

Table 4.2 Details of primers used to confirm siRNA knockdown by RT-PCR.

Duplex Name		Sequence
PPP2R1A	Sense	5'-rArGrGrCrGrGrArArCrUrUrCrGrArCrArGrUrA-3'
	Anti-Sense	5'-rArArArCrUrUrArArCrUrCrCrUrUrGrUrGrCrA'3
PPP2CA	Sense	5'-rGrGrArUrArGrCrArGrCrArArArCrArArUrCrArUrUrGrGrArG-3'
	Anti-Sense	5'-rCrUrCrCrArArUrGrArUrUrGrUrUrUrGrCrUrGrCrUrArUrCrCrUrU-3'
PPP1CA	Sense	5'-rCrArArGrArUrCrUrGrCrGrGrUrGrArCrArUrArUrUr-3
	Anti-sense	5'-rCrArArGrArGrArCrGrCrUrArCrArArCrArUrCrUrUr-3

Table 4.3 List of dicer siRNA used in this study and their sequences.

4.2.7 Transformation of *E. coli*

Chemically competent TOP10 *E. coli* (transformation efficiency of $1.6x10^7$ cfu/μg DNA) were transformed with 10 ng of DNA by heat shock (42^{0}C for 30 seconds) and immediately placed on ice for 5 min. A negative control (no DNA) was also included. Transformed cells were then allowed to recover for 1 h in BHI medium before being used to inoculate LB agar plates (1g/l LB agar, 100 μg/ml ampicillin) overnight at 37^{0}C in a shaking incubator. Single colonies were lifted and plated twice more to ensure transformation efficiency and antibiotic resistance. Finally, a single colony was chosen and grown in LB-medium (20g/l LB medium, 100 μg/ml ampicillin) at 37^{0}C with shaking (100 rpm, overnight). Plasmids were extracted and purified using PureYield™ Plasmid Midiprep System (Promega).

4.2.8 Overexpression of CIP2A

To assess the effect of overexpression of CIP2A on MMP-9 activity and invasion potential, Hep3B cells were seeded (2 x 10^5 cells per well) in 6 well in plates, and 24 h later transiently transfected with 1 μg of pcDNA3.1_CIP2aflag_WT plasmid (pCIP2A) or a pcDNA.3.1 (pDNA.3.1) control plasmid using PolyFect (Qiagen) in serum and antibiotic free DMEM. CIP2A over expression was confirmed 24 and 48 h post-transfection by qPCR and Western blot respectively. The effect of overexpression of CIP2A on MMP-9 abundance and activity was determined in conditioned media 48 h post transfection using ELISA and gelatin zymography.

4.2.9 Confirmation of CIP2A overexpression by Western Blot Analysis

Protein samples (10 μg) were boiled in Laemmli buffer supplemented with 2-β-mercaptoethanol (1% v/v). The samples were subjected to SDS-PAGE (10% gel) and semi-dry transferred onto PVDF membrane (Amersham, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS-T (Tris-base 10 mM, NaCl 100 mM HCl 1 M, and Tween-20 0.1% v/v) containing 5% w/v Marvel and probed with 1° antibody directed against CIP2A (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution) for 24 h at 4°C. Following extensive washing, membranes were incubated with a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP 1:2500 dilution). Signals were visualised by chemiluminescence detection (30% H_2O_2, 250 mM luminol in DMS0, 90 mM 4-iodophenylboronic acid in DMSO and 10 mM Tris-HCl) [373] and the signal recorded using a Fusion Fx imaging system. Membranes were stripped and re-probed with HRP-conjugated β-actin antibody as a loading control (1:7500, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

4.2.10 PP2A Phosphatase Activity Assay

PP2A activity was determined using a PP2A immunoprecipitation phosphatase assay kit (Millipore, USA). Briefly, cells were lysed on ice in a buffer comprised of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP40 containing a protease inhibitor cocktail. 50-100 μg of total protein was immunoprecipitate with anti-PP2A-C subunit antibody (4 μg; clone 1D6) along with protein A-agarose. The mix was incubated for 2 h at 4 °C, and the immunoprecipitate extensively washed before determination of the phosphatase activity as per the manufacturer's instructions. PP2A phosphatase activity was calculated by dividing the free phosphate of the test cells by that of the untreated cells, and expressed as a percentage. Furthermore, each assay run

contained a negative control (no lysates) as a quality control for phosphatase contamination.

4.2.11 MMP-9 Reporter Assay

Hep3B cells were transfected with the full length pGL3-MMP9$_{1285}$-Luc plasmid (400 ng/well) or mutated counterparts (pGL3-MMP9/NF-κB$_{(-600)}$-Luc, pGL3-MMP9/AP-1$_{(-79)}$-Luc or pGL3-MMP9/AP-1$_{(-533)}$-Luc) in conjunction with pRL-CMV (1 ng; internal standard) using PolyFect (Qiagen). Cells were subsequently exposed to OA (40 nM) or media for 24 h, before being harvested and lysed. Firefly and *Renilla* luciferase activity was evaluated using a Dual-Luciferase Reporter Assay System (Promega, USA).

4.2.12 MMP-9 ELISA

The effect of OA, silencing of PPP2CA by siRNA and over expression of CIP2A on MMP-9 abundance was determined in conditioned media using ELISA following the vendor's protocol (Quantikine/DuoSet; R&D Systems, UK). Culture media was reserved 24 h post-drug exposure and at 48 h post-transfection. All samples were stored at -80°C until required.

4.2.13 Cell Invasion Assays

Transwell inserts (6.5 mm, 8 μm pore; Corning, USA) were pre-treated with Matrigel (5 mg/ml; Sigma-Aldrich, Ireland) for 24 h. Hep3B cells ($2x10^4$ cells/well) were loaded into the upper compartment of the transwell and exposed to OA (40 nM) or media (control) under serum free conditions; 10% FBS was included in the lower chamber as chemoattractant. For CIP2A over-expression experiments, cells were transfected with pCIP2A for 48 h before being loaded into the upper compartment of the transwell. In all experiments, the filters were removed 24 h later; cells were fixed in 3% paraformaldehyde and stained with crystal violet. The matrigel membranes were imaged on a Cell Imaging System (EVOS® FL) for subsequent analysis. Each experiment was performed in triplicate.

4.2.14 Data Analysis

All data were normalised to the appropriate control and expressed as either an absolute value, a percentage or ratio, with the exception of the MMP-9 reporter data, which was expressed as the ratio of firefly luciferase to *Renilla* luciferase activity normalised to control (medium) or pGL3-$MMP9_{1285}$-Luc promoter activity as appropriate. Cell invasion assays were evaluated using ImageJ software [366]. Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett, Bonferroni) or by un-paired Student's t test, with the exception of the MMP-9 mRNA time course, which was analysed by 2-way ANOVA with *post hoc* analysis (Dunnett). Data are expressed as mean ± S.E.M., and a value of $P < 0.05$ is taken to indicate statistical significance.

4.3 Results

4.3.1 Effect of Protein Phosphatase inhibitors on Cell Viability and mRNA Profile

Okadaic acid and tautomycetin did not alter cell viability over the concentration range 0.1-100 nM (data not shown). However, cyclosporin A decreased cell viability at a concentration of 66 nM (45%) and higher.

The effect of pharmacological inhibition of PP1 (TMC), PP2A (OA) and PP2B (CSA) on a panel of markers representing extracellular matrix modulators, inflammation/growth factor releasers, transcription factors and a structural component (Table 4.4) were screened by qPCR. With regard to the extracellular matrix modulators, OA (40 nM) increased expression of MMP-9 and TIMP-1 mRNA by 1.90 ± 0.34 and 1.74 ± 0.31 fold respectively in Hep3B cells ($P < 0.05$; Table 3). In LX-2 cells MMP-9 expression was increased while that of TIMP-1 decreased (Table 4.3). MMP-9 and TIMP-1 expression were not altered by TMC or CSA (10 nM) in either cell line. In Hep3B and LX-2 cells expression of MMP-1, MMP-2, MMP-3, MMP-13, TIMP-2, TIMP-3 and TIMP-4 were unaltered by OA, TMC or CSA at the concentrations studied. OA augmented IL-8 mRNA expression by 1.57 ± 0.24 fold compared to control ($P < 0.05$; Table 4.4) in both cell lines, while having no effect on TGF-β1, IL-1β, ADAM-10, ADAM-15 or ADAM-17. None of the inflammation/growth factor releasers were altered by TMC or CSA irrespective of cell line. Of the three transcription factors studied, only JUN expression was increased by OA (7.15 ± 0.74 fold; $P < 0.05$; Table 4.4) in Hep3B and LX-2 cells. TMC and CSA did not modify mRNA expression of JUN, FOS or NFκB1.

On the basis of these data, subsequent experiments focussed on MMP-9 regulation.

4.3.2 Effect of Protein Phosphatase Inhibitors on Temporal Expression of MMP-9

To ensure that TMA can CSA did not have a transient effect on MMP-9 expression, the temporal response over 24 h was investigated. In Hep3B cells, OA (40 nM) elicited a time dependent increase in expression of MMP-9 mRNA that was detectable after 4 h. The response plateaued at 6 h and remained stable for the duration of the study period ($P < 0.05$; Figure 4.1). Although TMC (10 nM) caused a similar increase in MMP-9 expression (~1.4 fold) as OA, the response was transient, peaking at 4 h ($P < 0.05$; Figure 1), before quickly returning to baseline by 8 h, and remaining stable thereafter. In contrast, CSA did not alter expression of MMP-9 mRNA over the 24 h time course.

4.3.3 Effect of Protein Phosphatase Inhibitors on MMP-9 Abundance and the MMP-9:TIMP-1 ratio

OA increased secreted MMP-9 abundance to 413.9 ± 118 pg/ml from a basal level of 171.6 ± 35.88 pg/ml in Hep3B cells ($P < 0.05$; Figure 4.2A), and from 123.4 ± 14.43 pg/ml to 298.2 ± 24.83 pg/ml in LX-2 cells ($P < 0.05$; Figure 4.2B). TMC and CSA had no effect on secreted MMP-9 abundance in either cell line studied. TIMP-1 levels in the conditioned media were increased by OA to 5332 ± 430.4 pg/ml from 3043 ± 150.9 pg/ml in Hep3B cells ($P < 0.05$; Figure 4.2C). Interestingly, TIMP-1 abundance decreased from 1964 ± 318.9 pg/ml to 784.9 ± 150.5 pg/ml in conditioned media from LX-2 cells ($P < 0.05$; Figure 4.2D). While OA had little effect on the MMP-9:TIMP-1 ratio in Hep3B cells (Figure 4.2E), it was augmented by 3.72 ± 0.91 fold in LX-2 cells ($P < 0.05$; Figure 4.2F). TMC and CSA did not alter TIMP-1 abundance or the MMP-9:TIMP-1 ratio in either cell line.

4.3.4 Effect of PP2A and PP1 Subunit Knockdown on MMP-9 Activity

To ascertain PP2A and PP1 subunit involvement in MMP-9 regulation, Hep3B cells were transfected with siRNA targeting the catalytic (PPP2CA) and scaffold (PPP2R1A) subunits of PP2A, and the catalytic subunit of PP1 (PPP1CA). siRNA knockdown reduced basal mRNA expression of PPP2CA, PPP2R1A and PPP1CA by ~75% after 24 h compared to the mock-transfected and non-target transfected cells ($P < 0.05$; Figure 4.3A). Knockdown of PPP2CA increased detectable MMP-9 activity by ~1 fold and increased MMP-9 abundance in conditioned media by 581.6 ± 105.1 pg/ml compared to media from mock transfected cells ($P < 0.05$; Figure 4.3B,C). In contrast, knockdown of PPP2R1A and PPP1CA did not alter detectable MMP-9 activity or abundance (Figure 4.3B,C). Furthermore, knockdown of PPP2CA, PPP2R1A and PPP1CA had no effect on detectable MMP-2 activity as determined by zymography (data not shown). Transfection with the non-target siRNA had no effect on protein phosphatase subunit expression or MMP-9 activity/abundance.

4.3.5 Role of AP-1 and NF-κB Transcription Factor Binding Sites on MMP-9 Promoter Activity

In Hep3B-transfected cells, mutation of the AP-$1_{(-79)}$ but not AP-$1_{(-533)}$ binding motif attenuated basal MMP-9 promoter activity by ~55% compared to the p1285-luc (wild type) promoter construct ($P < 0.05$; Figure 4.4A,C). Similarly, mutation of the NF-$\kappa B_{(-600)}$ binding site decreased basal promoter activity by ~40% ($P < 0.05$; Figure 4.4A). OA (40 nM) increased p1285-luc MMP-9 promoter activity by 2.07 ± 0.33 fold compared to untreated cells ($P < 0.05$; Figure 4.4B). Mutation of the AP-$1_{(-79)}$ binding motif prevented OA-mediated induction of MMP-9 promoter activity, while mutation of the AP-$1_{(-533)}$ binding motif reduced the response by ~40% ($P < 0.05$; Figure 4.4B). When the NF-$\kappa B_{(-600)}$ binding site was mutated, the response to OA was similar to that

in cells transfected with the p1285-luc (wild type) promoter (Figure 4.4B).

4.3.6 Effect of CIP2A Overexpression on MMP-9 Activity and Abundance

To extend the physiological relevance of the OA and siRNA data, we investigated if overexpression of CIP2A, which is an endogenous inhibitor of PP2A, also regulates MMP-9 in Hep3B cells.

Transfection of Hep3B cells with a pCIP2A plasmid augmented CIP2A mRNA expression by 7 fold and protein expression by 4 fold ($P < 0.05$; Figure 4.5A,B) compared to mock transfected and empty vector (pDNA.3.1) transfected cells. CIP2A over expression increased total MMP-9 abundance from 361.3 ± 61.86 pg/ml to 882.8 ± 90.37 pg/ml and increased detectable MMP-activity compared to mock transfected and pDNA.3.1 transfected cells ($P < 0.05$; Figure 4.5C,D). Transfection of Hep3B cells with pDNA.3.1 did not alter CIP2A mRNA expression or total MMP-9 abundance compared to mock transfected cells (Figure 4.5A,C).

4.3.7 Inhibition of PP2A Phosphatase Activity

To establish that OA, CIP2A and PPP2CA silencing alter PP2A activity, a PP2A immunoprecipitation phosphatase activity assay was employed.

Exposure of Hep3B cells to OA (40 nM) for 24 h decreased PP2A activity by ~80% compared to untreated control cells ($P < 0.05$; Figure 4.6A). Overexpression of CIP2A attenuated PP2A activity by ~65% compared to mock transfected controls ($P < 0.05$; Figure 4.6B), the empty vector had no effect. SiRNA knockdown of PPP2CA inhibited PP2A activity by ~70% compared to mock and non-template siRNA transfected cells ($P < 0.05$; Figure 4.6C).

4.3.8 Effect of Okadaic Acid and CIP2A overexpression on Cell Invasion

To determine if pharmacological or endogenous inhibition of PP2A is functionally important, a Matrigel transwell cell invasion assay was utilised. Exposure of Hep3B cells to OA (40 nM) for 24 h augmented cell invasion through Matrigel coated transwell membranes by 3.7 ± 1.1 fold compared to the untreated control cells ($P < 0.05$; Figure 4.7A,C). Similarly, over expression of CIP2A increased cell migration by 3.22 ± 0.96 fold compared to mock transfected cells ($P < 0.05$; Figure 4.7B,D); transfection with the empty plasmid (pDNA.3.1) had no effect.

	OA		TMC		CSA	
	Hep3B	LX-2	Hep3B	LX-2	Hep3B	LX-2
MMP-1	↔	↔	↔	↔	↔	↔
MMP-2	↔	↔	↔	↔	↔	↔
MMP-3	↔	↔	↔	↔	↔	↔
MMP-9	↑ (1.9)	↑ (1.3)	↔	↔	↔	↔
MMP-13	↔	↔	↔	↔	↔	↔
TIMP-1	↑ (1.7)	↓ (-0.7)	↔	↔	↔	↔
TIMP-2	↔	↔	↔	↔	↔	↔
TIMP-3	↔	↔	↔	↔	↔	↔
TIMP-4	↔	↔	↔	↔	↔	↔
TGF-β1	↔	↔	↔	↔	↔	↔
IL-1β	↔	↔	↔	↔	↔	↔
IL-8	↑ (1.6)	↑ (1.9)	↔	↔	↔	↔
ADAM-10	↔	↔	↔	↔	↔	↔
ADAM-15	↔	↔	↔	↔	↔	↔
ADAM-17	↔	↔	↔	↔	↔	↔
JUN	↑↑ (7.0)	↑↑ (4.7)	↔	↔	↔	↔
FOS	↔	↔	↔	↔	↔	↔
NFκB1	↔	↔	↔	↔	↔	↔

Table 4.4 Effect of okadaic acid (OA, 40 nM), cyclosporin A (CSA, 10 nM) and tautomycetin (TMC, 10 nM) on mRNA expression in Hep3B and LX-2 cells. Data are depicted as a fold change from control summarised as an increase (↑), decrease (↓) or no change (↔). The fold change is given in brackets (shading $P < 0.05$).

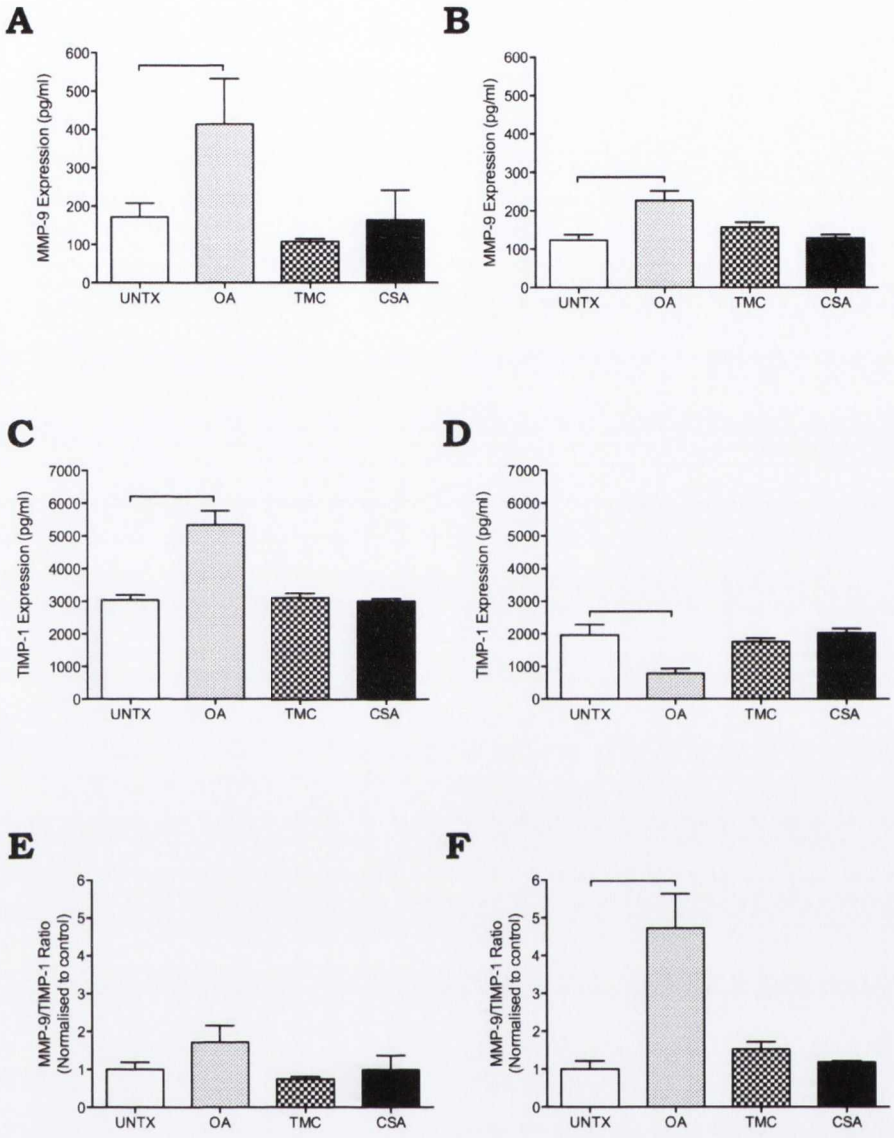


Figure 4.1: Effect of protein phosphatase inhibitors on MMP-9 (A, B) and TIMP-1 abundance (C, D,) and MMP-9:TIMP-1 ratio (E, F) in Hep3B (A, C, E) and LX-2 (B, D, F) cells. Cells were exposure to okadaic acid (OA, 40 nM), tautomycetin (TMC, 10 nM), cyclosporin A (CSA, 10 nM) or media (UNTX) for 24 h and abundance determined in conditioned media by ELISA. Data are presented as pg/ml or as a ratio (mean ± S.E.M) and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate statistical significance at $P < 0.05$ (n=6).

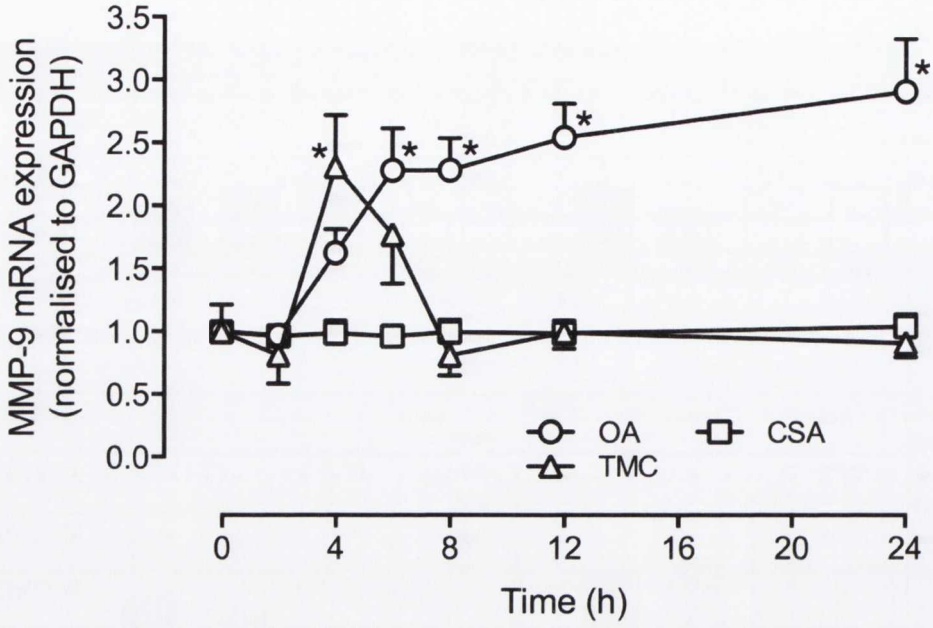


Figure 4.2: Effect of phosphatase inhibitors on the temporal expression of MMP-9 mRNA determined by qPCR (comparative quantification) in Hep3B cells over 24 h following exposure to okadaic acid (OA, 40 nM), tautomycetin (TMC, 10 nM) and cyclosporin A (CSA, 10 nM). Data are normalised to baseline (mean ± S.E.M) and were analysed by two-way ANOVA with *post hoc* analysis (Dunnett). * $P < 0.05$ (n=6).

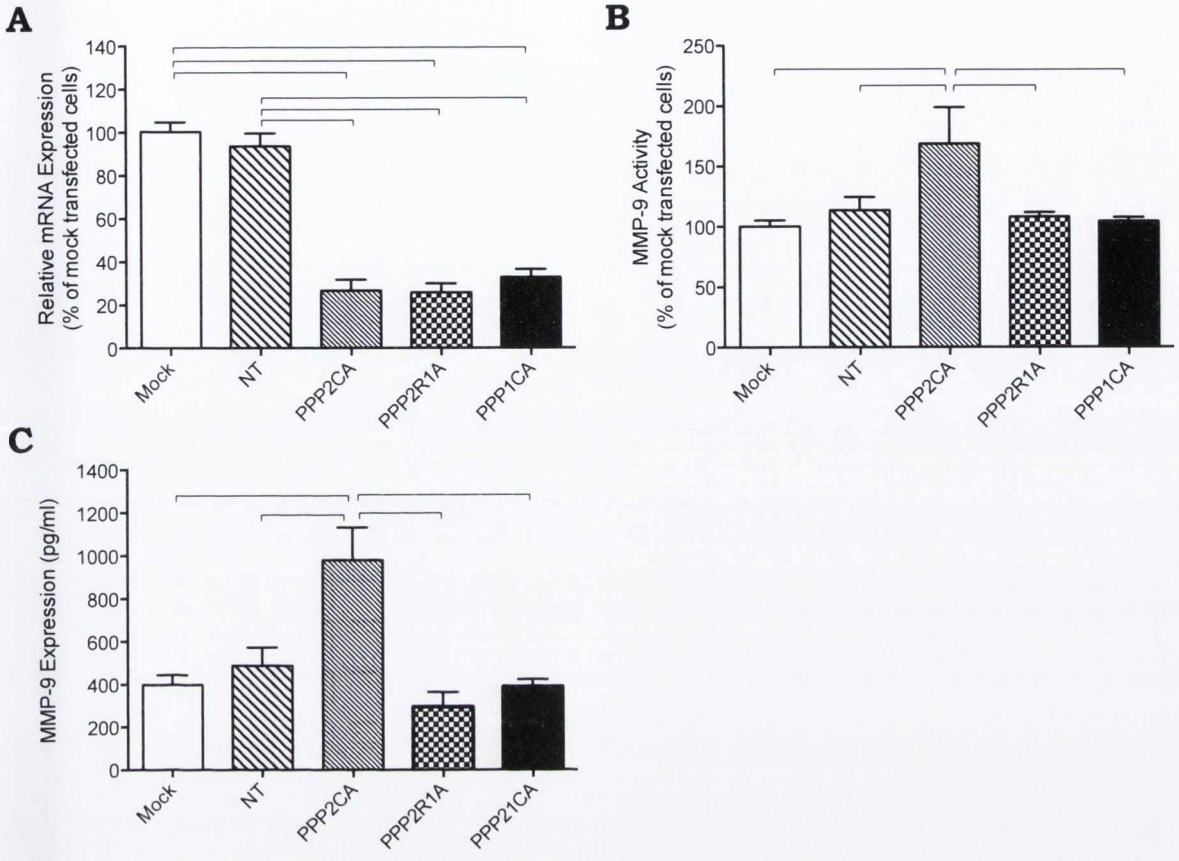


Figure 4.3: Effect of transfection of Hep3B cells with PP2CA (PP2A catalytic subunit), PPP2R1A (PP2A regulatory subunit), PPP1CA (PP1 catalytic subunit) and non-target (NT) siRNA on: basal subunit mRNA expression (A), detectable MMP-9 activity (B) and MMP-9 abundance (C). Data are presented as a percentage of mock-transfected cells or in pg/ml (mean ± S.E.M) and were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=5).

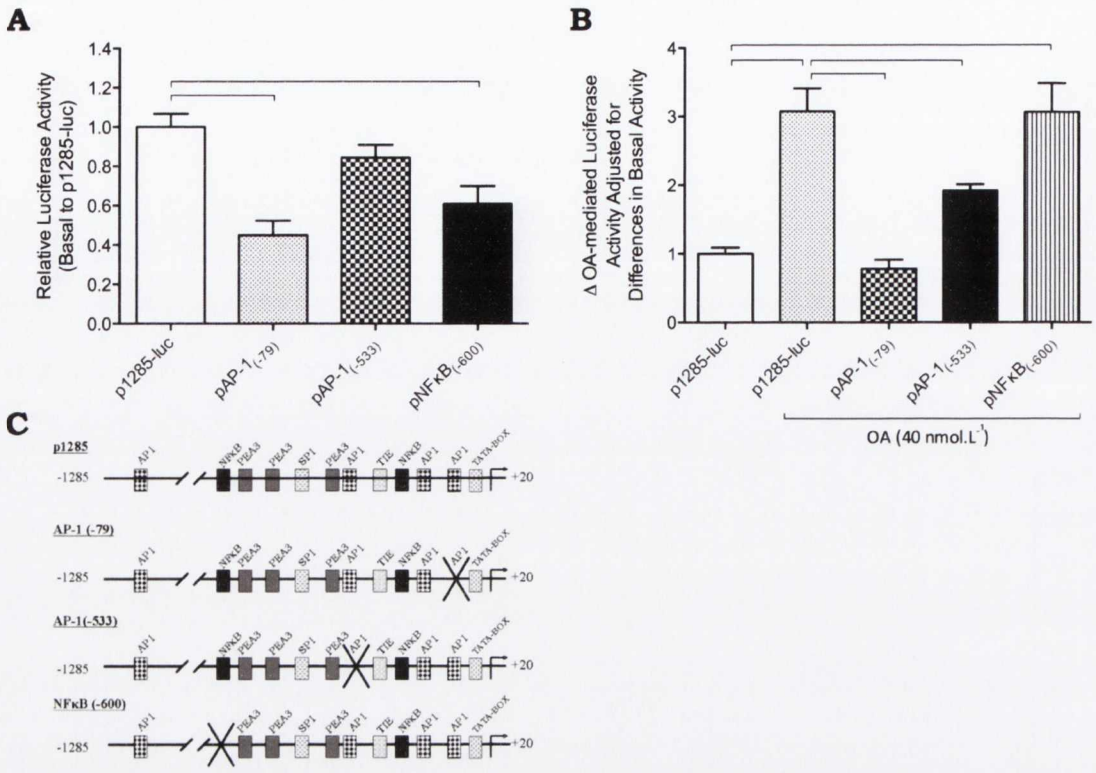


Figure 4.4: Effect of mutation of the AP-1 and NF-κB binding motifs on MMP-9 promoter activity under: basal conditions (A) and following exposure to okadaic acid (40 nM, B). A diagrammatic representation of the MMP-9 promoter region detailing the location of the mutated binding motifs (C). Hep3B cells were transfected with p1285-luc, p1285-luc AP-$1_{(-79)}$, p1285-luc AP-$1_{(-533)}$ and p1285-luc NF-$\kappa B_{(-600)}$ promoter constructs and luciferase activity determined in cell lysates. Basal promoter activity for each mutant was normalised to the p1285 construct, and their effect on the OA response expressed as a change from the p1285-luc construct under un-stimulated conditions. Data are presented as mean ± SEM, and were analysed by one-way ANOVA with Dunnett or Bonferroni *post hoc* analysis as appropriate. Horizontal bars indicate statistical significance at $P < 0.05$ (n=6).

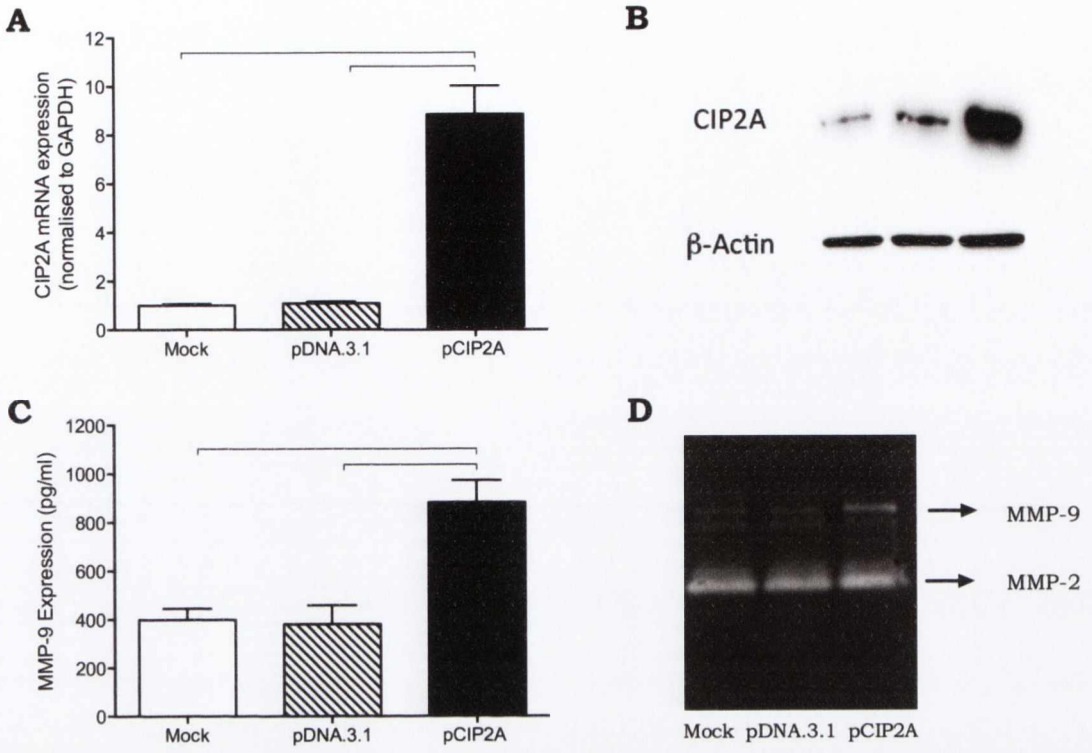


Figure 4.5: Effect of transfection of Hep3B cells with a CIP2A expression plasmid on: CIP2A mRNA and protein expression (A, B) and detectable MMP-9 abundance and activity (C, D). Mock transfection and transfection with the empty backbone plasmid were included as controls. Data are presented as a fold increase from mock-transfected cells or in pg/ml (mean ± S.E.M.) and were analysed by one-way ANOVA with post hoc analysis (Dunnett). Horizontal bars indicate statistical significance at $P < 0.05$ (n=4).

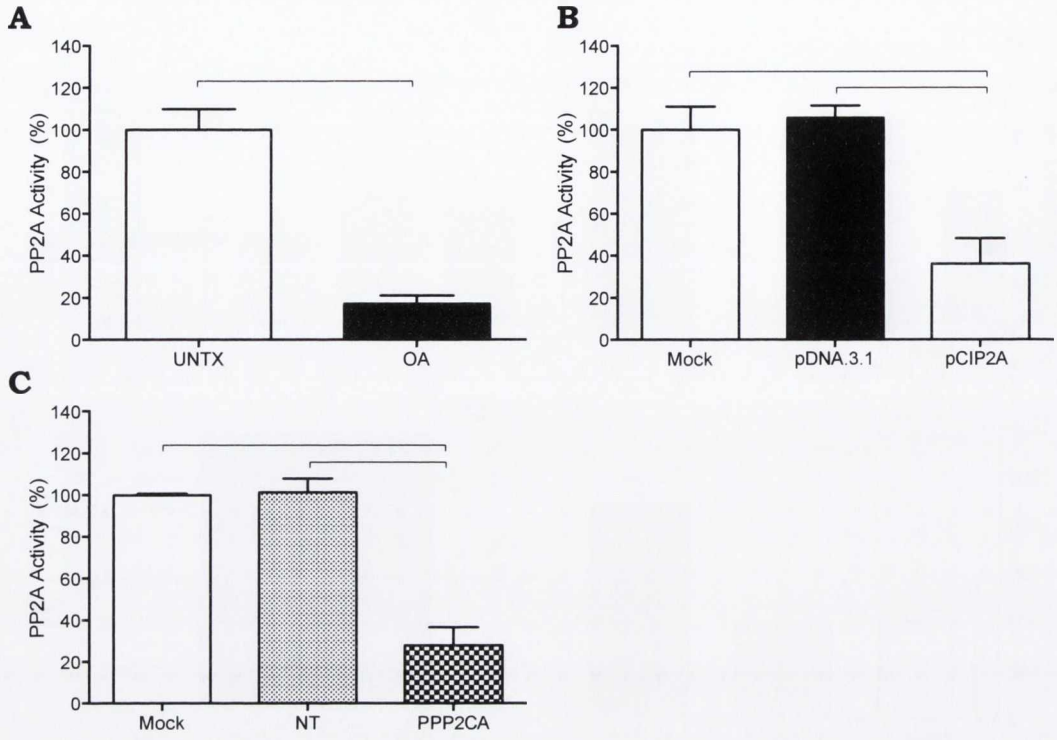


Figure 4.6: Effect of okadaic acid (OA, 40 nM, A), PPP2CA siRNA silencing (B) and CIP2A overexpression (C) on PP2A phosphatase activity in cellular lysates from Hep3B cells. Mock transfections and transfection with the CIP2A backbone plasmid (pDNA.3.1) and non-target siRNA (NT) were included as control groups. Data are presented as a percentage of the untreated (UNTX) or mock-transfected cells (mean ± S.E.M.), and were analysed by one-way ANOVA with post hoc analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

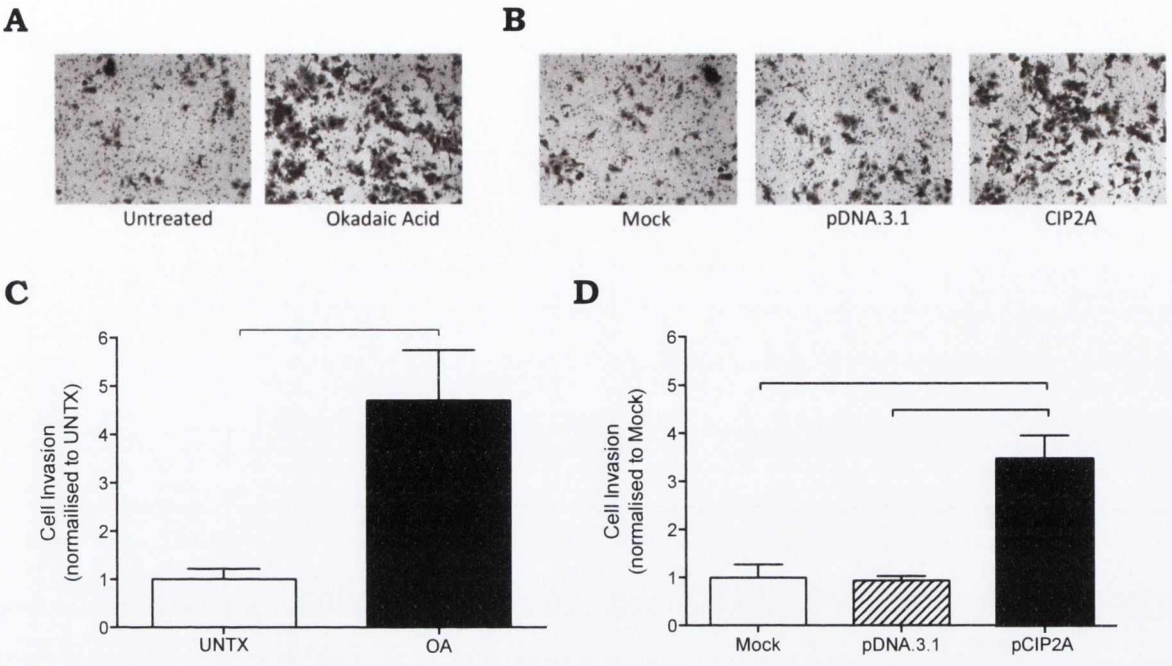


Figure 4.7: Effect of okadaic acid (A, C) and CIP2A (B, D) on Hep3B cell invasion across a Matrigel coated transwell membrane. Cells exposed to okadaic acid (OA, 40 nM) or transfected with a CIP2A expressing plasmid and images captured on a Cell Imaging System (EVOS® FL); media (UNTX) and empty vector (pDNA.3.1) were included as controls. Image analysis was preformed using ImageJ software. Data are presented normalised to either the untreated (UNTX) or pDNA.3.1 transfected groups (mean ± S.E.M), and were analysed by paired Student t test or one-way ANOVA with post hoc analysis (Bonferroni) as appropriate. Horizontal bars indicate statistical significance at $P < 0.05$ (n=5). Representative images of migration across the transwell inserts are inset (A, B).

4.4 Discussion

The present study provides evidence that OA increases expression of MMP-9, IL-8 and JUN mRNA in Hep3B and LX-2 cells, but differentially regulates TIMP-1 expression, while TMC and CSA had no effect at 24 h. Interestingly, in time course experiments, TMC transiently increased MMP-9 mRNA expression, while OA elicited a sustained response. Furthermore, OA increased MMP-9 and TIMP-1 abundance in Hep3B, while increasing MMP-9 but decreasing TIMP-1 levels in LX-2 cells. As such, the MMP-9:TIMP-1 ratio was changed towards a degradative phenotype in LX-2 cells. Expression of other members of the MMP/TIMP/ADAM family and inflammatory / growth factors such as TGFβ1, IL-1β and NFκB1 were unaffected by OA, TMC or CSA. Knockdown of PPP2CA increased MMP-9 activity and abundance, while knockdown of the PP2A scaffolding subunit (PPP2R1A) or the catalytic subunit of PP1 (PPP1CA) had no effect. At the promoter level, OA-mediated induction of MMP-9 required functional AP-1 but not NF-κB binding motifs. More importantly, overexpression of CIP2A, an endogenous inhibitor of PP2A, increased MMP-9 abundance and activity, and increased Hep3B invasiveness across a collagen matrix, as did OA.

Although the ECM provides structural support to cells, it also contributes to fundamental cell behaviour as diverse as cell proliferation, adhesion and migration, to cell differentiation and cell death; in part due to its ability to act as a reservoir for growth factors and cytokines [462-464]. It is the pleiotropic nature of the ECM and it's dynamic remodelling that makes it an important component of cancer. While numerous studies have investigated kinase dependent regulation of tumour remodelling and invasion [465, 466], there is a paucity of data on the role of serine/threonine protein phosphatases in this regard. This is surprising given the importance of tumour dissemination as an indicator of clinical outcome in hepatocellular carcinoma, and the

emergence of protein phosphatases as novel "druggable" targets [132]. On the basis of this, we screened key factors associated with ECM synthesis and degradation (Table 4.4) to ascertain if they are modulated by protein phosphatases in hepatocellular carcinoma. The work is based upon Hep3B cells, which are widely utilised as an *in vitro* model of hepatocellular carcinoma.

With regard to the ADAMs we assessed the effects of pharmacological inhibition of PP2A, PP2B and PP1 on ADAM-10, ADAM-15 and ADAM17 expression. These were specifically chosen as their expression is increased in HCC and are associated with tumour metastasis and proliferation. Our data clearly demonstrates that OA, TMC and CSA do not alter expression of ADAM-10, -15 or -17 in Hep3B or LX-2 cells. While there is little to compare this to in the literature, a recent study found OA to increase ADAM-10 mRNA expression in neuronal cells from rat [467].

In addition to the above, inflammation also plays its role in shaping the ECM in the tumour microenvironment. For example in patients with hepatocellular carcinoma, serum levels of IL-1β and IL-8 are elevated and associated with poor prognosis [468]. However, we report that IL-1β expression is not affected by OA, CSA or TMC in either Hep3B or LX-2 cells. This is consistent with OA having little to no effect on IL-1β expression in THP-1 cells, but is at variance to other studies demonstrating increased expression in rat astrocytes [469] and enhancement of PMA-induced IL-1β expression in THP-1 monocytes [470]. Furthermore, CSA reduces IL-1β expression in transplanted islets of langerhan and in a brain injury model in rats [471, 472].

Interestingly, in the present study, inhibition of PP2A by OA increased IL-8 expression in Hep3B and LX-2 cells. This supports and extends previous studies in human lung epithelial and human promyelocytic leukaemia cells [344-346]. However, inhibition of PP2B and PP1 had no effect, despite previous studies showing CSA to attenuate PMA induced

IL-8 expression through functional NF-κB and AP-1 sites on the IL-8 promoter [473] in human glioblastoma cells.

With regard to the matrix metalloproteinases system pharmacological inhibition of PP2A (OA), increased TIMP-1 expression in Hep3B cells while decreasing it in LX-2 hepatic stellate cells. This likely reflects cell line differences as OA increased TIMP-1 mRNA expression in HT-1080 fibrosarcoma cells [341] while PPP2CA siRNA knockdown decreases it in human HA22T hepatocellular carcinoma cells [474]. In the current study, we did not find any evidence that pharmacological inhibition of PP2B (CSA) and PP1 (TMC) alter TIMP-1 expression in the cell lines used. While there is no published literature on TMC regulating TIMP-1, there are conflicting studies with regard to CSA. For example CSA decreases TIMP-1 mRNA but not protein expression in gingival fibroblasts [475], but increases it in MRC-5 fibroblasts and HK-2 renal tubular epithelial cells [476]. However, these studies are limited in that the concentrations of CSA used are well above that associated with selective inhibition of PP2B. Furthermore, we are the first to report that pharmacological inhibition of PP2A, PP2B and PP1 do not affect TIMP-2, -3 or -4 expression in our cell lines, although silencing of CIP2A increases TIMP-4 mRNA expression in MCF-7 cells [477].

When we investigated the effect of OA, CSA and TMC on MMP expression it was found that expression of MMP-1, 2, 3 and 13 in Hep3B and LX-2 hepatic stellate cells was unaltered. In contrast, OA increases, MMP-1 promoter activity in murine and human fibroblasts [341, 342], MMP-2 expression and mRNA stability in U937 cells exposed to amsacrine [478], MMP-3 in HT1080 cells [342] and MMP-13 in murine fibroblasts [479]. The reported literature is less consistent with regard to CSA as it decreases MMP-1 in human gingival fibroblasts but increases it in HUVEC cells [475, 480]. In the present study, TMC did not alter expression of MMP-1, 2, 3 or 13 in either Hep3B or LX-2 hepatic stellate cells. While this has not been assessed before, activation of PP1 decreases MMP-1 expression in fibroblasts [337].

With regard to MMP-9 the story is quite different. As previously reported by our group, OA increases MMP-9 expression and activity in murine fibroblasts. We extend previous work to show that TMC and CSA do not affect MMP-9 expression or abundance in either cell line. Although, when the temporal response was investigated, TMC elicited a rapid but transient increase in MMP-9 expression, indicating that PP1 may be involved; OA produced a sustained response. The transient effect of TMC is likely an "off target" response, as knockdown of the catalytic subunit of PP1 did not alter MMP-9 activity or abundance. Interestingly, the authors postulate that the increase in MMP-9 mRNA expression by TMC is as a result of TMC inhibitory capacity towards PP2A [74]. In contrast PPP2CA knockdown increases MMP-9 activity and abundance in Hep3B cells and HA22T cells [474], but did not alter MMP-9 activity in murine fibroblasts [339]. This may simply reflect species differences or regulation via other PP2A subunits, but this was not investigated in our earlier paper. However, in Hep3B cells knockdown of the PPP2R1A scaffolding subunit of PP2A is not involved in regulating MMP-9.

The MMP-9 promoter contains multiple transcription factor binding sites including three AP-1 sites and one NF-κB site [481, 482]. Mutation of the proximal AP-$1_{(-79)}$ site or NF-$\kappa B_{(-600)}$ attenuated basal MMP-9 promoter activity indicating the importance of these motifs in maintaining constitutive activity of the MMP-9 promoter. Indeed, mutation of the proximal AP-$1_{(-79)}$ site in MDA-MB-231.HTB-94 and HIG-82 cells diminishes basal MMP-9 promoter activity [483, 484]. Moreover, mutation of the proximal AP-$1_{(-79)}$ and distal AP-$1_{(-533)}$ binding site indicating that they are required for activation of the p1285-luc promoter by OA in Hep3B cells, while the NF-κB motif is not. Previous studies have shown OA to enhance expression of JunB and trans-activation of AP-1 complexes containing c-Jun and JunB to regulate MMPs [342, 485].

While OA is a marine toxin widely used as a pharmacological inhibitor of PP2A, few studies have investigated the effects of CIP2A and SET-

mediated inhibition of PP2A. This may be fundamentally different as OA is a small molecule, which binds to His191 and Gln122 at the hydrophobic end of a surface pocket of the catalytic subunit of PP2A, while SET interacts through interactions with the catalytic subunit [224, 486]; the precise interaction between CIP2A and PP2A remains to be determined as not crystal structure yet exist, CIP2A has been found to interact with the PR65 (scaffold; A) subunit of PP2A by immunoprecipitation [227]. While over-expression of SET decreases MMP-9 mRNA expression in neck squamous cell carcinoma [487] we show that overexpression of CIP2A also increases MMP-9 activity and abundance in Hep3B cells. This was associated with decreased PP2A activity and increased invasiveness of Hep3B cell across a Matrigel membrane, which mimicked the effect of OA and silencing of the PP2A catalytic subunit. This is consistent with over-expression of CIP2A promoting cell invasion in lung adenocarcinoma [251] and silencing reducing RCC migration and invasion [488]. Importantly, the increase in cell invasiveness in the present study is likely due to MMP-9 rather than MMP-1, -2, -3 and -13 mediated degradation collagen type IV in the matrigel basement membrane, as their expression was unaltered. Clinically, our data may in part explain why increased CIP2A expression in many cancers including hepatocellular carcinoma [358] is associated increased tumour aggressiveness and poor prognosis [488-490].

Though it is recommended that pooled siRNA is used when undertaking knockdown experiments, our Dicer-substrate-siRNA (DsiRNA) proved to be efficient at gene knockdown of the various subunits of PP2A and PP1. DsiRNAs are chemically synthesized 27mer duplex RNAs that have increased potency in RNA interference compared to traditional, 21mer siRNAs [491]. Though they have been found to have a greater efficiency at gene silencing, the authors acknowledge that a pooled DsiRNAs would have been the more appropriate choice due to decrease in off-target knockdown. However, this was not achieved due to cost limitations with this study.

In conclusion, the increase in MMP-9 activity and total protein via PP2A catalytic subunit suppression required both AP-1 binding sites on the promoter and reduced PP2A activity. Pharmacological effects resulting from OA exposure was mimicked using both siRNA knockdown and by the over-expressing an endogenous PP2A inhibitor, CIP2A. This study directly links loss of PP2A activity with an increase in cell invasion and ECM remodelling, with MMP-9 being the predominant driving force. Our study indicates that other members of the MMP and ADAM family are not affected by PP2A, PP1 or PP2B inhibition. Interestingly, reduced phosphatase activity does not alter TGF-β or IL-1β, both of which are key in driving the inflammatory aspect of ECM remodelling. Other factors that influence tumour ECM remodelling such as the pro-inflammatory chemokine IL-8 and TIMP-1 may too have a role to play in aiding MMP-9 mediated remodelling in Hep3B cells. These functional consequences of PP2A inhibition resulting in an increase in MMP-9, makes targeting PP2A and attractive option in the development of a therapy for hepatocellular carcinoma.

Chapter 5

PP2A Regulation of Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP-1)

5.1 Introduction

The extracellular matrix (ECM) is a dynamic structure that not only provides a scaffold for organising tissue architecture but also contributes signals that regulate cell function. Remodelling of the ECM is controlled, in part, by the actions of members of the matrix metalloproteinase (MMP) family. It is postulated that matrix remodelling is the result of a shift in the balance between active MMPs *vs.* tissue inhibitors of metalloproteinase (TIMPs), which regulate local MMP activity by inhibiting their enzymatic function [282]. However, recent studies have shown secondary moonlighting functions of TIMP-1 within tumours; TIMP-1 regulates apoptosis, tumour metabolism, and confers drug resistance to tumour cells [292, 492, 493]. Overexpression of TIMP-1 is found in pancreatic cancer and lung adenocarcinoma [296, 494], while high serum levels of TIMP-1 are linked with poor prognosis in patients with breast cancer and HCC [444, 495].

It appears that the role of TIMP-1 on tumour progression is dependent on the type of cancer. To date, there are a limited number of studies on the role of TIMP-1 in HCC progression. Overall, low levels of TIMP-1 correspond to an increase in mean survival time, while high TIMP-1 expression is linked to poor prognosis [492, 496]. TIMP-1 is also a contributory factor in the formation of the liver cancer-associated metastatic niche formation [291]. The pro-metastatic and oncogenic functions of TIMP-1 have been linked to tumour proliferation and evasion of apoptosis through MMP-independent CD63-TIMP-1 signalling [497, 498]. Interestingly, this signalling mechanism of CD63-TIMP-1 confers resistance to anoikis in melanoma cells by activating PI3-K signalling pathways, independently of Akt phosphorylation through activation of β1-intergin signalling [293]. These secondary functions of TIMP-1 and its potential MMP-independent role in HCC tumour progression highlight the need for a greater understanding of its signalling and transcriptional regulation.

Little is know about the signalling pathways and the transcriptional regulation of TIMP-1 in hepatic cells. The difficulty in understanding TIMP-1 regulation is that it differs depending upon the stimuli and expressing cell type. In fibroblasts transforming growth factor-β (TGF-β) increases TIMP-1 expression through the induction of activator protein-1 (AP-1) [499]. Histone deacetylase and extracellular regulated kinase (ERK) signalling may also be required for expression of TIMP-1 in fibroblasts [500]. ERK1/2 or p38 kinase (p38K), but not c-Jun N-terminal kinase (JNK), is required for oncostatin M-induced expression of TIMP-1 in murine fibroblast [501]. In rat granulosa cells, PKA, p38 and ERK1/2-selective inhibitors block human chorionic gonadotropin-induced TIMP-1 expression [502]. As well as MAP kinase dependent signalling, TIMP-1 is regulated through inflammatory mechanisms. In astrocytes, knockdown of the p65 unit of nuclear factor NF-κB transcription machinery blocks IL-1β-induced expression of TIMP-1 [503]. LPS and TLR4 dependent signalling has differential effects on TIMP-1; no effect on secreted TIMP-1 expression in cardiomyoblasts [504], decreased expression in primary skin fibroblasts [505], while increasing TIMP-1 in keratinocytes [506] and murine mesangial cells [507].

The transcriptional regulation of TIMP-1 is a complex processes. Structural features of the human, murine, and rat TIMP-1 gene promoters have been described [508]. All three promoters lack a classical TATA box but contain an evolutionary conserved 22-bp serum response element located 75 bp upstream of the major transcription start site [288, 508, 509]. The serum response element, which is critical for responsiveness of the promoter to growth factors, cytokines, and viruses, is composed of binding sites for AP-1 (Fos/Jun), signal transducer and activator of transcription, and Pea3 (Ets) transcription factors. Like our previous studies describing MMP-9, the AP-1 site within the serum response element is critical for basal and inducible transcription of the TIMP-1 promoter [288]. Activation of the AP-1

complex by JunB or c-Jun by transfection with over expression plasmids increases MMP-1 promoter activity in NIH3T3 cells [501]. A commonality with regards the TIMP family is the presence of SP-1 binding sites in the promoter regions of TIMP-1, 2, 3, 4 [288, 510-512]. However, TIMP-1 is unique in being the only member of the family to possess a CCAAT enhancer-binding protein region within its promoter [288].

There is a paucity of data to the role of protein phosphatase in the regulation of TIMP-1, and what is know is contradictory. Okadaic acid increases TIMP-1 mRNA expression in HT-1080 cells through a suspected JunB mediated event [342], while low concentrations of okadaic acid and PPP2CA siRNA knockdown decreases TIMP-1 expression in HA22T cells [474]. In chapter four, we described an increase in TIMP-1 mRNA in Hep3B cells and a decrease in LX-2 cells following exposure to OA. As with our previous study dealing with PP2A regulation of MMP-9, we sought to investigate the role of PP2A/OA in regulating TIMP-1 using a variety of molecular techniques such as RT-PCR, ELISA, Bioinformatics and Luciferase Reporter Assays. Thus, the aims of this study were to investigate:

1. The effects of okadaic acid, LPS, TNF-α and PMA on TIMP-1 expression in hepatocellular and hepatic stellate cell lines.
2. Investigate the role of NF-κB, PI3-K, JNK and MEK signalling in OA mediated TIMP-1 regulation.
3. Delineate PP2A subunit involvement in TIMP-1 regulation.
4. Compare TIMP-1 promoter sequences and demarcate potential putative transcription factor binding sites and assess their role in PP2A mediate TIMP-1 regulation.

5.2 Methods

5.2.1 Materials

Okadaic acid (OA) was purchased from Calbiochem (Germany). Human recombinant TNF-α was purchased from Prospec (Israel). LPS from *Escherichia coli* and PMA (Phorbol 12-myristate 13-acetate) were purchased from Sigma-Aldrich (Ireland). NF-κB inhibitors, NFKB III and BAY 11-7088 were obtained from Sigma-Aldrich (Ireland) and Calbiochem (Germany) respectively. LY-294002, PD-0325901 and SP-600125 were all obtained from Sigma-Aldrich (Ireland). LX-2 hepatic stellate cells were a gift from Prof Scott L. Friedman, Mount Sinai School of Medicine, USA, while Hep3B and Huh-7 cells were a gift from Dr Steven Gray, Trinity College Dublin, Ireland. DNA primers were obtained from Metabion (Germany) and Integrated DNA Technologies (Belgium). siRNA duplexes were acquired from Integrated DNA Technologies (Belgium). All other reagents unless specified were obtained from Sigma-Aldrich (Ireland).

5.2.2 Cell Culture

Human epithelial hepatocellular carcinoma (Hep3B), hepatocellular carcinoma (Huh-7) and hepatic stellate (LX-2) cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and 4 mM L-glutamine, at 37°C in an humidified atmosphere containing 5% CO_2. All experiments were performed under serum free conditions. siRNA knockdown and DNA transfection were performed under antibiotic free conditions.

5.2.3 Plasmids

TIMP-1 reporters construct (pGL3-TIMP1-1718/+988-Luc (p-TIMP-1-1718/+988), pGL3-TIMP1-1718/+96-Luc (p-TIMP-1-1718/+96), pGL3-TIMP1-738/+96-Luc (p-TIMP1-738/+96), pGL3-TIMP-1 -102/+96-Luc

(p-TIMP-1 -102/+96) and pGL3-TIMP-1 -102/+96$_{(AP\text{-}1\ mutant)}$-Luc (p-TIMP-1 -102/+96$_{(AP\text{-}1\ mutant)}$ were kind gifts from Prof Ian Clark, University of East Anglia, UK. The plasmid pRL-CMV was purchased from Promega (Ireland). The CIP2A expression plasmid, pcDNA3.1_CIP2aflag_WT, was kindly provided by Prof J Westermarck, University of Turku, Finland. The SET expression plasmid pcDNA-SET-FLAG-HA was a gift from Prof Judy Lieberman, Harvard Medical School, USA. The TIMP-1 expression plasmid p6610 MSCV-IP N-HAonly TIMP1 was a gift from Prof Peter Howley, Harvard Medical School, USA. All other plasmid constructs used were designed using MatInspector software (See 5.2.9) and synthesised and validated by GenScript (USA).

5.2.4 Transformation of *E. coli*

Chemically competent TOP10 *E. coli* (transformation efficiency of $1.6x10^7$ cfu/μg DNA) were transformed with 10 ng of DNA by heat shock (42^{0}C for 30 seconds) and immediately placed on ice for 5 min. A negative control (no DNA) was also included. Transformed cells were allowed to recover for 1 h in BHI medium before being used to inoculate LB agar plates (1g/l LB agar, 100 μg/ml ampicillin) overnight at 37^{0}C in a shaking incubator. Single colonies were lifted and plated twice more to ensure transformation efficiency and antibiotic resistance. Finally, a single colony was chosen and grown in LB-medium (20 g/l LB medium, 100 μg/ml ampicillin) at 37^{0}C with shaking (100 rpm, overnight). Plasmids were extracted and purified using PureYield™ Plasmid Midiprep System (Promega).

5.2.5 Semi-Quantitative Real-Time PCR

Cells were grown in 12-well plates (8×10^4 cells per well) and total RNA isolated using TRI Reagent™ (Sigma-Aldrich, Ireland). Following treatment with DNase I (Sigma-Aldrich, Ireland), RNA was reverse transcribed using random hexamers and ReverseAid reverse transcriptase (Thermofisher, Dublin, Ireland). TIMP-1, CIP2A, SET and

GAPDH mRNA expression was analysed by semi-quantitative PCR using target specific primers (Table 1) and GoTaq qPCR Master Mix from (Promega, Ireland) on a Mx3000P qPCR system (Agilent Technology, Germany). Gene expression was quantified using the comparative Ct method [2-ΔΔCt; 460]. For each primer pair, a no template control and no RT control was included. Authenticity of the primer sequences was verified by nucleotide search (Primer-BLAST; NCBI), while the size and specificity of the amplicons were checked by gel electrophoresis with UV trans-illumination (Fusion Fx imaging system, Vilber Lourmat, Marne-la-Vallée Cedex, France).

Gene ID	NM ID	Forward Sequence	Reverse Sequence
TIMP-1	NM_003254.2	CATCCGGTTCGTCTACACCC	TCTGCAGTTTGCAGGGGATG
PPP2CA	NM_002715.2	GCCTCTGCGAGAAGGCTAAA	GAATGGTGATGCGTTCACGG
PPP2R1A	NM_014225.5	AAAGGGACGGAGCCAAGATG	GGGTAGTGAAGGTTCCCAGC
CIP2A	NM_020890.2	TGGACCCACAAATCACCTCG	ATTACCTCCAAGTGCCGCAA
SET	NM_001122821.1	TTAGTGACTGGGGAAGCGTG	CCTTTACTCGGACACCGACC
GAPDH	NM_002046.5	CAAGAGCACAAGAGGAAGAGAG	CTACATGGCAACTGTGAGGAG

Table 5.1: List of primers and their sequences used in this study.

TIMP-1, PPP2CA, PPP2R1A primers were obtained from Metabion while CIP2A, SET and GAPDH were obtained from IDT.

5.2.6 siRNA Knockdown of PP2A Catalytic and Scaffolding Subunits

Hep3B cells, LX-2 and Huh-7 were seeded in 24-well plates (4 x 10^4 cells per well) 24 h prior to transfection with siRNA targeting PPP2CA (10 nM; catalytic subunit of PP2A), PPP2R1A (10 nM; scaffolding subunit of PP2A), Integrated DNA Technology, USA) or non-target siRNA (Fisher, USA) using HIPerFect as per the manufacturer's instructions (Qiagen, UK); a mock transfection was also included as a control. For optimum transfection efficiency, the siRNA oligonucleotides and transfection reagent were incubated together for 30 min prior to Hep3B, LX-2 and Huh-7 transfection. Knockdown of PPP2CA, PP2R1A was confirmed by qPCR as described. The culture medium was collected 48 h later, and TIMP-1 abundance was determined using ELISA.

siRNA duplex		Sequence
PPP2R1A	Sense	5'-rArGrGrCrGrGrArArCrUrUrCrGrArCrArGrUrA-3'
	Anti-Sense	5'rArArArCrUrUrArArCrUrCrCrUrUrGrUrGrCrA'3
PPP2CA	Sense	5'-rGrGrArUrArGrCrArGrCrArArArCrArArUrCrArUrUrGrGrArG-3'
	Anti-Sense	5'-rCrUrCrCrArArUrGrArUrUrGrUrUrUrGrCrUrGrCrUrArUrCrCrUrU-3'

Table 5.2: List of dicer siRNA used and their sequences

5.2.7 Overexpression of CIP2A and SET

To assess the effect of overexpression of CIP2A and SET protein on TIMP-1 expression levels in a model of HCC, Hep3B cells were seeded (2 x 10^5 cells per well) in 6 well in plates, and 24 h later transiently transfected with 1 μg of pcDNA3.1_CIP2aflag_WT plasmid (pCIP2A), pcDNA-SET-FLAG-HA (pSET), p6610 MSCV-IP N-HAonly TIMP1 or (pTIMP1) or pcDNA.3.1 (pDNA.3.1) control plasmid using PolyFect (Qiagen) in serum and antibiotic free DMEM. CIP2A, SET and TIMP-1 over expression was confirmed 24 and 48 h post-transfection by qPCR while ELISA assessed TIMP-1 protein expression at 48 h. The effect of overexpression of CIP2A and SET on TIMP-1 abundance was determined in conditioned media 48 h post transfection using TIMP-1 ELISA.

5.2.8 TIMP-1 ELISA

The effect of OA, signal transduction inhibitors, silencing of PPP2CA and PPP2R1A by siRNA and overexpression of CIP2A and SET on TIMP-1 abundance was determined in conditioned media using ELISA following the vendor's protocol (DuoSet; R&D Systems, UK). Culture media was reserved 24 h post-drug exposure and at 48 h post-transfection. All samples were stored at -80°C until required.

5.2.9 Identification Of Putative Transcription Factor Binding Sites (TFBS) in the TIMP-1 promoter

For identification and promoter sequence comparison, the Eukaryotic Promoter database was used to identify the TIMP-1 promoter sequence [513]. The sequence was then analysed by MatInspector, which is software that looks for putative binding sites in DNA sequences according to position specific score matrix (PSSMs). Comparative promoter analyses were then performed by input of these sequences in FASTA format into MatInspector using optimised default matrix similarity thresholds. Similar and/or functionally related TFBS were grouped into "matrix families" and graphical representations of common TFBS were generated. The 'V$' prefix to the individual matrices are representative of the Vertebrate MatInspector matrix library. The "core sequence" of a matrix is defined as the (usually four) highest conserved positions of the matrix that is provided in upper case letters in our Tables. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix exactly matches the sequence. Only matches that contain the "core sequence" of the matrix with a score higher than the core similarity are listed in the output. The core similarity used in this study was 0.8. Following identification of the putative transcription factor, the TIMP-1 promoter sequence from -890 to +96 was synthesised by GenScript and cloned into a pUC57 vector with Hind III and XhoI restriction sites. The -890/+96 pTIMP-1 plasmid was sub-cloned into a pGL3-basic vector (pGL3-TIMP-890/+96). Following identification of putative CREB (-231), AP-1 (-351) and PEA3 (-460) transcription factor binding sites by MatInspector, three mutant p-890/+96 pTIMP-1 plasmids were generated; pGL3-TIMP-890/+96 $_{\text{CREB (-231)}}$, pGL3-TIMP-890/+96 $_{\text{AP-1 (-351)}}$ and pGL3-TIMP-890/+96 $_{\text{PEA3 (-460)}}$ using GenScript custom gene synthesis. The resulting plasmids were transformed using *E. coli* TOP10 (Invitrogen, USA), followed by plasmid preparation and transformation. Plasmids were isolated using PureYield™ Plasmid Midiprep System (Promega).

5.2.10 TIMP-1 Dual-Reporter Luciferase Assay

Hep3B cells were transfected with 500 ng/well of the full length pGL3-TIMP1-1718/+988-Luc plasmid or truncated (pGL3-TIMP1-1718/+96-Luc, pGL3-TIMP1-738/+96, pGL3-TIMP-890/+96 (-890/+96), TIMP-1 or pGl3-TIMP-1 -102/+96-Luc in conjunction with pRL-CMV (1 ng; internal standard) using PolyFect (Qiagen) and basal promoter activity assessed in Hep3B cells. In complementary experiments, cells were transfected with pGL3-TIMP-890/+96, pGL3-TIMP1-738/+96, pGL3-TIMP-890/+96 $_{\text{CREB (-231)}}$ (-890/+96 $_{\text{CREB (-231)}}$), pGL3-TIMP-890/+96 $_{\text{AP-1 (-351)}}$ (-890/+96 $_{\text{AP-1 (-351)}}$) or pGL3-TIMP-890/+96 $_{\text{PEA3 (-460)}}$ (-890/+96 $_{\text{PEA3 (-460)}}$) and exposed to OA (40 nM) or media for 24 h or co-transfected with pCIP2A or pDNA.3.1, before being harvested and lysed. Firefly and *Renilla* luciferase activity was evaluated using a Dual-Luciferase Reporter Assay System (Promega) to measure transcriptional activity with the aid of a luminometer (Thermo Fisher Scientific, Dublin, Ireland). Reporter construct responses (firefly) were normalised to internal standard (Renilla) and fold increases calculated relative to controls.

5.2.11 Statistic and data analysis

All data were normalised to the appropriate control and expressed as either an absolute value, a percentage or ratio, with the exception of the TIMP-1 reporter data, which was expressed as the ratio of firefly luciferase to *Renilla* luciferase activity normalised to control (medium) or as a ratio expressed mock construct (ΔTIMP-1). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett, Bonferroni). Data are expressed as mean ± S.E.M., and a value of $P < 0.05$ is taken to indicate statistical significance.

5.3 Results

5.3.1 Differential regulation of TIMP-1 expression and abundance by PMA, LPS, TNF-α and Okadaic acid in Hep3B, Huh-7 and LX-2 cell lines

PMA (50 ng/ml) increased TIMP-1 expression by 2.73 ± 0.19 fold, 2.65 ± 0.14 fold and 2.9 ± 0.35 fold in Hep3B cells, Huh-7 cells and LX-2 cells respectively ($P < 0.05$; Figure 5.1A-C). Exposure to LPS (50 ng/ml) augmented TIMP-1 expression by 1.9 ± 0.16 fold in Hep3B cells, 1.66 ± 0.14 fold in Huh-7 cells and 3.67 ± 0.3 fold LX-2 cells ($P < 0.05$; Figure 5.1A-C). Interestingly, TNF-α (10 ng/ml) did not alter TIMP-1 mRNA expression in either Hep3B or Huh-7 cells (Figure 5.1A,B), but increased it by 1.86 ± 0.15 fold in LX-2 cells ($P < 0.05$; Figure 5.1C). Okadaic acid (OA; 40 nM) increased TIMP-1 expression by 1.58 ± 0.37 fold in Hep3B cells, 1.31 ± 0.2 in Huh-7 (Figure 5.1A,B), but decreased it by 0.74 ± 0.08 fold in LX-2 cells ($P < 0.05$; Figure 5.1C).

At the protein level TIMP-1 abundance under basal conditions was 871.01 ± 102 pg/ml in Hep3B cells, 544.25 ± 48.23 pg/ml in Huh-7 cells and 1079.92 ± 83.55 pg/ml in LX-2 cells (Figure 5.2; $P < 0.05$). PMA (50 ng/mL) increased secreted TIMP-1 abundance to 1802.46 ± 227.90 pg/ml in Hep3B cells, 1156.14 ± 157.65 pg/ml in Huh-7 cells and to 2061.11 ± 129.28 pg/ml in LX-2 cells ($P < 0.05$; Figure 5.2). LPS (50 ng/m) increased TIMP-1 secretion to 1985.47 ± 116.59 pg/ml in Hep3B cells, 1275.76 ± 88.21 pg/ml in Huh-7 and 3019.28 ± 65.29 pg/ml in LX-2 cells ($P < 0.05$). TNF-α had no effect on TIMP-1 abundance in conditioned media from Hep3B or Huh-7 cells (Figure 5.2) but increased it in LX-2 cells to 1948.42 ± 191.82 pg/ml ($P < 0.05$; Figure 5.2). Okadaic (40 nM) acid increased TIMP-1 abundance in Hep3B and Huh-7 ($P < 0.05$; Figure 5.2). However, it decreased TIMP-1 abundance in conditioned media from LX-2 cells to 497.1 ± 71.83 pg/ml (Figure 5.2).

5.3.2 Concentration dependent effect of okadaic acid on TIMP-1 expression and abundance in Hep3B, Huh-7 and LX-2 cell lines

To assess the concentration dependence of the okadaic acid response, each hepatic cell line was exposed to increasing concentrations of OA (0-100 nM). In Hep3B cells, 1 nM of OA decreased expression and abundance of TIMP-1 by 0.70 ± 0.03 fold and 293.8 ± 79.28 pg/ml respectively ($P < 0.05$; Figure 5.3A and 5.4A). This effect was lost at a concentration of 10 nM and 20 nM OA. OA at a concentration of 40nM and higher, increased TIMP-1 expression and abundance by 1.47 ± 0.32 fold and 593.1 ± 89.35 pg/ml respectively ($P < 0.05$; Figure 5.3A and B).

In Huh-7 cells, OA at a concentration of 20 nM and below did not alter TIMP-1 expression or abundance, while both were increased at concentrations of 40 nM and greater ($P < 0.05$; Figure 5.3B and 5.4B).

In LX-2 cells, OA decrease expression of TIMP-1 by ~0.6 fold at concentrations of 10-40 nM. The abundance of TIMP-1 in condition media was reduced by 275-640 pg/mL at concentrations of 1-40 nM ($P < 0.05$; Figure 5.3C and 5.4C). At the highest concentration studied OA (100 nM) did not affect expression or abundance of TIMP-1 in LX-2 cells.

5.3.3 PP2A catalytic and scaffolding subunit regulate TIMP-1 Expression in Hep3B, Huh-7 and LX-2 cells

To establish if the catalytic subunit (PPP2CA) and scaffolding subunit (PPP2R1A) of PP2A are involved in regulating TIMP-1, a siRNA silencing approach was utilised. Transfection of Hep3B, Huh-7 ad LX-2 cells with siRNA targeting PPP2CA and PPP2R1A decreased mRNA expression by ~70-80% compared to mock/NT-siRNA transfection controls ($P < 0.05$; Figure 5.5A-C). NT targeting siRNA and mock transfection had no effect on expression of PPP2CA or PPP2R1A in any of the cell lines studied.

In Hep3B, Huh-7 and LX-2 cells knockdown of the PPP2CA subunit decreased mRNA expression of TIMP-1 by 0.59 ± 0.08, 0.53 ± 0.06 and 0.64 ± 0.1 fold respectively compared to mock transfected cells ($P < 0.05$; Figure 5.6A;). Knockdown of the PPP2R1A subunit did no affect mRNA expression of TIMP-1 in any of the cell lines studied. However, there was a tendency for silencing of PPP2R1A to decrease mRNA expression of TIMP-1 in LX-2 cells (Figure 6.5A). NT targeting siRNA and mock transfection had no effect on TIMP-1 mRNA expression in any cell line studied.

Similarly, silencing of the PPP2CA subunit decreased TIMP-1 abundance in conditioned media from Hep3B, Huh-7 and LX-2 cell lines ($P < 0.05$; Figure 5.6B). Silencing of the PPP2R1A subunit did not alter TIMP-1 abundance in conditioned media from Hep3B or Huh-7 cell lines, but did decrease it in conditioned media from LX-2 cells ($P < 0.05$; Figure 5.6B). NT-targeting siRNA and mock transfection did not alter TIMP-1 abundance in conditioned media from any cell line.

Taken together these data suggest a commonality between the three cell lines in that the catalytic subunit of PP2A decreases TIMP-1 mRNA expression and abundance. Based upon this, we focused our attention on the Hep3B cell line in subsequent experiments.

5.3.4 Effects of CIP2A and SET overexpression on expression of TIMP-1 in Hep3B cells

As OA and knockdown of the catalytic subunit of PP2A differentially regulated TIMP-1 expression, we investigated the role CIP2A and SET in regulation of TIMP-1. Transfection of Hep3B cells with a CIP2A expression plasmid increased mRNA expression of CIP2A by 10.96 ± 0.41 ($P < 0.05$; Figure 5.7A). Similarly, transfection of the same cell line with a SET augmented mRNA expression of SET by 22.65 ± 0.33 fold ($P < 0.05$; Figure 5.7B). Mock transfection and transfection with the empty plasmid (pDNA3.1) had no effect on mRNA expression of either CIP2A or SET.

CIP2A overexpression in Hep3B cells decreased TIMP-1 abundance in conditioned media by 0.65 ± 0.09 fold, while SET overexpression attenuated it by 0.83 ± 0.02 fold ($P < 0.05$; Figure 5,7C) compared to mock transfected cells. Transfection with pDNA3.1 or mock transfection had no effect on TIMP-1 abundance in conditioned media from Hep3B cells. Interestingly, when OA (40 nM) is added 24 h post transfection, TIMP-1 abundance in condition media was higher in CIP2A, pSET, pDNA3.1 and mock transfected cells compared to their corresponding counterpart in the absence of OA ($P < 0.05$; Figure 5.7D).

5.3.5 TIMP-1 signal transduction regulation in Hep3B cells

To identify key signalling components through which OA increases TIMP-1 expression, Hep3B cells were exposed to a panel of cell signalling inhibitors. DMSO (0.01% or 0.1% v/v) did not have any effect on basal TIMP-1 abundance in conditioned media from Hep3B cells compared to untreated cells (Figure 5.8A,D). However, cells exposed NF-κB III inhibitor (10 μM) or BAY 11-7085 (10 μM, NF-κB and/or IK-κB inhibitor) reduced TIMP-1 levels by 0.43 ± 0.1 and 0.45 ± 0.04 fold respectively in conditioned media ($P < 0.05$; Figure 5.8A). In contrast Hep3B cells exposed to LY-294002 (10 μM), PD-0325901 (10 nM) and SP600125 (10 μM) did not alter basal TIMP-1 abundance in conditioned media (Figure 8C).

NF-κB III (10 μM) and BAY 11-7085 (10 μM) did not alter the increase in TIMP-1 abundance following exposure to OA indicating that NF-κB signalling is not involved (Figure 5.8B). In contrast, LY-294002 (10 μM; PI3K inhibitor) prevented OA from increasing TIMP-1 abundance in Hep3B cells, while PD-032590 (10 nM; MEK inhibitor) attenuated the response ($P < 0.05$; Figure 5.8D). Interestingly, SP600125 (10 μM; c-Jun inhibitor) in combination with OA not only inhibited the ability of OA to increase TIMP-1 abundance in conditioned medium from Hep3B cells, but also elicited an additional inhibitory effect (~0.5 fold; $P < 0.05$).

5.3.6 Bioinformatic Identification of Putative Transcription Factor Binding Sites within the TIMP-1 Promoter

Using bioinformatics, we compared three potential TIMP-1 promoter sequences as described in Materials and Methods. We identified a region between the transcription initiation start site and -890 upstream of this as having the highest number of conserved putative transcription factor binding sites (Figure 5.9). An alphabetically listed of the conserved vertebrate (V$) matrix families and identified their putative sequences are presented in Table 5.3 and Figure 5.9. Within this region, 75 putative transcription factor-binding sites were identified across the three promoter sequences (Supplemental table 5.1). Key conserved matrix families included binding sites for AP-1, cyclic AMP-responsive element binding proteins, CART proteins, oestrogen response elements, human ETS1 factors, SMAD, and EGRF transcriptional factor-binding sites. Many of the identified transcription factor-binding sites have not previously been shown to be present in the TIMP-1 promoter. Within the list of 75 putative transcription factor binding sites identified, 3 were selected as being of particular interest with regard to PP2A mediate regulation of TIMP-1. These were a cyclic AMP-responsive element (CREB) at position -231, a member of the AP-1 complex JUNB/D at position -351 and a PEA3 complex at position -460. These sites were mutated to assess their role OA-mediated regulation of the TIMP-1 promoter.

5.3.7 Characterisation of the TIMP-1 promoter

TIMP-1 has multiple transcription start sites and GC boxes within its promoter region and as stated previously lacks a well-defined TATA box region (Figure 5.9). To identify key regions that regulate basal TIMP-1 promoter activity as series of truncated TIMP-1 promoter constructs were use.

Basal TIMP-1 reporter activity was 0.77 ± 0.11 fold lower in Hep3B cells transfected with the full-length TIMP-1 reporter plasmid (pGL3-TIMP1-1718/+988-Luc) compared to the pGL3-TIMP1-1718/+96-Luc construct. This would indicate the presence of an inhibitory element in an 892 bp region downstream of position +96. Removal of 826 bases between -1718 and -890 increased TIMP-1 reporter activity by 0.62 ± 0.19 fold increase compared to the pGL3-TIMP1-1718/+96-Luc construct ($P < 0.05$; Figure 5.10A). This was not affected by removing an additional 153 bases (p-738/+96), However, shortening the promoter region by a further 636 bases (p-102/+96), reduced TIMP-1 promoter activity by 0.79 ± 0.07 fold compared to the pGL3-TIMP1-1718/+96-Luc construct (Figure 5.10A; $P < 0.05$).

As the region between -890 and the initiation transcription start site was identified as a "hot" spot for transcription factor binding and was shown to be functionally important, the roles of key motifs within this area were studied. Mutation of $CREB_{(-231)}$ binding site ($p\text{-}890/+96_{CREB\ (-231)}$) reduced basal promoter activity 60 ± 0.07 % compared to the p-890/+96 construct (Figure 5.10B; $P < 0.05$). Mutation of neither the $p\text{-}890/+96_{AP\text{-}1\ (-351)}$ nor $-890/+96_{PEA3\ (-460)}$ altered TIMP-1 promoter activity (Figure 5.10B). As several studies indicate that AP-1 binding motifs proximal to the initiation start site are key to regulation promoter activity, we looked at this in a truncated construct. The $p\text{-}102/+96_{(AP\text{-}1\ Mutant)}$ decreased TIMP-1 promoter activity compared to the p-102/+96 construct confirming the importance of the AP-1 binding motif (-97) to TIMP-1 basal promoter activity (Figure 5.10C; $P < 0.05$).

5.3.8 PP2A inhibition and TIMP-1 promoter activity

OA (40 nM) increased TIMP-1 -890/+96 and -738/+96 promoter activity by 1.28 ± 0.12 and 1.44 ± 0.14 fold respectively (Figure 5.11; $P < 0.05$). With regard to the -890/+96 promoter construct, mutation of the AP-1 motif at position -351 and the CREB motif at position -231 attenuate the OA by 78% and 95% respectively ($P < 0.05$). Mutation of the PEA3 motif at position -460 (p-890/+96$_{PEA3\ (-460)}$) had no effect on the OA response compared to the -890/+96 construct (Figure 5.11). In Hep3B cells transfected with the p-102/+96 promoter construct, OA increased promoter activity by 1.1 ± 0.13 fold (Figure 5.11; $P < 0.05$). This effect was attenuated by 91% on mutation of the AP-1 binding site (Figure 5.11; $P < 0.05$).

As OA and CIP2A differentially modulate TIMP-1 expression (5.3.4) we investigated if CIP2A mediates its effects through regions and motifs. Over expression of CIP2A in Hep3B cells decreased TIMP-1 promoter activity (p-890/+96) by 0.56 ± 0.07 fold compared to non-CIP2A overexpressing cells (Figure 5.12; $P < 0.05$). Interestingly, truncating the -890/+96 construct by 152 bp (p-738/+96 construct) obliterated the ability of CIP2A to decrease TIMP-1 promoter activity ($P < 0.05$; Figure 5.12) following co-transfection with CIP2A, postulating a role for a particular transcription factor-binding motif between position -890 and -738 within the TIMP-1 promoter modulating CIP2A expression of TIMP-1. Mutation of AP-1$_{(-351)}$ or PEA3$_{(-460)}$ binding motifs had no effect on CIP2A mediated TIMP-1 promoter activity. However, mutation of the CREB $_{(-231)}$ binding motif negated the effect of CIP2A (Figure 5.12; $P < 0.05$).

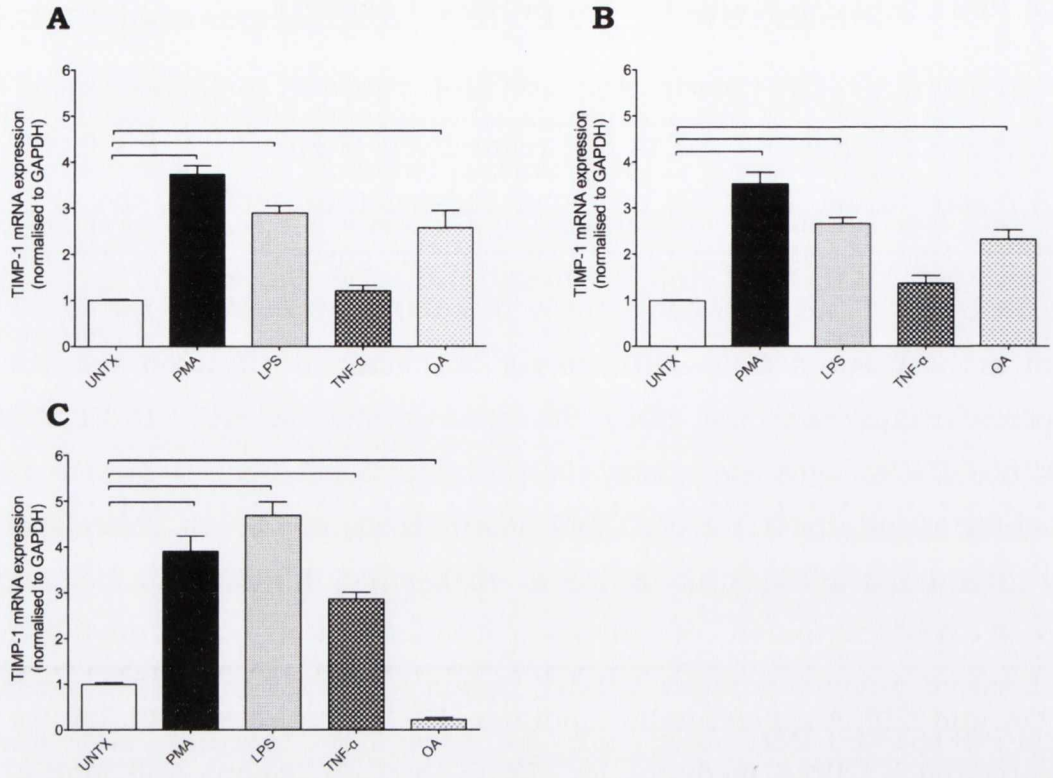


Figure 5.1: TIMP-1 mRNA expression in hepatic cell lines

(A) Hep3B, (B) HUH-7 and (C) LX-2 mRNA expression Cells were seeded $8x10^4$ cells/well in a 12 well plate and allowed to attach in complete media for 24 h. Media was changed to serum free and cells were treated with PMA (50 ng/ml), LPS (50 ng/ml), TNF-α (10 ng/ml) and okadaic acid (OA; 40 nM) for 24 h. TIMP-1 mRNA was determined by qPCR (comparative quantification), and data normalised to GAPDH (mean ± S.E.M). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate significance $P < 0.05$ (n=5).

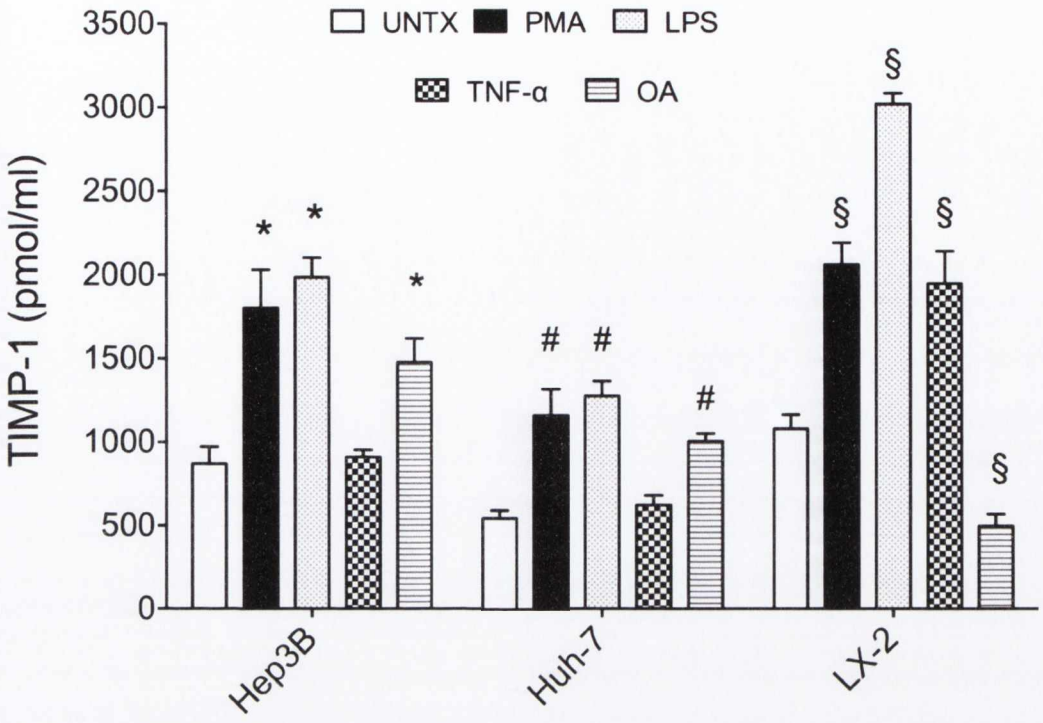


Figure 5.2: TIMP-1 abundance in hepatic cell lines

TIMP-1 secreted TIMP-1 protein expression in Hep3B, Huh-7 and LX-2 cells. Cells were seeded 8 x 10^4 cells/well in a 12 well plate and allowed to attach in complete media for 24 h. Media was changed to serum free and cells were treated with PMA (50 ng/ml), LPS (50 ng/ml), TNF-α (10 ng/ml) and okadaic acid (OA; 40 nM) for 24 h. Secreted TIMP-1 was quantified using ELISA and presented as pg/ml (mean ± S.E.M). Data were analysed by two-way ANOVA with *post hoc* analysis (Dunnett's). *, # and § indicate significance $P < 0.05$ (n=4).

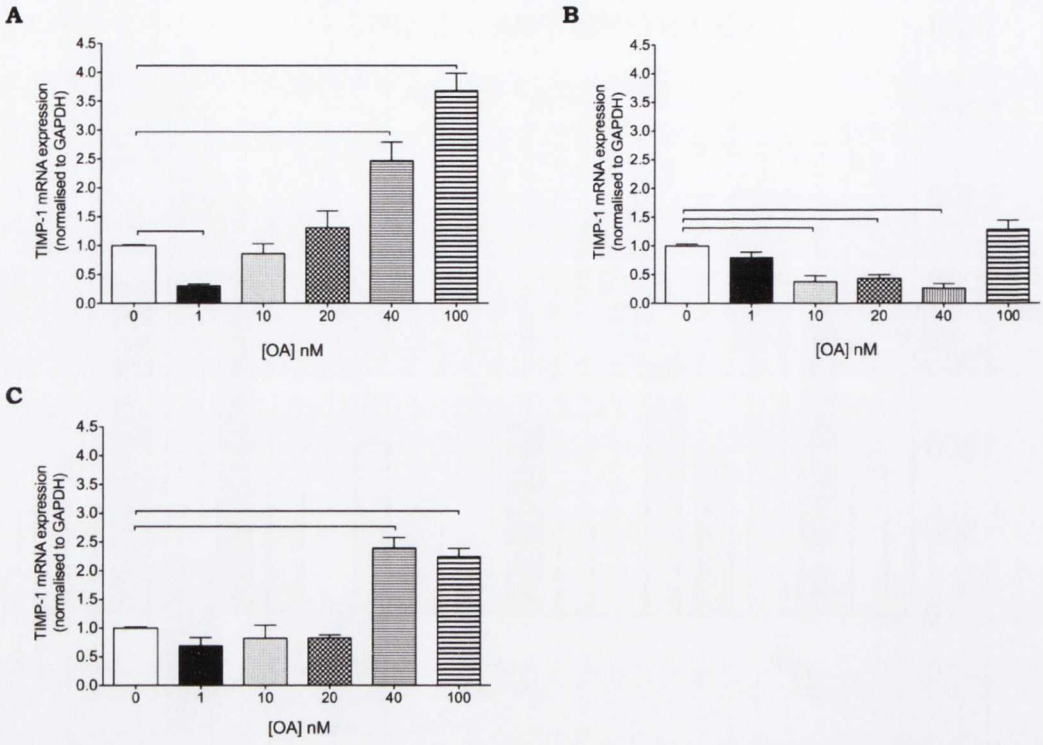


Figure 5.3: Concentration dependent effects of OA on TIMP-1 mRNA expression in hepatic cells

Effect of increasing concentrations of OA (0-100 nM) on TIMP-1 mRNA expression in (A) Hep3B, (B) Huh-7, (C) LX-2 cells. Cells were exposed to OA for 24 h and TIMP-1 mRNA determined by qPCR (comparative quantification) in Hep3B, Huh-7 and LX-2 cells following exposure. Data are normalised to GAPDH (mean ± S.E.M) were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate significance $P < 0.05$ (n=4-6).

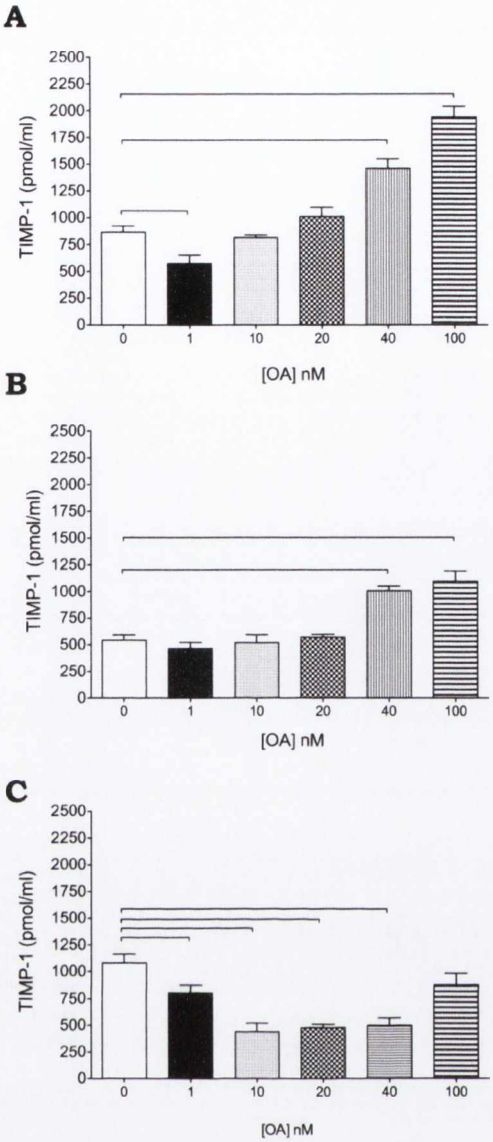


Figure 5.4: Concentration dependent effects of OA on TIMP-1 protein expression in hepatic cells

Effect of increasing concentrations of OA (0-100 nM) on secreted TIMP-1 protein expression in (A) Hep3B, (B) Huh-7, (C) LX-2 cells. Cells were exposed to OA for 24 h. Supernatants were collected, aliquoted and stored at -80^{0C} until analysis by TIMP-1 ELISA. Data are presented as pg/ml (mean ± S.E.M) and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate significance $P < 0.05$ (n=4-7).

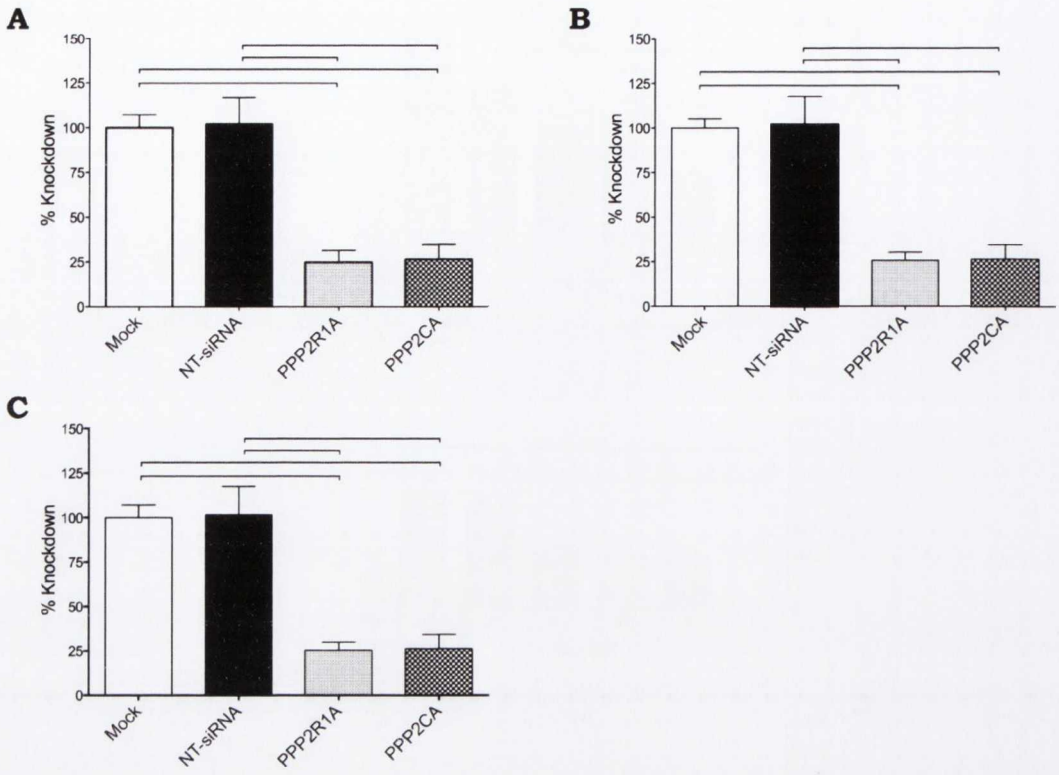


Figure 5.5: Effect of siRNA knockdown of PPP2CA and PPP2R1A on basal mRNA expression

(A) Hep3B, (B) Huh-7 and (C) LX-2 cells were transfected with PPP2CA (PP2A catalytic subunit A), PPP2R1A (PP2A regulatory subunit), and non-target (NT) siRNA for 24 h. A mock transfection (no siRNA) was included as a control. Knockdown was confirmed by RT-PCR and mRNA expression of PPP2CA and PPP2R1A were normalised to baseline and GAPDH. Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

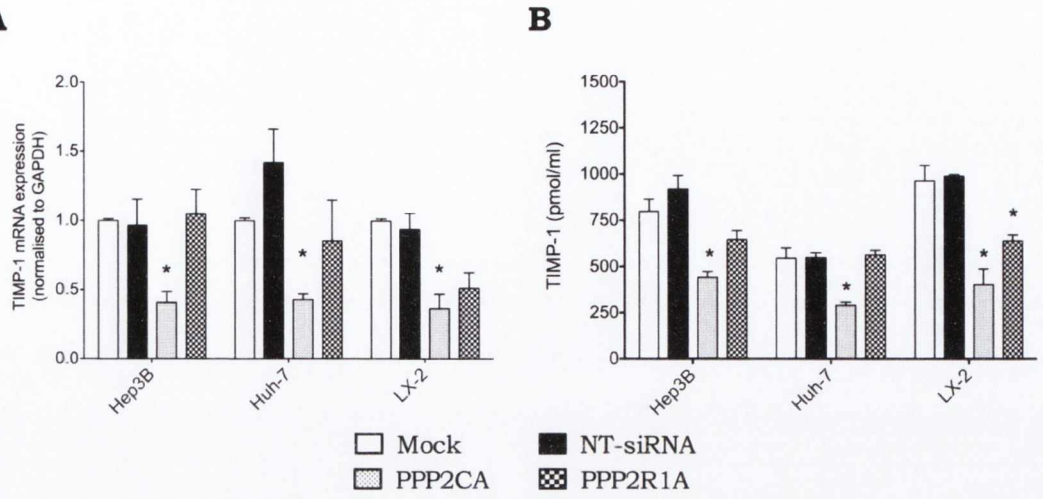


Figure 5.6: Delineation of PP2A subunit involvement in TIMP-1 regulation

The effect of PP2A knockdown on TIMP-1 (A) mRNA expression and (B) protein expression in Hep3B, Huh-7 and LX-2 cell line. Cells were transfected with PPP2CA, PPP2R1A and non-target (NT) siRNA for 24 h. A mock transfection (no siRNA) was included as a control and the effect of knockdown on TIMP-1 expression was confirmed by RT-PCR. (B) For TIMP-1 protein determination, media was replaced after 24 h and TIMP-1 expression was determined 48 h post-transfection. PCR data were normalised to baseline and GAPDH (mean ± S.E.M). Comparative cell line TIMP-1 protein levels were measured by ELISA and presented as pg/ml (mean ± S.E.M). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). * Indicates statistical significance at $P < 0.05$ (n=3-4).

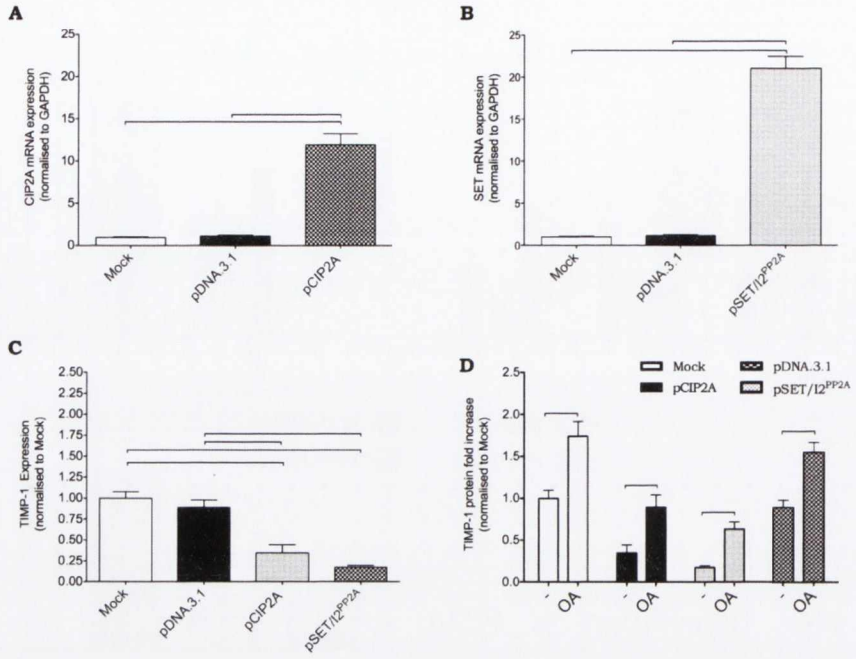


Figure 5.7: Effect of overexpression of PP2A inhibitor proteins on TIMP-1 expression in Hep3B cells. Cells were transfected with 1 μg plasmid DNA (pCIP2A, pSET/I2^{PP2A}) or 1 μg empty vector plasmid (pDNA.3.1) for 48 h. The cell supernatant was collected, aliquoted and stored at 80^{0C} until analysis by human TIMP-1 ELISA. TriReagent was used to extract RNA from cells. (A) Confirmation of relative CIP2A mRNA expression compared to mock and empty vector (DNA.3.1) controls. (B) Confirmation of relative SET mRNA expression compared to mock and empty vector (DNA.3.1) controls. (C) Effect transfection of pCIP2A and pSET/I2^{PP2A} on TIMP-1 protein expression. (D) Effect of OA exposure on pCIP2A and pSET/I2^{PP2A} mediated TIMP-1 protein expression. Cells were exposed to OA (40 nM) 24 h post transfection and incubated for a further 24 h. Cell supernatant was collected, aliquoted and stored at 80^{0C} until analysis by human TIMP-1 ELISA. Data expressed as folds increase compared to mock transfection and empty vector control and were analysed by one-way ANOVA with post hoc analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3-4).

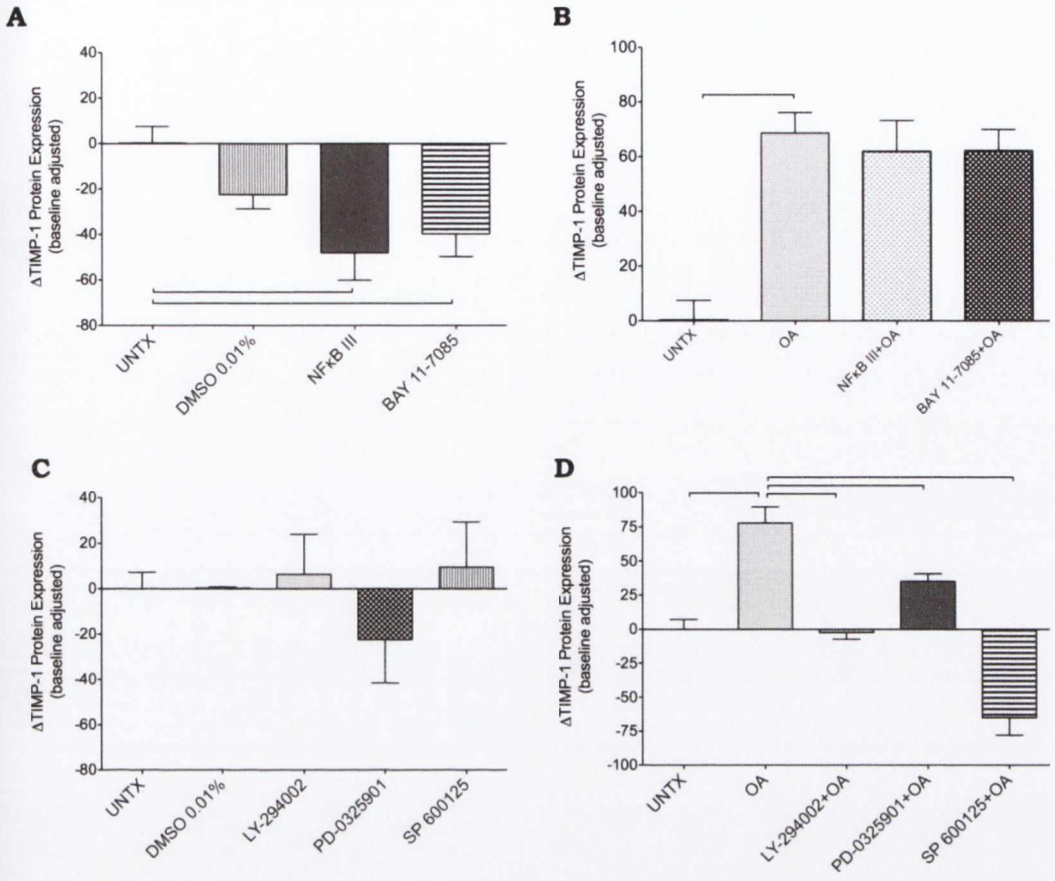


Figure 5.8: Effect of cell signalling inhibitors on OA mediated TIMP-1 expression in Hep3B cells

(A) Effect of NF-κB inhibition on basal TIMP-1 abundance and (B) OA mediated increase. Cells were treated with NF-κB inhibitor III (10 μM) and BAY 11-7085 (10 μM) 30 min prior to exposure to OA for 24 h. (C) Effect of PI3K (LY-294002), MEK-1 (PD-0325901) and c-Jun (SP 600125) inhibition on basal TIMP-1 expression and (D) OA mediated increase of TIMP-1 protein levels. Cells were treated LY-294002 (10 μM), PD-0325901 (10 nM) and SP 600125 (10 μM) 30 min prior to exposure to OA for 24 h. Data expressed as a fold increase compared to untreated and were analysed by one-way ANOVA with post hoc analysis (Dunnett's). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

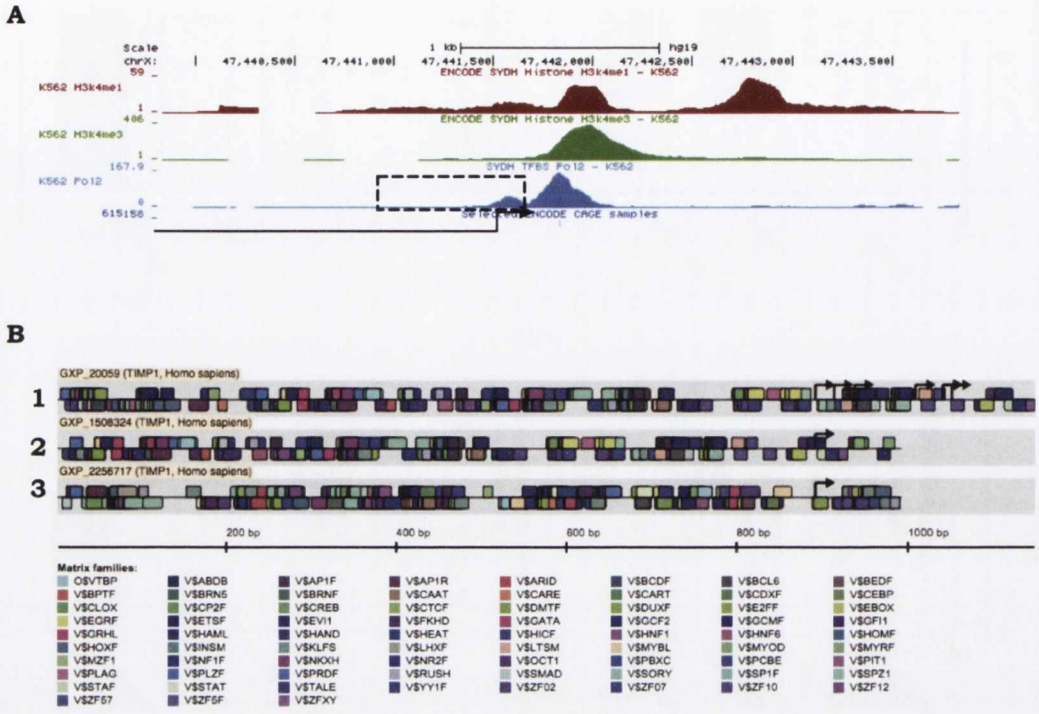


Figure 5.9: Bioinformatic analysis of the TIMP-1 promoter

(A) TIMP-1 promoter region was identified and mapped using eukaryotic promoter database and visualized using UCSC Genome Browser. The promoter region was identified at chromosome X position47, 430,835-47,443,835. The square box highlights the region of interest for MatInspector analysis. See links more information regarding the TIMP-1 promoter region. http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&position=chrX%3A47439816-47443815&hgsid=439417715_muOKEAnVAqAhinaaZJH9Nrr2nHCd

(B) The three known TIMP-1 promoter sequences were compared using MatInspector. A 1 kb fragment of (1) GXP_20059, (2) GXP_1508324 and (3) GXP_2256717 promoter sequences were compared and putative transcription factor binding sites assessed. (2) GXP_1508324 was the transcript on which our promoter plasmids were based from Clark IM, *et al* (1997).

Transcription Factor	Position	Sequence
AP-1	-95	ggatGAGTaatgc
E2F	-134	aagggGCGGgccgacga
CREB	-231	tagtgacTGACgtgga
CCAAT	-322	ctatCCAAtcaccca
AP-1	-351	gtttgaGTCAtag
HOMO box	-362	ctgaacTAATttgggtttg
FKHD	-425	catacaaAACAagggat
ERG	-460	ggcagagaGGAAacaaattaa
SHOX2	-461	ctacttAATTtgtttcctctc
NFAT	-462	gagaGGAAacaaattaagt
GATA	-497	gggaGATAaggca
SWI/SNF	-585	tgCCATgtatt
NF1	-618	caggtggccaggagCCAAagc
HOMO box	-705	agcttTGAGtgagataaac
GATA	-710	gtgaGATAaacta
Myb	-761	tcatccgtaaAACGggaataa
EVI1	-768	ttttacgGATGaggaaa
CCAAT	-772	cggatgagGAAAtat
ETS1	-782	gtaaaacgGGAAtaagaaccg

Table 5.3: MatInspector identified TIMP-1 transcription sites and sequences.

List of transcription factors identified on the TIMP-1 promoter and their position within the p-890/+96 promoter.

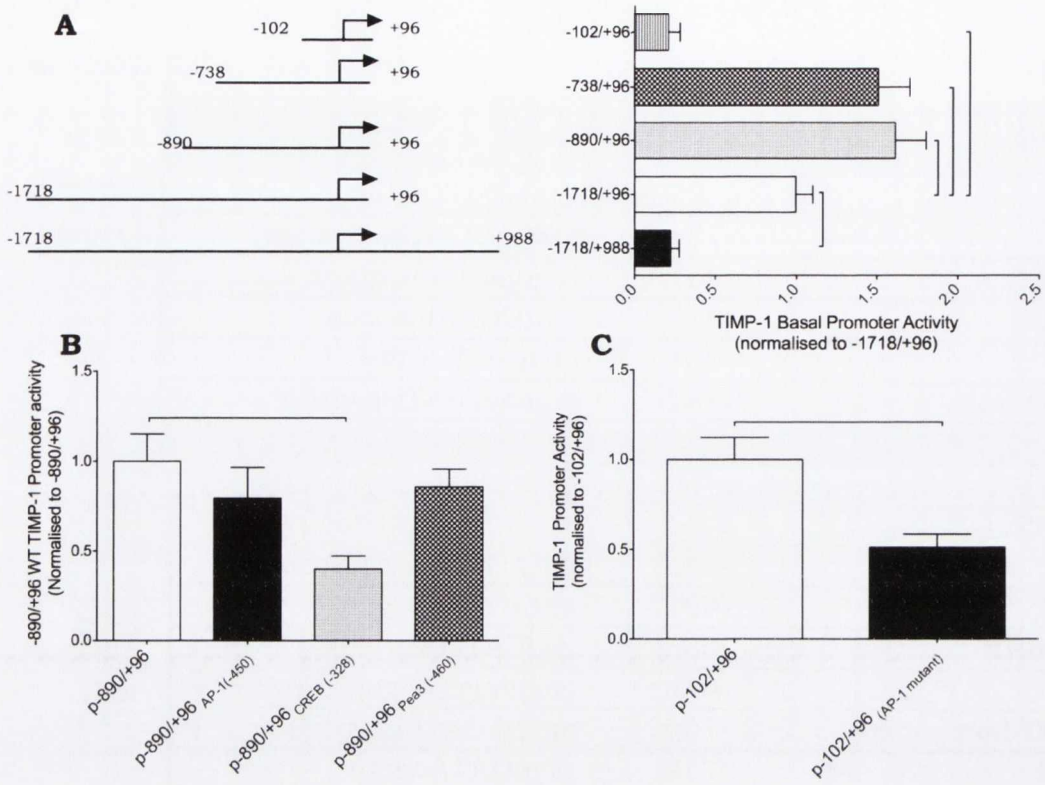


Figure 5.10: Effect of truncation and mutation of AP-1, CREB and PEA3 binding motifs on TIMP-1 promoter activity

(A) Proximal and distal truncation of the TIMP-1 promoter. Hep3B cells were transfected with p-1718/+988, p-1718/+96, p-890/+96, p-890/+96 AP-1 (-351), p-890/+96 CREB (-231), p-890/+96 Pea3 (-460), p-738/+96, p-102/+96 and p-102/+96 AP-1 (mutant) for 24 h and luciferase activity was determined in the cell lysates. Basal activity was assessed in comparison to the full-length p-1718/+988 TIMP-1 wild-type promoter (A), p-890/+96 (B) and p-102/+96 (C). Promoter activity was normalised to the individual control and expressed as a ratio to their relevant construct. Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate statistical significance at $P < 0.05$ ($n \geq 5$).

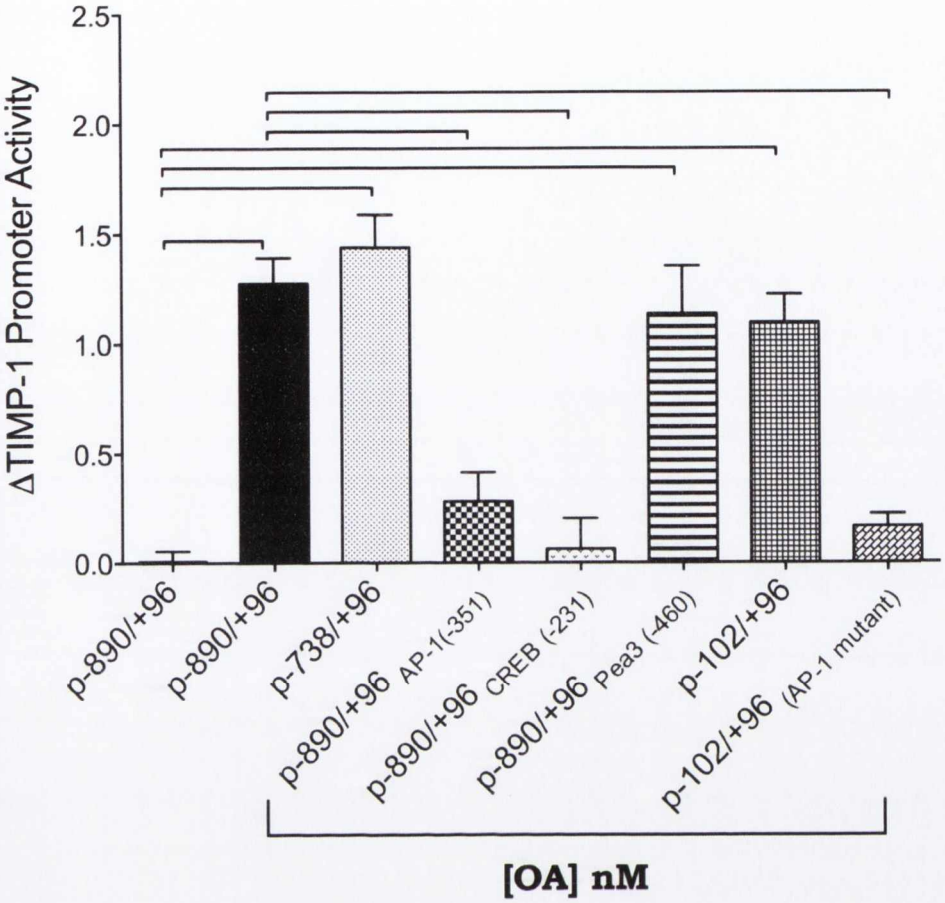


Figure 5.11: Effect of OA on mutant TIMP-1 promoter constructs

Hep3B cells were transfected with p-890/+96, p-890/+96 AP-1 (-351), p-890/+96 CREB (-231), p-890/+96 Pea3 (-460), p-738/+96, p-102/+96 and p-102/+96 AP-1 (mutant) for 24 h with or without OA (40 nM) and luciferase activity was determined in the cell lysates. Promoter activity was normalised to the appropriate plasmid control and expressed as a ratio to the untreated p-890/+96 construct (ΔTIMP-1). Data were analysed by one-way ANOVA with post hoc analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=5).

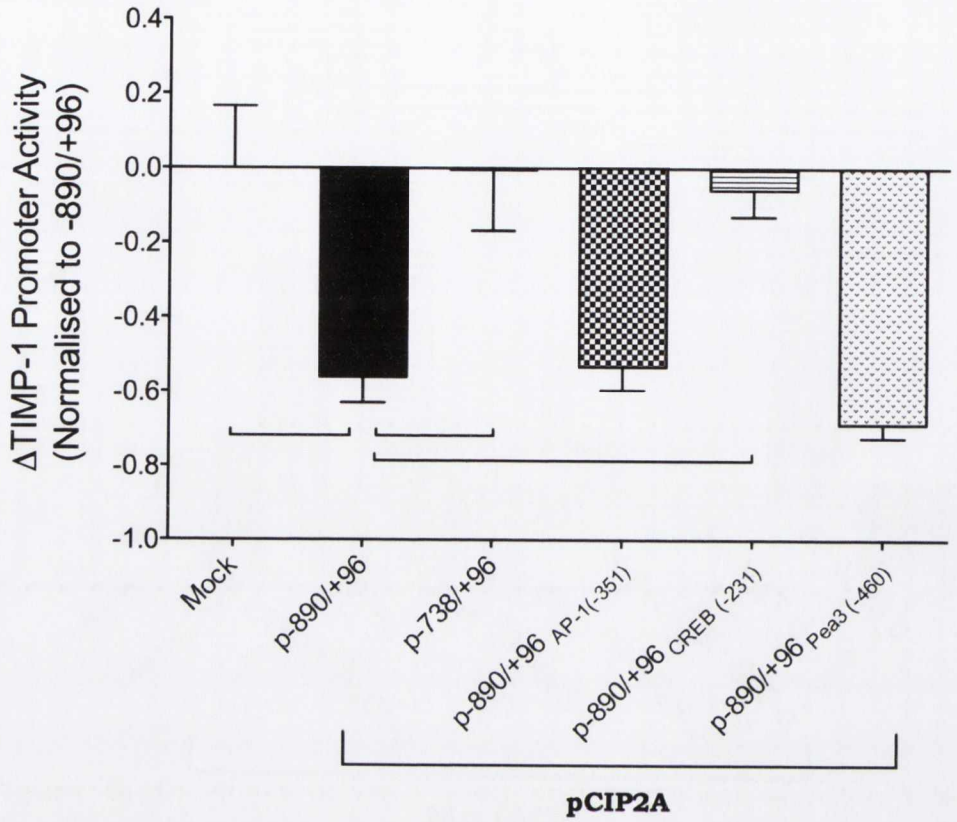


Figure 5.12: Effect of co-transfection of CIP2A on mutant TIMP-1 promoter

Hep3B cells were transfected with p-890/+96, p-890/+96 AP-1 (-351), p-890/+96 CREB (-231), p-890/+96 Pea3 (-460), p-738/+96 and pCIP2A for 24 h and luciferase activity was determined in the cell lysates. Promoter activity was normalised to their relevant mock control construct and expressed as a ratio to the mock p-890/+96 construct (ΔTIMP-1). Data were analysed by one-way ANOVA with post hoc analysis (Dunnett's). Horizontal bars indicate statistical significance at $P < 0.05$ (n=4).

5.4 Discussion

The present study provides evidence for PP2A regulation of TIMP-1 in three hepatic cell lines albeit with disparities between each of the cell lines used. Okadaic acid increases TIMP-1 mRNA and protein expression in Hep3B and Huh-7 cells but decreases it in LX-2 cells. In contrast, loss of the catalytic subunit of PP2A decreases TIMP-1 expression and secretion in all three-cell lines. In keeping with this, overexpression of CIP2A and SET/^{I2}PP2A (endogenous inhibitors of PP2A) decreased TIMP-1 expression in Hep3B cells. Through the use of pharmacological inhibition, PI3-K, JNK and MEK, but not NFκB signalling is implicated in OA's ability to augment TIMP-1 expression in Hep3B cells. Using a bioinformatic approach in conjunction with reporter assays, it was found that $CREB_{(-231)}$, $AP\text{-}1_{(-450)}$ and $AP\text{-}1_{(proximal)}$ binding motifs are critical of OA to induce TIMP-1 promoter activity. With regard to the effect of CIP2A on TIMP-1 expression, a region between -890 and -738 is crucial for down regulating TIMP-1 promoter activity along with a $CREB_{(-231)}$ binding motif (Figure 5.13).

The role of TIMP-1 in cancer remains the subject of conflicting reports, processing both antitumour and tumour proliferative activity [514]. Within the liver, mainly activated hepatic stellate cells produce TIMP-1 or Kupffer cells and plays a pivotal role in matrix remodelling during liver injury and repair. High levels of TIMP-1 in cancer promotes cancer associated fibroblast recruitment and activation of pro-survival signalling through TIMP-1s trophic signalling [353, 492, 515, 516].

Pro-inflammatory signals such as TNF-α and LPS are well documented to regulate TIMP-1 expression [517-519]. LPS-dependent signalling has differential effects on TIMP-1; decreased expression in primary skin fibroblasts [505], while increasing TIMP-1 in keratinocytes [506] and murine mesangial cells [507]. We have shown that LPS stimulation of Hep3B, Huh-7 and LX-2 results in an increase in TIMP-1 expression and abundance. PMA stimulation was also found to increase TIMP-1 in

all three hepatic cell lines. This is in keeping with previous studies while have found increased TIMP-1 expression increased following PMA exposure [520, 521]. While TNF-α increased TIMP-1 expression in LX-2 cells, it had no effect on TIMP-1 in either of our HCC cell lines. HCC cell lines do not respond well to TNF-α stimulation [522], with Hep3B cells among the most receptive to the cytokine [523]. Hepatic stellate cells found with high levels of TIMP-1 expression are hepatoprotective, preventing HSC apoptosis through inaction of MMPs [524, 525].

OA exposure decrease in TIMP-1 expression in LX-2 cells, similar to that reported in HA22T cells [474], while TIMP-1 was increased in Hep3B Huh-7 cells which is comparable to that reported in for HT-1080 cells [342]. Interestingly in Hep3B cells, OA caused a biphasic change in TIMP-1 expression with low concentrations of OA decreasing TIMP-1 expression, while higher concentrations increased it. While it could be suggested that at higher concentrations, OA becomes non-selective and inhibits PP1 or PP4, this is unlikely as the concentrations used in the present study are consistent with those reported for selective inhibition of PP2A [526]. Interestingly, knockdown of PPP2CA in all three hepatic cell lines reduced TIMP-1 expression and abundance. This is in keeping with the previous study, which found a decrease in TIMP-1 expression following PPP2A knockout [474]. In LX-2 cells, knockdown of PPP2R1A also decreased TIMP-1, implicating a potential role for the scaffolding subunit in PP2A-mediated TIMP-1 expression in LX-2 cells. Over expression of CIP2A and SET in Hep3B cells also decreased TIMP-1 expression.

To address the disparity between pharmacological inhibition and knockdown, Hep3B cells were transfected with CIP2A and SET overexpression plasmids. Over expression of CIP2A and SET decreased TIMP-1 expressing, further validating a role for molecular inhibition of PP2A. This is the first study to our knowledge linking TIMP-1 expression with CIP2A or SET protein overexpression. A previous study

has report knockdown of CIP2A increases TIMP-4 expression in breast cancer cells[477].

Due to the disparity between OA and PP2A knockdown, we exposed cells to okadaic 24h post CIP2A and SET plasmid transfection. Intriguingly, OA (40 nM) increased TIMP-1 expression independent of CIP2A and SET protein inhibition of PP2A. To assess this disparity further, we investigated potential signalling pathways that may influence OA/TIMP-1 regulation. Pharmacological inhibition of NF-κB reduced basal TIMP-1 expression in Hep3B cells. Inhibition of NF-κB signalling has been found to lower TIMP-1 abundance, while hyper-activation of NF-κB has been found correlated with increased TIMP-1 expression in tumours[527]. NF-κB is required for IL-1β upregulation of TIMP-1 in astrocytes [503]. Okadaic acid results in the activation of PI3-K signalling in cells [528]. Inhibiting PI3-K using LY-294002 impeded okadaic acid mediated TIMP-1 expression in Hep3B cells. Prior exposure of Hep3B cells to PD-0325901, a MEK inhibitor also negated the OA effect. Both PI3-K and MEK belong to the Ras-dependent signalling pathways that are controlled by PP2A signalling [129]. TIMP-1 has been found to be a positive inducer of Ras-dependent signalling [529, 530]. ERK2, which lies down-stream from MEK, was found to play a crucial role in Ras-TIMP-1 signalling. Interestingly, PP2A is both a positive and negative regulator of Ras-dependent signalling [531]. Although PP2A is known to regulate some of the RAS effector pathways, it has not been systematically assessed how these proteins functionally interact. Whether higher concentrations of OA interacts differently with upstream factors of these pathway remains to be determined.

Pharmacologically inhibition of c-Jun by SP600125 results in the okadaic acid increase in TIMP-1 being reversed, causing a decrease in TIMP-1 mimicking PPP2CA knockdown and CIP2A overexpression. The authors postulated that at the higher concentrations, OA over stimulates c-Jun phosphorylation for a longer period of time in

comparison to molecular inhibition. OA has been found to stimulate c-Jun phosphorylation in concentration and time dependent, with higher concentrations of OA increasing the rate of c-Jun activation [532, 533]. Thus, we can postulate that the differential response it due to OA's induction of c-Jun phosphorylation and its cell line specificity. More experimental evidence is needed however to prove this hypothesis. Comparing c-Jun phosphorylation between the three cell lines in response to OA could elucidate this hypothesis further.

To elucidate further our understanding of TIMP-1 regulation we compared TIMP-1 promoter sequences and demarcated potential putative transcription factor binding sites and assessed their role in basal and PP2A mediate TIMP-1 regulation. Firstly, basal TIMP-1 regulation is complex. The 2.7 kb promoter region of TIMP-1 was found to contain multiple transcription start sites. TIMP1 is both constitutively expressed and inducible which may explain why TIMP-1 lacks a TATA box and multiple transcription start sites [534]. Loss of the downstream component of the TIMP-1 (+988) promoter resulted in an increase in TIMP-1 promoter activity compared -1718/+96 region. The reason for this is unknown. However, it is conjectured that the multiple transcription start sites are repressive in nature as an ATG start sequence point mutations on the 3' end through intron 1 of the TIMP-1 promoter results in increased TIMP-1 promoter activity [535]. We have shown that truncation of the intron 1 region increases TIMP-1 activity in Hep3B cells. This repressive nature of the TIMP-1 promoter is not just 3' dependent and both the -738/+96 and the -890/+96 promoter constructs had approximately a fold increase in activity compared to the -1718/+96 identifying potential upstream regulatory elements between regions -1718 and -890. The identification of these repressor elements is as yet unknown, however, recent findings have found MMP-3 to have a repressor element in a similar region [536].

OA increased in both -890/+96 and -738/+96 TIMP-1 promoter activity. Mutation of the AP-1 site at -351 reduced OA's increase in TIMP-1

promoter activity, however it did not abolish it. Mutation of the proximal AP-1 binding site at position -102 resulted in both inhibition of the OA increase and basal TIMP-1 promoter activity. Previous studies have identified the importance of the AP-1 site at -102 in basal TIMP-1 regulation [288]. However, our study also builds on this previous work as we identified the importance of cyclic AMP-response element (CREB) at position -231 in basal promoter and OA modulation of promoter activity which was not identified by Clark *et al.* CREB's importance in the regulation of basal activity of various genes has been reported [537, 538]. CREB or cAMP response binding element is phosphorylated following activation of PKA [539] and has been found activated following PP2A inhibition by OA [540]. CIP2A's regulation of TIMP-1 is mediated through this -231 putative CREB binding domain. However, further experiments are needed to confirm the existence of the CREB binding region and to deduce further its role in TIMP-1 regulation. Loss of the -890 and -738 region of the TIMP-1 promoter negated the CIP2A mediated decrease in TIMP-1 promoter activity. Bioinformatic analysis of this region identified ETS1, myb-like transcriptional site and a CCAAT/Enhancer Binding Protein (C/EBP) region, all of which may play a role in CIP2A mediated decrease in TIMP-1 promoter activity.

In conclusion, we have demonstrated PP2A regulation of TIMP-1 is complex; disparities exist between the roles of pharmacological and molecular inhibition of PP2A has on TIMP-1 regulation in hepatic cell lines. Okadaic acid-dependent increase in TIMP-1 is mediated through PI3K and MEK signalling, while its decrease is linked to an inhibition of c-jun. Loss of PP2A catalytic subunit or overexpression of either SET or CIP2A protein decrease TIMP-1 expression, with the later mediated through CREB or a distal unknown transcription factor site. Together these data provide evidence for PP2A mediate regulation of TIMP-1 in hepatic cells and highlights the need for a better understanding of its regulation in cancer.

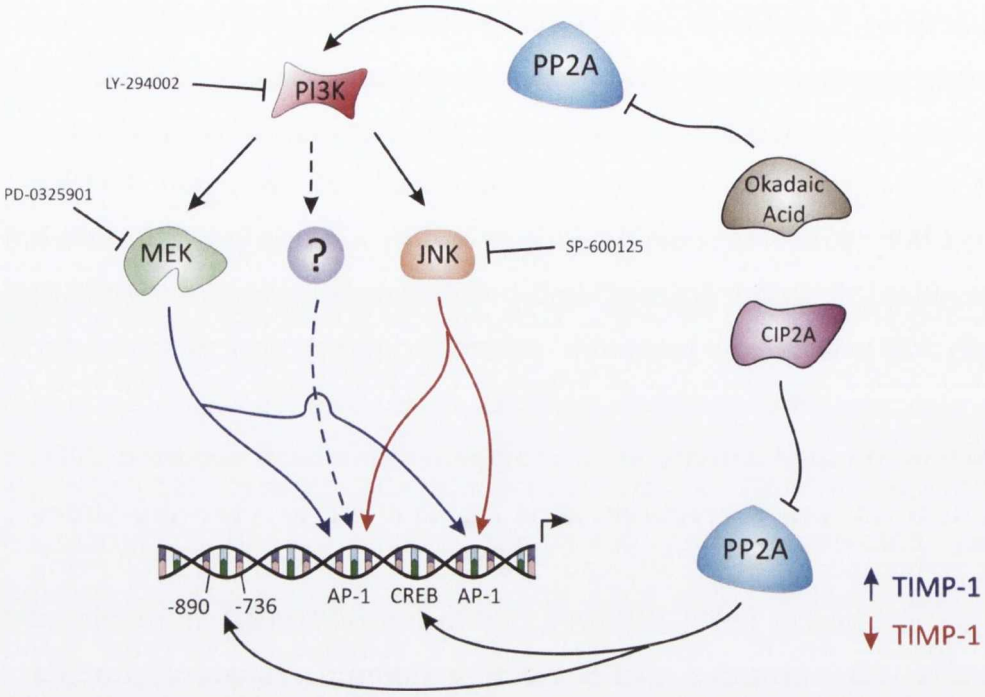


Figure 5.13: Overview of PP2A regulation of TIMP-1

TIMP-1 is dual regulated by PP2A. Okadaic acid increases TIMP-1 through PI3K and MEK signalling. This increase requires functional AP-1 and CREB transcription factor binding site. Blocking of JNK signalling reverses the OA effect. Overexpression of CIP2A or knockdown of PP2Ac decreases TIMP-1 expression and requires CREB and the -890 to-736 region of the TIMP-1 promoter.

Chapter 6:

Role of Tumour Associated Macrophages in Altering PP2A Activity in HCC

6.1 Introduction

Within the general classification of solid cancers, the tumour microenvironment contains many different inflammatory cells that contribute to the inflammatory process affecting degradation and regeneration of the extracellular matrix (ECM) [541]. Macrophages constitute a major component of the infiltrating cell population, and are referred to as tumour-associated macrophages (TAM). The inflammatory milieu within the tumour stroma guides the TAMs into one of two main phenotypes. Classically, the M1 phenotype induced by interferon-γ (INF-γ) or LPS, possesses pro-inflammatory and antitumour properties, while the M2 phenotype, induced by anti-inflammatory cytokines such as interleukin (IL) -4 or IL-13, supports the growth of malignant cells [542, 543]. However, the M1 and M2 phenotypes should be considered at the extremes of a spectrum of continuous differentiation [544]. TAMs in general are classified as "M2-like" as the tumour microenvironment lacks M1-derived signals such as IFN-γ or bacterial components. Tumour cells produce M2 polarisation factors such as IL-10, which limits a Th1 response against tumour cells. This promotes the infiltration of Th2 lymphocytes, which are a fundamental source of IL-4 and IL-13 [545, 546]. Additionally, other cell types within the microenvironment aid in macrophage editing: neoplastic cells and fibroblasts that produce high levels of TGF-β, polarising macrophages into a tissue remodelling phenotype [547].

TAMs facilitate tumour remodelling by secreting high levels of MMP-1, MMP-2, MMP-3, MMP-9, VEGF and IL-8 into the microenvironment, which aid in the dissemination of the tumour and promote formation of the metastatic niche [548, 549]. Interestingly, there is interplay between these factors as MMP-mediated degradation of the ECM not only releases some of the growth factors (TGF-β, TNF-α, Fas Ligand, heparin bound-epidermal growth factor), but is also involved in their activation [550]. Taken collectively, TAMs aid tumour growth by facilitating the

formation of the metastatic niche by enabling an environment conducive for tumour cell proliferation and migration.

The tumour microenvironment is also influenced by metabolic factors. As a consequence of defects in cellular respiration, tumours have increased glucose uptake and lactate formation even under normoxic conditions. This results from overexpression of glycolytic enzymes and metabolite transporters as a consequence of oncogenic alterations, which switches tumour cell metabolism from oxidative phosphorylation (OXPHOS) towards an altered glycolytic state [551, 552]. Alterations in pathways involving c-Myc, NF- κB, Akt/protein kinase B, epidermal growth factor (EGF), insulin-like growth factor I, phosphoinositol 3 kinase (*PI3K*), mTOR, KRAS, AMPK, and hypoxia-inducible factor 1α (HIF-1α) are drivers of this process [3, 552]. Glycolysis forces tumour cells to produce high levels of lactate, which acidifies the tumour microenvironment. This acidification leads to not only the activation of pro-survival stress signals, but also assists in tumour evasion of the immune system [553]. A recent study has shown lactic acid to polarise macrophages to TAMs and promote arginase 1 activity and expression, a key marker of anti-inflammatory macrophages [317]. This is a unique observation as previous report found lactic acid-producing bacteria to evade immune detection by polarising macrophages to an M2 phenotype, thus creating an anti-inflammatory environment similar to that of the tumour microenvironment [554].

Previously in chapter 3, we identified phosphorylation of Arginase 1 (ARG-1), lactate dehydrogenase A (LDH-A) and pyruvate kinases 2 (PKM2) in Hep3B cells following exposure to okadaic acid, suggesting a potential role for PP2A in macrophage polarisation/immune surveillance. Several lines of evidence support this observation: (i) PP2A can regulate pro-inflammatory macrophage signalling through the regulation of c-Jun phosphorylation and IL-1β production [470]; (ii) LPS reduces PP2A activity in macrophages [555], while *H. pylori* increases CIP2A expression in gastric cancer [249, 250]; and (iii) the HPV E6

upregulates CIP2A expression through IL-10 in lung adenocarcinoma [251]. This has lead us to postulate a potential role for PP2A in persistent or "smouldering inflammation", which may result in further tumour oncogenesis due to reduced PP2A activity and increased expression of PP2A inhibitory proteins. However, the role of PP2A in regulating macrophage polarisation in cancer and the effect of macrophages on CIP2A expression has yet to be addressed.

Thus, the aims of the present study were to:

1. Establish an *in vitro* model of the tumour microenvironment to investigate paracrine interactions relevant to hepatocellular carcinoma;
2. Investigate the effect(s) of macrophage polarisation on: (i) activity of PP2A, (ii) expression of key genes associated with tumour growth, immune response and metabolism, and (iii) the role of PP2A and CIP2A in modulation of extracellular matrix remodelling and end-stage glycolysis associated with tumour metabolism;
3. Explore whether Hep3B cells assist in the "re-education" of macrophages through modulation of the PP2A system;
4. Assess if macrophages alter the phenotypic behaviour of Hep3B cells (cell invasion).

6.2 Methods

6.2.1 Materials

L-(+)-Lactic acid was obtained from Sigma-Aldrich (Ireland). Human IL-4, IL-10, IL-13 and INF-γ were obtained from Prospec (Israel). Hep3B and THP-1 cells were a gift from Dr Steven Gray, Trinity College Dublin, Ireland. DNA primers were purchased from Integrated DNA Technologies (Belgium). All other materials were obtained from Sigma-Aldrich (Ireland) unless otherwise specified.

6.2.2 Cell Culture

6.2.2.1 Hep3B cell culture

Hep3B cells were cultured in DMEM or Roswell Park Memorial Institute media (RPMI) 1640 (supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and 4 mM L-glutamine) at 37°C in an humidified atmosphere containing 5% CO_2 as described previously (Section 3.2.2). Hep3B experiments were carried out in serum free conditions.

6.2.2.3 THP-1 cell culture

THP-1 cells were cultured in RPMI 1640 (supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and 4 mM L-glutamine), at 37°C in a humidified atmosphere containing 5% CO_2. THP-1 cells were differentiated to a macrophage (Mϕ) phenotype by exposure to PMA (320 nM) for 24 hours. A M1 phenotype was generated by differentiating THP-1 cells with PMA (320 nM) for 24h during which time LPS (100 ng/ml) and IFN-γ (20 ng/ml) were added for the last 18 h (Figure 6.1). THP-1 cells were differentiated to the M2 phenotype following exposure to PMA (320 nM) for 24 h, along with IL-4 (20 ng/ml) and IL-13 (20 ng/ml) for the last 18 h.



Figure 6.1 THP-1 cell polarisation protocols

6.2.2.3 Co-culture experiments

For co-culture experiments, THP-1 macrophages, (1×10^6 cells) were seeded into the upper insert of a six-well Transwell plate (0.4 μM pore size, Corning, Lowell, MA) and polarised as above for 24h. The polarised THP-1 cells in the upper insert were washed with PBS and media changed to serum free RPMI media (100 U/mL penicillin and 100 mg/mL streptomycin and 4 mM glutamine). Hep3B (2×10^5) cells were seeded into the lower chamber in serum free RPMI media (100 U/mL penicillin and 100 mg/mL streptomycin and 4 mM L-glutamine) 24 h post THP-1 polarisation. Apart from normal cell husbandry, all experiments were carried out in serum free conditions.

6.2.3 Semi-Quantitative Real-Time PCR

Total cellular RNA was isolated using TRI Reagent™ (Sigma Aldrich). Following treatment with DNase I (Sigma Aldrich), RNA was reverse transcribed using random hexamers and ReverseAid reverse transcriptase (Thermofisher, Dublin, Ireland). mRNA expression was determined using semi-quantitative PCR using target specific primers (Table 6.1, IDT) and GoTaq qPCR Master Mix from (Promega, Ireland) on a Mx3000P qPCR system (Agilent Technology). Gene expression was quantified using the comparative Ct method [2-ΔΔCt; 460]. For each primer pair, a no template control and no RT control was included. Authenticity of the primer sequences was verified by nucleotide search (Primer-BLAST; NCBI), while the size and specificity of the amplicons were checked by gel electrophoresis with UV trans-illumination (Fusion Fx imaging system, Vilber Lourmat, Marne-la-Vallée Cedex, France).

Gene	NM ID	Sequence
TNFA	NM_000594	Fw: CCCAGGGACCTCTCTCTAATCA
		Rv: GGTTTGCTACAACATGGGCTACA
IL1B1	NM_214055	Fw: GCAACAAGTGGTGTTCTC
		Rv: CAGATTCTTTTCCTTGAGGC
NOS2	NM_214055	Fw: GTCAGACTCACCATCCTCTTT
		Rv: GCAGCTCAGCCTGTACTTATC
IL6	NM_000600.3	Fw: ATGTAGCCGCCCCACACAGA
		Rv: ATTTGCCGAAGAGCCCCTCAG
IL8	NM_000584.3	Fw: CCACCGGAAGGAACCATCTC
		Rv: TTCCTTGGGGTCCAGACAGA
IL10	NM_000572.2	Fw: GCCTAACATGCTTCGAGATC
		Rv: CTCATGGCTTTGTAGATGCC
IL13	NM_002188.2	Fw: CACGGTCATTGCTCTCACTT
		Rv: CTTCTGGTTCTGGGTGATGTT
TGFB1	NM_000660.5	Fw: CCCACAACGAAATCTATGAC
		Rv: TGTATTTCTGGTACAGCTCC
MMP9	NM_004994.2	Fw: GAACTTTGACAGCGACAAGAAG
		Rv: CGGCACTGAGGAATGATCTAA
PPP2CA	NM_002715.2	Fw: GCCTCTGCGAGAAGGCTAAA
		Rv: GAATGGTGATGCGTTCACGG
MYC	NM_002467.4	Fw: GCCACGTCTCCACACATCAG
		Rv: TCTTGGCAGCAGGATAGTCCTT
CIP2A	NM_020890.2	Fw: TGGACCCACAAATCACCTCG
		Rv: ATTACCTCCAAGTGCCGCAA
LDHA	NM_005566.3	Fw: AGATTCCAGTGTGCCTGTATG
		Rv: ACCTCTTTCCACTGTTCCTTATC
PKM2	NM_002654.4	Fw: CCACCCAATCAAGGGAAGAA
		Rv: GGACAGAGTACACACAGGAAAG
HIF1A	NM_001530	Fw: GAAAGCGCAAGTCTTCAAAG
		Rv: TGGGTAGGAGATGGAGATGC
ARG1	NM_001244438.1	Fw: AGATTCCAGTGTGCCTGTATG
		Rv: ACCTCTTTCCACTGTTCCTTATC
VEGFA	NM_001171623.1	Fw: CTCAGAAGCAGGTGAGAGTAAG
		Rv: CTACATGGCAACTGTGAGGAG
GAPDH	NM_002046.5	Fw: CAGGACATTGCTGTGCTTTG
		Rv: CTACATGGCAACTGTGAGGAG

Table 6.1 List of primers used in this study. All primers were obtained from Integrated DNA Technologies (IDT).

6.2.4 Determination of protein concentration (Bradford-assay)

The protein concentration in cell lysates was determined using Bradford reagent in accordance with the 96-well plate assay protocol supplied by the company (Sigma-Aldrich). In brief, a BSA standard curve over the concentration range 0.1-1.4 mg/ml was prepared by serial dilution. Samples, standards and blank (lysis buffer) were added to a 96-well plate. Bradford reagent (250 µl) was added to each well, mixed for 30 seconds and incubated for 20 min, absorbance was measured at a wave length of 650 nm (BioTek, EL 808, Bedfordshire, UK). All samples were analysed in duplicate. Protein concentration of the samples was determined by interpolation of mean absorbance against protein concentration of the standards.

6.2.5 Cell viability

Cell viability was assessed as described previously (Section 3.2.3). Briefly, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) was added to Hep3B cells incubated following exposure to THP-1 media or Hep3B cells co-culture with THP-1 cells. At the end of the incubation period, the culture medium was removed, and the purple formazan deposits were dissolved in DMSO. Formazan production was quantified by spectrophotometry at a wavelength of 540 nm with a reference wavelength of 650 nm (BioTek, EL 808, Bedfordshire, UK). Samples were normalised to untreated and expressed as a percentage.

6.2.6 PP2A activity assay

PP2A activity was determined using a PP2A immunoprecipitation phosphatase assay kit (Millipore, USA). Hep3B and THP-1 cells were grown to 60-70% confluency in T-75 flasks (Nunc) or six-well Transwell plates (0.4 µM pore size, Corning, Lowell, MA) as described above in section 6.2.2.3. THP-1 cells were polarised to Mϕ, M1 and M2 as described previously. Hep3B cells exposed to OA (40 nM) or media for

24 h. Cells were lysed on ice in a buffer comprised of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% (v/v) NP40 containing a protease inhibitor cocktail. 50-100 μg of total protein was immunoprecipitated with anti-PP2A-C subunit antibody (4 μg; clone 1D6) along with protein A-agarose. The mix was incubated for 2 h at 4 °C, and the immunoprecipitate extensively washed with PBS before determination of the phosphatase activity as per the manufacturer's instructions. Briefly, PP2A phosphatase activity was calculated by dividing the free phosphate of the test cells by that of the untreated cells, and expressed as a percentage. Each assay run contained a negative control (no lysates) as a quality control for phosphatase contamination.

6.2.7 Gelatin Zymography

Hep3B cells previously co-cultured with THP-1 cells for 24 h were seeded (2 x 10^5 cells) into 6 well plates in complete RPMI 1640 media. MMP-9 and MMP-2 activity was measured by gelatin zymography as previously described [461]. In brief, conditioned media denatured in a non-reducing Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 0.1% phenol red, 50% glycerol, 5% ficoll 400 and 5% lithium dodecyl sulphate; final concentrations) was loaded onto 8% SDS-polyacrylamide gels co-polymerised with gelatin (0.1% w/v). Following electrophoresis, gels were washed in 2.5% Triton X-100 and incubated for 24 h at 37°C in development buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM NaCl, pH 7.6). Gels were stained with 0.25% (w/v) Coomassie blue R-250 in a glacial acetic acid: methanol: distilled H_2O mixture (1:3:6). Following destaining, gels were imaged, and lytic activity quantified by densitometry using a Fusion Fx imaging system.

6.2.8 Western blot

Cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail SIGMADFAST™ (Sigma Aldrich). Protein samples (10 μg) were boiled in Laemmli buffer supplemented with 2-β-mercaptoethanol (1% v/v). The samples were subjected to SDS-PAGE (10% gel) and semi-dry transferred (Transfer buffer: Tris-base 50 mM, Glycine 40 mM, methanol 20% v/v, SDS 0.037%, dH_2O) onto PVDF membrane (Amersham, Buckinghamshire, UK). Membranes were blocked for 2 h in TBS-T (Tris-base 10 mM, NaCl 100 mM, HCl 1M, and Tween-20 0.1% v/v) containing 5% w/v Marvel and probed with 1° antibody directed against CIP2A (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 dilution), PP2Ac (PP2A catalytic subunit (clone 1D6) (Millipore; 1:1000 dilution) for 24 h at 4°C. Following extensive washing with TBS-T, membranes were incubated with a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP 1:2500 dilution). Signals were visualised by chemiluminescence detection (30% H_2O_2, 250 mM luminol in DMS0, 90 mM 4-iodophenylboronic acid in DMSO and 10 mM Tris-HCl) [373] and the signal recorded using a Fusion Fx imaging system. Membranes were stripped and re-probed with HRP-conjugated β-actin antibody as a loading control (1:7500, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

6.2.9 Overexpression of CIP2A

To assess the effect of overexpression of CIP2A in Hep3B cells on lactate production cells were seeded (2 x 10^5 cells per well) in 6 well plates, and 24 h later transiently transfected with 1 μg of pcDNA3.1_CIP2aflag_WT plasmid (pCIP2A), pcDNA.3.1 (pDNA.3.1) control plasmid or no DNA control (mock) using PolyFect (Qiagen) in serum and antibiotic free DMEM. CIP2A overexpression was confirmed 24 h post-transfection by qPCR and lactate concentration was determined 48 h post-transfection.

6.2.10 Determination of lactate concentration in culture medium post drug treatment and co-culture

Lactate concentration was measured using a Lactate Assay kit (Sigma-Aldrich) as per the manufacturer's instructions. Conditioned media (Hep3B, THP-1 (Mϕ, M1 and M2) and co-culture media were diluted 1:100 with lactate assay buffer and prepared as triplicates for the colorimetric determination of lactate. The absorbance was measured at 570 nm 10 min after sample preparation. Background absorbance was subtracted. The mean values for lactate concentration were determined and expressed in mM. For calculation of lactate concentration, the equation below was used:

$$S_a/S_v = C$$

S_a = Amount of lactate in unknown sample (nM) from standard curve.

S_v = Sample volume (µl) added into the wells.

C = Concentration of lactate in sample.

6.2.11 Cell invasion

Transwell inserts (6.5 mm, 8 µm pore; Corning, USA) were pre-treated with Matrigel (5 mg/ml; Sigma-Aldrich, Ireland) for 24 h. Hep3B cells (2 x 10^4 cells) that were previously co-cultured with polarised THP-1 cells for 24 h were seeded into the upper compartment of the transwell. Hep3B cells (2 x 10^4 cells) not co-cultured were exposed to IL-10 (20 ng/ml), 15 mM lactate or media (control) under serum free conditions; a chemoattractant (10% FBS media) was included in the lower chamber. Inserts were removed 24 h later and cells were fixed in 3% paraformaldehyde, followed by staining with crystal violet (0.05%). The matrigel membranes were imaged on a Cell Imaging System (EVOS® FL) for subsequent analysis. Each experiment was performed in triplicate.

6.2.12 Data analysis

All data were normalised to the appropriate control and expressed as an absolute value, a percentage or ratio and analysed by one-way ANOVA with *post hoc* analysis (Dunnett, Bonferroni) or non-paired Student t-test. Data are expressed as mean ± S.E.M., and a value of $P < 0.05$ is taken to indicate statistical significance.

6.3 Results

6.3.1 Characterisation of THP-1 cell polarisation

The expression of key pro-inflammatory and anti-inflammatory markers in THP-1 derived macrophages was determined by RT-PCR. TNFA expression was increased in M1 macrophages compared to Mϕ by 3.9 ± 0.78 fold (Table 6.2; $P < 0.05$), while it was lower in M2 macrophages (0.71 ± 0.04 fold; $P < 0.05$). M1 macrophages expressed high levels of IL1B1 compared to Mϕ macrophages (8.26 ± 2.4 fold), while M2 macrophages had a low expression of IL1B compared to Mϕ macrophages (-0.88 ± 0.06 fold; $P < 0.05$). NOS2 expression was higher in M1 macrophages than Mϕ macrophages (3.01 ± 0.71 fold; $P < 0.05$), while being lower in M2 macrophages compared to Mϕ macrophages (-0.56 ± 0.07 fold; $P < 0.05$). M1 macrophages had high mRNA expression of IL6 (4.50 ±0.72 fold) and IL8 (70.23 ± 10.25 fold) compared to Mϕ ($P < 0.05$). M2 macrophages expressed low levels of IL6 (-0.89 ± 0.04 fold) compared to Mϕ ($P < 0.05$). M2 macrophages expressed high levels of TGFB1 (5.95 ± 2.0 fold), IL-10 (1.52 ± 0.33 fold), IL13 (3.25 ±1.68 fold) and ARG1 (753 ± 150.2 fold; $P < 0.05$) compared to Mϕ macrophages. With regard to PPP2CA expression, this was increased in M1 (1.42 ± 0.23 fold; $P < 0.05$) and M2 (3.8 ± 0.47 fold; $P < 0.05$) macrophages compared to Mϕ. The expression of CIP2A was similar across all macrophage subtypes. Myc expression was increased in M2 (4.12 ± 0.9 fold; $P < 0.05$) but not in M1 macrophages compared to Mϕ.

6.3.2 Influence of THP-1 co-culture on Hep3B proliferation

To assess whether co-culture with THP-1 cells affects Hep3B proliferation, Hep3B cells were exposed to condition media from Mϕ, M1 and M2 polarised THP-1 cells or co-cultured with pre-polarised THP-1 cells for 24 h. Hep3B cells exposed to conditioned media from Mϕ reduced ($P < 0.05$; Figure 6.2A) proliferation by 55.08 ± 6.16%

compared to Hep3B cells alone. Similarly, conditioned media from M1 macrophages reduced Hep3B cell proliferation by 67.4 ± 3.8% compared to Hep3B cells alone ($P < 0.05$). Conditioned media from M2 macrophages did not alter Hep3B proliferation (Figure 6.2A).

Hep3B cells co-cultured with Mϕ macrophages showed a 38.22 ± 5.1% decrease in cell proliferation compared to non co-cultured Hep3B cells, while in cells co-cultured with M1 macrophages it decreased by 31.48 ± 3.8% ($P < 0.05$). Hep3B cells co-cultured with M2 macrophages increase cell proliferation by 35.6 ± 6.3% compared to monocultured Hep3B cells (Figure 6.2B; $P < 0.05$). As co-culture altered cell proliferation differently than conditioned media, presumably as a result of paracrine signalling between the two cell lines, all subsequent experiments were conducted using the co-culture model.

6.3.3 Effect of THP-1 cell culture on Hep3B PP2A activity

To assess the influence of THP-1 derived monocyte subsets on PP2A activity in Hep3B cells, THP-1 cells were polarised for 24 h before co-culture with Hep3B cells. PP2A activity in Hep3B cells co-cultured with Mϕ cells was 25 ± 1.48% lower than in monocultured Hep3B cells (Figure 6.3A; $P < 0.05$). Although co-culture with M1 macrophages did not alter PP2A activity in Hep3B cells, co-culture with M2 cells reduced activity by 29± 2.58% compared to Hep3B cells alone (Figure 6.3B; $P < 0.05$).

6.3.4 Effect of macrophage co-culture on PP2A expression in Hep3B cells

To establish if the change in PP2A activity is reflected by a change in protein expression, the expression of the PP2A catalytic subunit (PP2Ac) in Hep3B cells was investigated. Hep3B cells co-cultured with Mϕ, M1 or M2 macrophages did not alter PP2Ac protein expression (Figure 6.4). As PP2Ac protein expression was unaffected, we next investigated the effects of co-culture on CIP2A expression in Hep3B cells.

6.3.5 Effect of macrophage co-culture on CIP2A expression in Hep3B cells

CIP2A expression was explored using RT-PCR and Western blotting. Hep3B cells co-cultured with Mϕ or M1 macrophages did not alter CIP2A mRNA expression compared to Hep3B cells alone. However, it was increased by 1.26 ± 0.14 fold (Figure 6.5A; $P < 0.05$) in Hep3B cells co-cultured with THP-1 derived M2 macrophages. Although the increase in CIP2A mRNA expression was reflected at the protein level in Hep3B cells co-cultured with M2 macrophages (Figure 6.5B; $P < 0.05$), co-culture with Mϕ and M1 macrophages also increases CIP2A protein expression. The rank order of the CIP2A expression level being Hep3B < M1/Hep3B < Mϕ /Hep3B < M2/Hep3B.

Given the key role of TAMs, PP2A and CIP2A in cancer, we undertook a series of experiments to ascertain if co-culture of different macrophage-subtypes alters expression of key remodelling, inflammatory and metabolic mediators in Hep3B cells.

6.3.6 Effect of macrophage co-culture on MMP-9 expression and activity in Hep3B cells

MMP-9 mRNA expression was increased by 4.06 ± 0.5 fold in Hep3B cells co-cultured with M2 macrophage compared to Hep3B cells alone (Figure 6.6A; $P < 0.05$). Co-culture with Mϕ and M1 had a tendency to increase MMP-9 mRNA by 1.59 ± 0.13 and 1.04 ± 0.7 fold respectively but this failed to reach significance (Figure 6.6A).

Co-culture with Mϕ, M1 and M2 macrophages increased Hep3B MMP-9 activity by 1.52 ± 0.25, 1.65 ± 0.17 and 3.66 ± 0.63 fold respectively (Figure 6.6C,B; $P < 0.05$). MMP-2 activity was unaltered in Hep3B cells upon co-culture with THP-1 derived macrophage.

6.3.7 Effect of macrophages on cytokine and growth factor expression in Hep3B cells

M2 macrophages increased VEGFA, IL-8 and IL-10 mRNA expression in Hep3B cells by 5.8 ± 0.98, 2.37 ± 0.74 and 1.88 ± 0.53 fold respectively (Figure 6.7A,B,C; $P < 0.05$). Co-culture with Mϕ or M1 macrophages did not alter VEGF, IL-8 or IL-10 expression in Hep3B cells.

6.3.8 Effect of macrophage co-culture on expression of metabolic genes in Hep3B cells

As okadaic acid, a PP2A inhibitor, increased phosphorylation of LDHA and PKM2 (Study 3), we investigated if macrophages altered their expression in Hep3B cells. Mϕ or M1 did not alter expression of LDHA or PKM2 in Hep3B (Figure 6.8A,B). However, LDHA expression was increased by 1.02 ± 0.39 fold in Hep3B cells co-cultured with M2 macrophages (Figure 6.8A; $P < 0.05$). Although, M2 macrophages did not alter PKM2 expression in Hep3B cells co-cultured with macrophages, there was a tendency for it to be increased (Figure 6.8B). As HIF1α mediates metabolic function in tumours it was included in the analysis [556]. HIF1A mRNA expression was increased by 2.53 ± 0.59 fold in Hep3B cells co-cultured with M2 macrophage compared to Hep3B cells alone (Figure 6.8C; $P < 0.05$). Its expression was unaltered by co-culture with Mϕ or M1 macrophages.

6.3.9: Effect of PP2A inhibition and co-culture with TPH-1 derived macrophages on lactate production by Hep3B cells

Based upon the effects on co-culture on expression of genes associated with metabolism, the involvement of PP2A and co-culture on lactate production was assessed. In Hep3B cells exposed to okadaic acid (40 nM) for 24 h, lactate levels in the culture medium increased from 7.72 ± 0.6 mM to 14.82 ± 1.2 mM (Figure 6.9A; $P < 0.05$). Similarly, overexpression of CIP2A in Hep3B cells increased ($P < 0.05$) lactate production to 13.06 ± 1.02 mM from 7.06 ± 0.16 mM in mock

transfected Hep3B cells. Mock transfection or transfection with the empty plasmid had no effect on lactate production (Figure 6.9A). Under co-culture conditions, lactate production was greater in the media from Hep3B cells co-cultured with M2 macrophages than those co-cultured with Mϕ macrophages (Figure 6.9B; $P < 0.05$). Lactate production was similar in media from Hep3B cells co-cultured with M1 or Mϕ macrophages.

6.3.10 Effect of co-culture of Hep3B cells with THP-1 derived macrophages on PP2A activity

PP2A activity was decreased by 70 ± 4.05% in M1 polarised macrophages compared to Mϕ, but increased by 67 ± 4.4% in M2 macrophages (Figure 6.10; $P < 0.05$). As M2 macrophages decreased PP2A activity in Hep3B cells (Figure 6.3) and increased Hep3B proliferation, we assessed if Hep3B cells had a reciprocal effect on PP2A activity in M2 macrophages. PP2A activity was attenuated by 64 ± 5.9% in M2 macrophages co-cultured with Hep3B cells compared to Mϕ monocultures (Figure 6.10; $P < 0.05$).

6.3.11: Effect of Hep3B cells on PP2A and CIP2A expression in M2 macrophages

To investigate the decrease in PP2A activity further we addressed if co-culture altered expression of the PP2CA catalytic subunit and its endogenous inhibitor CIP2A. In M2 macrophages co-cultured with Hep3B cells PPP2CA mRNA expression was decreased by 0.39 ± 0.42 fold (Figure 6.11A; $P < 0.05$), while CIP2A expression increased by 3.87 ± 0.99 compared to M2 macrophages in monoculture (Figure 6.11B; $P < 0.05$).

6.3.12 Effect of Hep3B cells on M2 macrophage gene expression

As co-culture with Hep3B cells altered PPP2ACA and CIP2A expression in THP-1derived M2 macrophages, we investigated if co-culture would

alter expression of other regulators of the tumour microenvironment. Co-culture with Hep3B cells increase in MMP-9 mRNA expression by 3.1 ± 0.3 fold in M2 macrophages (Figure 6.12A; $P < 0.05$). VEGFA increased 1.82 ± 0.43 fold in M2 macrophages co-cultured with Hep3B cells compared to M2 macrophages alone (Figure 6.12B; $P < 0.05$). IL-8 and IL-10 expression was increased 4.7 ± 0.53 and 0.6 ± 0.23 fold respectively, in M2 macrophages co-cultured with Hep3B cells (Figure 6.12C,D; $P < 0.05$). IL-10 mRNA expression increased by 0.6 ± 0.23 fold in M2 macrophages co-cultured with Hep3B cells (Figure 12D; $P < 0.05$). LDHA mRNA expression was increased 0.6 ± 0.19 fold in M2 macrophages in co-cultured (Figure 6.12E; $P < 0.05$), while arginase 1 (ARG1) expression increased by 2.48 ± 0.49 fold (Figure 6.12F; $P < 0.05$). Myc mRNA expression increased 1.86 ± 0.69 fold (Figure 6.12G; $P < 0.05$) in M2 macrophages co-cultured with Hep3B cells.

6.3.13 Effect of co-culture and Lactate on Hep3B invasion

Hep3B cells co-cultured with M2 macrophages 24 h prior to invasion-increased invasion through the matrigel-coated membrane by 2.14 ± 0.31 ($P < 0.05$; Figure 6.13A,B). Interestingly, lactic acid (15 mM) also augmented invasion through the matrigel-coated membrane by a factor of 2.15 ± 0.19 fold.

Gene	Mϕ	M1	M2
TNFA	1 ± 0.8	4.9 ± 0.78*	-0.71 ± 0.04
IL1B1	1 ± 0.8	9.26 + 2.4*	-0.88 ± 0.06*
NOS2	1 ± 0.1	4.01 ± 0.71*	-0.56 ± 0.07*
IL6	1 ± 0.12	5.5 ±0.72*	-0.89 ± 0.04*
IL8	1 ± 0.4	71.23 ± 10.25*	1.17 ± 0.22
TGFB1	1 ± 0.23	1.21 ±0.1	6.95 ± 2.0*
IL10	1 ± 0.21	1.01± 0.12	2.52 ± 0.33*
IL13	1 ± 0.2	1.25 ±0.15	4.25 ± 1.68*
ARG1	1 ± 0.1	1.29 ± 0.47	754 ± 150.2*
PPP2CA	1 ± 0.21	2.42 ± 0.23*	4.8 ± 0.47*
CIP2A	1 ± 0.11	1.2 ± 0.1	1.31 ± 0.05*
MYC	1± 0.19	1.1 ± 0.45	5.12 ± 0.9*

Table 6.2: Expression of inflammatory markers in THP-1 derived macrophage subsets

THP-1 cells were polarised with PMA (Mϕ), PMA + LPS/INF-γ (M1) or PMA + IL-4/IL-13 to generate Mϕ, M1 and M2 subsets. The mRNA expression profile was determined by RT-PCR. Data are normalised to expression in Mϕ (mean ± S.E.M) and were analysed using one-way ANOVA with *post hoc* analysis (Dunnett). * Indicates significance at $P < 0.05$ compared to Mϕ (n≥3).

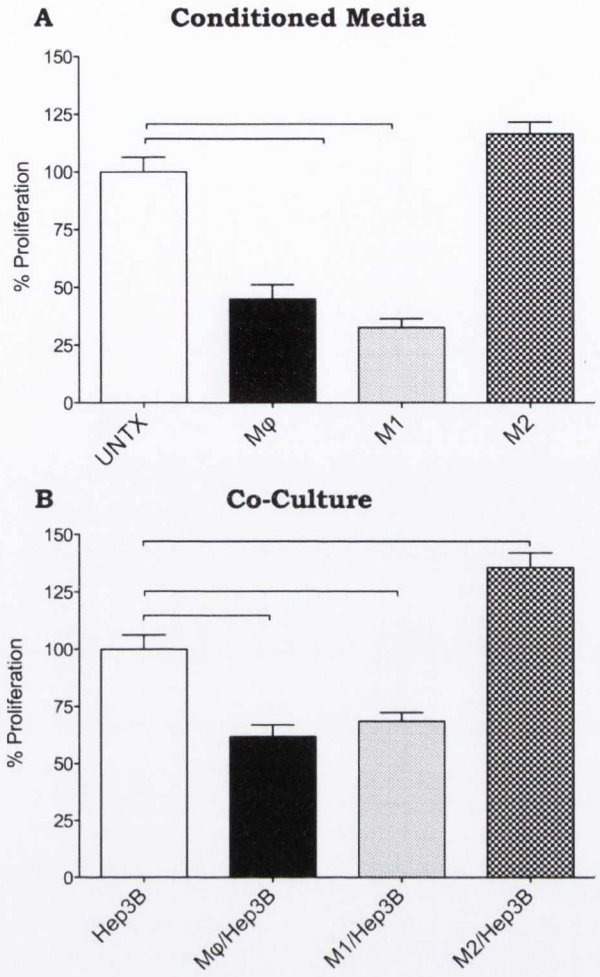
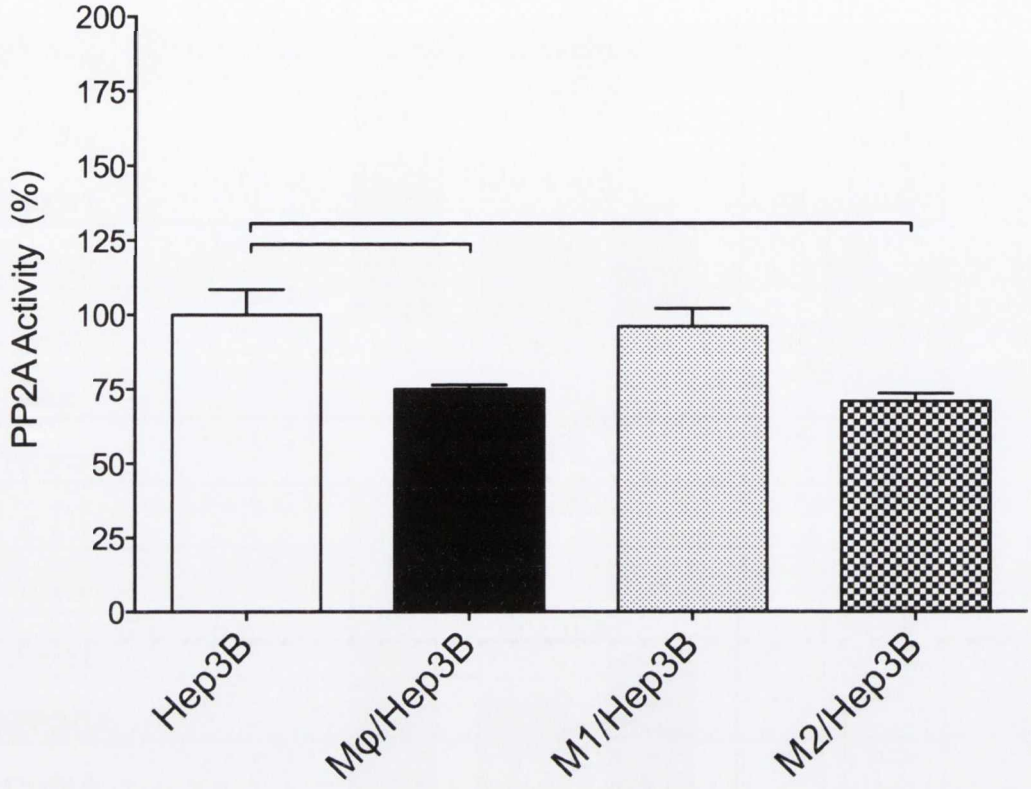


Figure 6.2: Effect of macrophage conditioned media and co-culture on Hep3B cell viability.

(A) Hep3B cells were exposed to conditioned media from Mϕ, M1 and M2 macrophages for 24 h. Hep3B cell viability was measured by MTT assay. (B) Hep3B cells were cultured with pre-polarised THP-1 macrophages for 24h before viability was assessed by MTT. Experiments were carried out in duplicate and data are expressed as a percentage (mean ± S.E.M.). Data were analysed using one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate significance $P < 0.05$ (n=5).



F

igure 6.3: PP2A phosphatase activity in Hep3B cells following co-culture with THP-1 cells.

PP2A activity was in Hep3B cells following co-culture with polarised THP-1 cells. Data are expressed as a percentage of phosphatase activity in Hep3B cells alone. Data are presented as mean ± S.E.M. and were analysed using one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

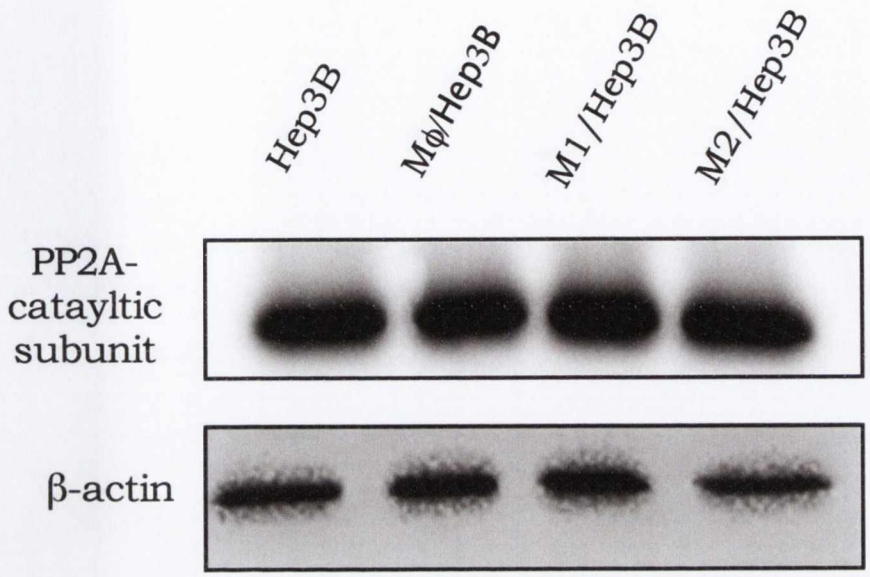


Figure 6.4: Effect of THP-1 co-culture on PP2Ac expression in Hep3B cells

Hep3B cells were co-cultured with Mϕ, M1 and M2 macrophages for 24 h. Whole cell protein was extracted and probed (10 μg) with and antibody directed against PP2Ac overnight. Afterwards, membranes were stripped and probed with an anti-β-actin antibody as a loading control. Images are representative of three individual experiments.

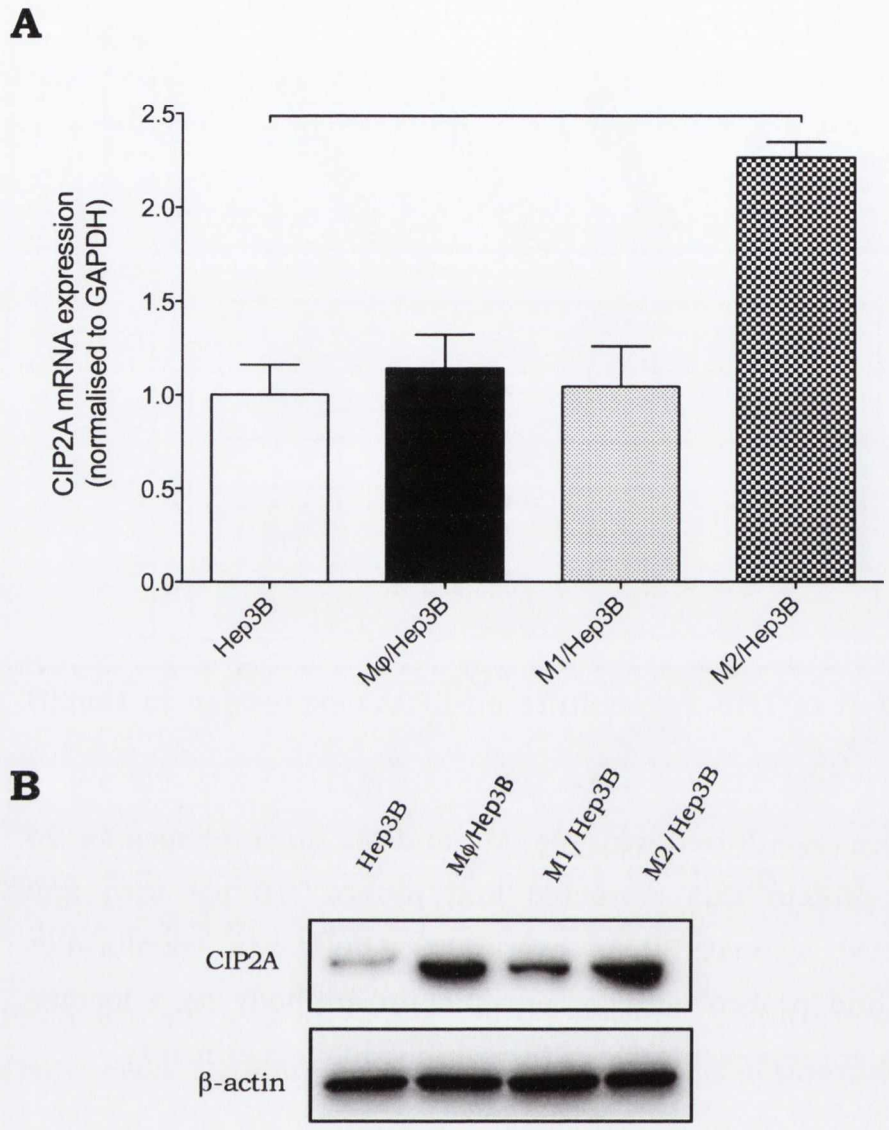


Figure 6.5: Influence of macrophage co-culture on CIP2A expression in Hep3B cells.

(A) CIP2A mRNA expression and (B) a representative Western blot of CIP2A protein expression in Hep3B cells following co-culture with THP-1 derived macrophage subtypes. RT-PCR data are normalised to basal expression of CIP2A in Hep3B cells. Data are presented as mean ± S.E.M. and were analysed using one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate significance at $P < 0.05$ (n=3-4).

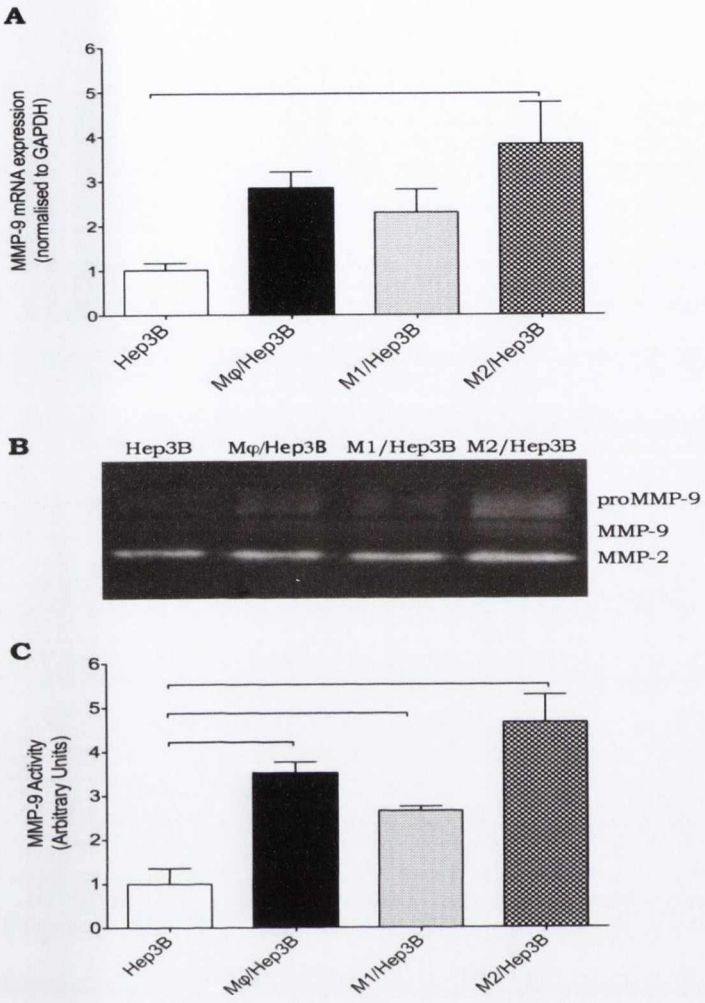


Figure 6.6: Effect of macrophage co-culture on MMP-9 mRNA expression and activity in Hep3B cells. Mϕ, M1 and M2 macrophage were co-cultured with Hep3B and MMP-9 mRNA expression determined in Hep3B cells using RT-PCR (A). Representative zymogram of MMP-2/9 activity in Hep3B cells following co-culture is presented in (B). Arbitrary MMP-9 activity in Hep3B cells following co-culture (C). Data are normalised to basal expression and activity in Hep3B cells under monoculture. Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate significance at $P < 0.05$ (n=3-4).

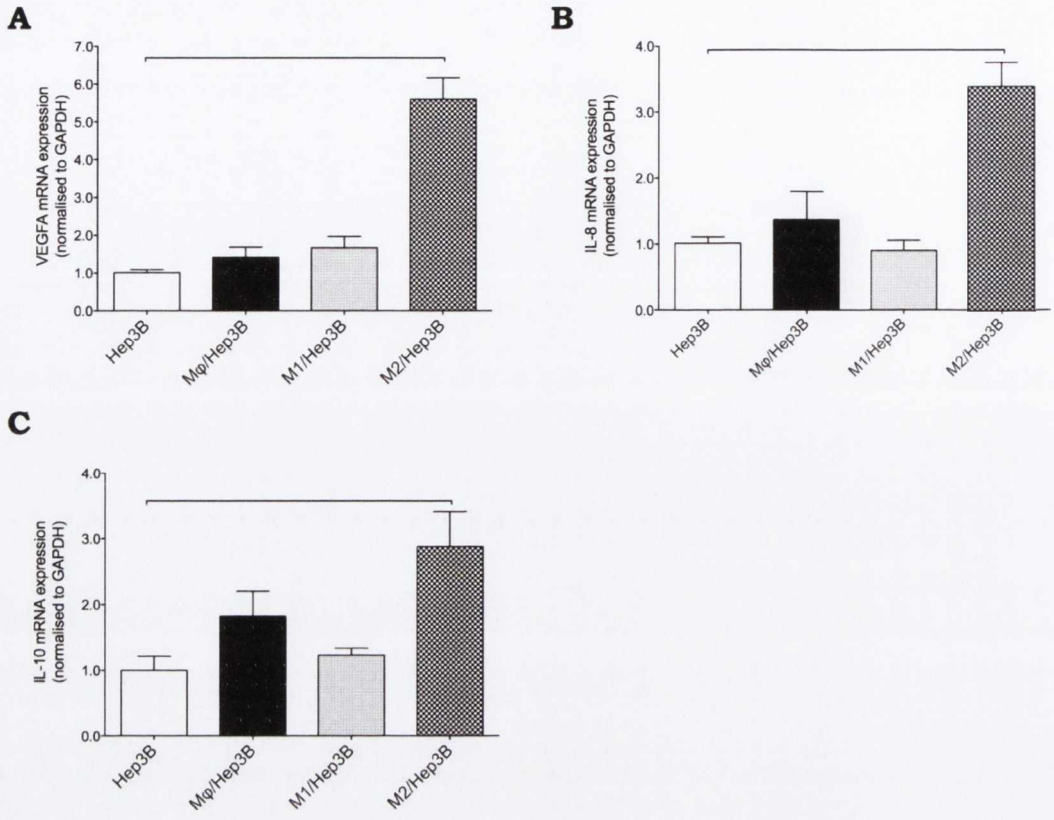


Figure 6.7: Effect of macrophage co-culture on cytokine and growth factor expression in Hep3B cells

Expression of (A) VEGFA, (B) IL-8 and (C) IL-10 in Hep3B cells under monoculture and co-culture with THP-1 derived macrophages. mRNA expression was assessed using RT-PCR, normalised to GAPDH and presented relative Hep3B cells in monoculture. Data are presented as mean ± S.E.M. and were analysed using one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate significance at $P < 0.05$ (n=4).

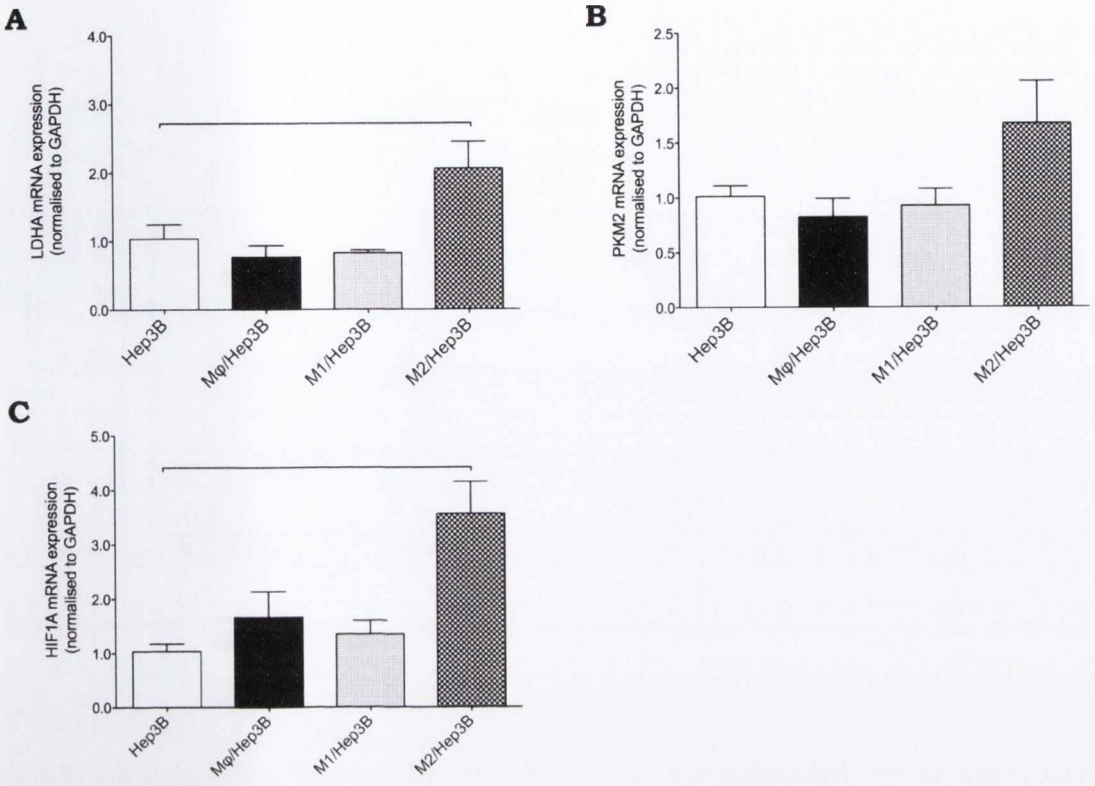


Figure 6.8: Effect of macrophage co-culture on expression of genes associated with metabolism in Hep3B cells

A) LDHA, (B) PKM2 and (C) HIF1A mRNA expression was determined using RT-PCR in Hep3B cells under monoculture and when co-cultured with macrophages. RT-PCR data are normalised to basal expression in Hep3B cells. Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate significance at $P < 0.05$ (n=3).

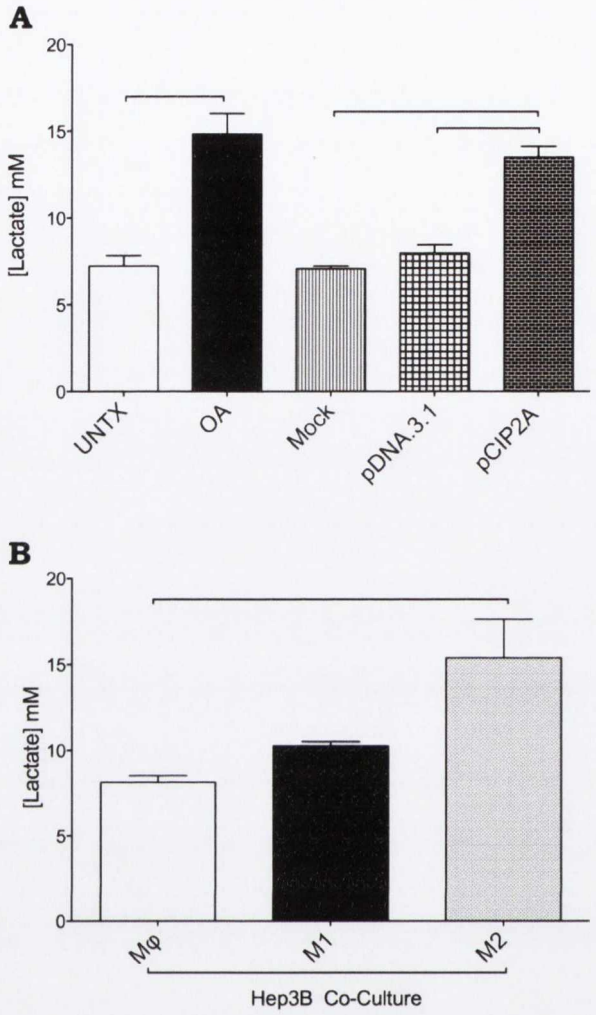


Figure 6.9: Effect of PP2A inhibition and co-culture with TPH-1 derived macrophages on lactate production by Hep3B cells

Hep3B cells were exposed to (A) okadaic acid (OA; 40 nM), for 24 h, transfected with pDNA.3.1 and pCIP2A for 48 h and (B) co-cultured with Mϕ, M1 and M2 macrophages for 24 h. Secreted lactate levels were determined using a colorimetric assay. Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate significance $P < 0.05$ (OA experiments: n=5; transfection and co-culture experiments: n=3).

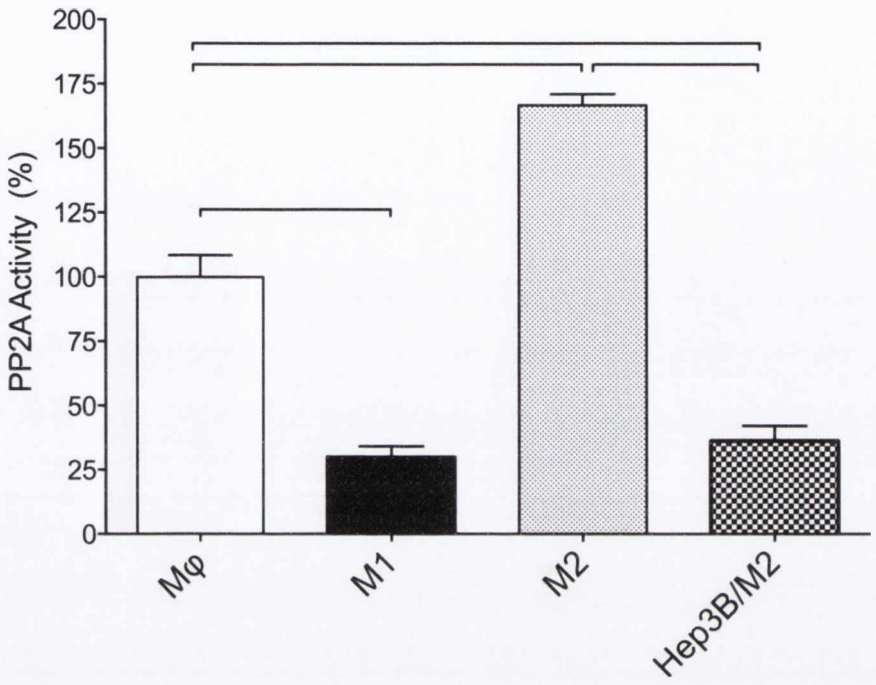


Figure 6.10: PP2A activity in THP-1 derived macrophages and M2 macrophages co-cultured with Hep3B cells

PP2A activity was determined in polarised THP-1 macrophages following polarisation and in M2 macrophages following co-culture with Hep3B cells. Data are presented as a percentage of phosphatase activity in Mϕ cells. Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

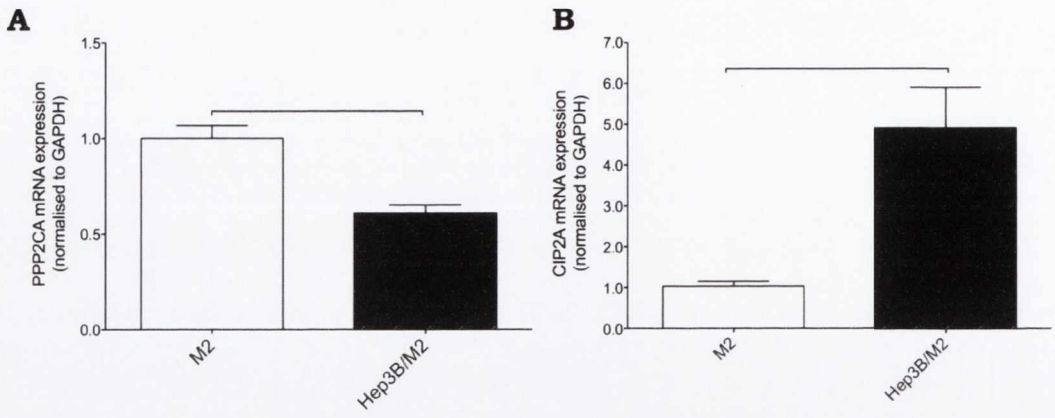


Figure 6.11: Effect of Hep3B cells on PPPCA and CIP2A mRNA expression in THP-1 derived M2 macrophages

A) PPP2CA, and (B) CIP2A mRNA expression was determined using RT-PCR in M2 macrophages under monoculture and when co-cultured with Hep3B cells. RT-PCR data are normalised to basal expression in M2 cells. Data are presented as mean ± S.E.M. and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate significance at $P < 0.05$ (n=5).

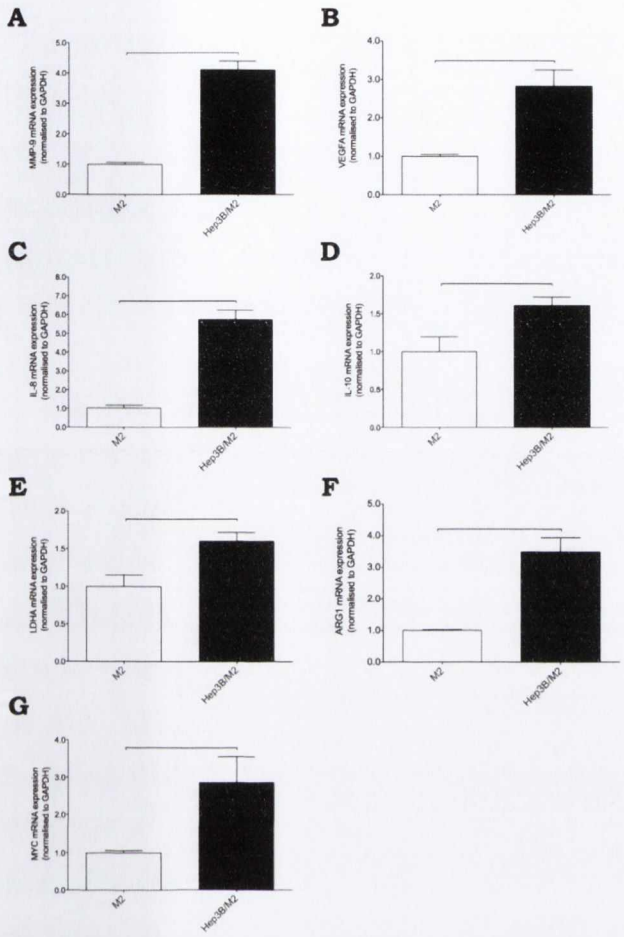


Figure 6.12: Effect of co-culturing Hep3B cells with THP-1 derived M2 macrophages on regulators of the tumour microenvironment.

Expression of A) MMP-9, (B) VEFA, (C) IL-8, (D) IL-10, (E) LDHA, (F) ARG1 and (F) Myc were determined in M2 macrophages in monoculture and when co-cultured with Hep3B cells. mRNA expression was analysed using RT-PCR, normalised to GAPDH and presented relative to expression in monocultured M2 cells. Data are presented as mean ± S.E.M. and were analysed using an unpaired Student's t-test. Horizontal bars indicate significance at $P < 0.05$ (n=3-4).

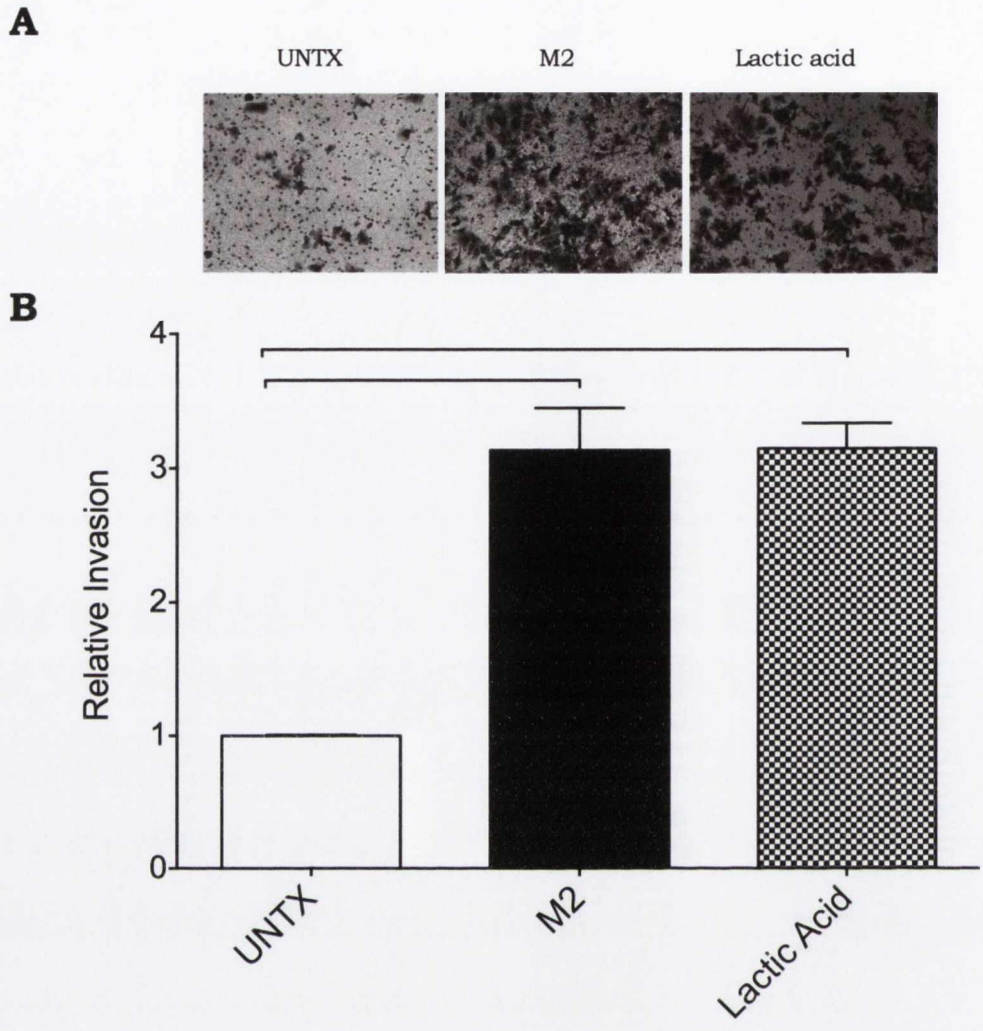


Figure 6.13: Effect of M2 macrophages and exposure to lactic acid on Hep3B invasion. Hep3B cells were co-cultured with M2 polarised macrophages for 24h or exposed to lactic acid (15 mM) for 24 h before images were captured on a Cell Imaging System (EVOS® FL). Representative images are presented (A) along with relative quantification of cell invasion (B). Image analysis was preformed using ImageJ software. Data are presented normalised to UNTX (mean ± S.E.M), and were analysed by one-way ANOVA with *post hoc* analysis (Dunnett). Horizontal bars indicate statistical significance at $P < 0.05$ (n=3).

6.4 Discussion

The present study provides evidence for macrophage-mediated loss of PP2A activity and increase in CIP2A expression in Hep3B cells. The loss of PP2A activity increases lactate production from cells, altering expression of key metabolic and remodelling genes in both immune and tumour cells when co-cultured.

Several studies have investigated the effect of growth factors and co-culturing as an *in vitro* model of the tumour microenvironment. More importantly, to the best of our knowledge a co-culture model using THP-1 cells has not been used within the context of PP2A activity and expression in hepatocellular carcinoma. THP-1 cells are acknowledged as a represent model to study macrophage biology [557, 558]. Previous studies have used polarised THP-1 macrophages to investigate the effect of Mϕ, M1 and M2 subsets on tumour cell invasion [557, 559]. In keeping with these studies the THP-1 derived M1 and M2 macrophages display a pro-inflammatory and anti-inflammatory expression profile respectively. In particular M1 macrophages were high gene expressers of TNFA, IL1B1, IL6 and IL8, while M2 macrophages had low expression of pro-inflammatory markers and high expression of TGFB1, IL13, IL10 and ARG1.

A recent study using a combination of primary macrophages and THP-1 cells found alternatively activated M2 macrophages promote tumour growth and invasiveness through the macrophage chemokine CCL22 promoting epithelial-mesenchymal transition in hepatocellular carcinoma [560]. A marked increase in cell proliferation is observed with M2-polarised-THP-1 cells co-cultivated with HCC cells (MHCC97L) resulting in an increase in tumour cell numbers by 1.3 fold [560]. In the present study, conditioned media from THP-1 derived Mϕ and M1 but not M2 macrophages reduced Hep3B cell proliferation, which is consistent with findings from a previous study by Engstrom *et al.*, [561]. Although a similar effect was noted on co-culture of Mϕ and M1

macrophages with Hep3B cells, co-culture with M2 macrophages increased Hep3B proliferation, similar to that reported by Yeung *et al.* [560].

To test our hypothesis that the increase in cell proliferation is resulted in altered PP2A function, a PP2A immunoprecipitation activity assay was used following co-culture with THP-1 derived Mϕ, M1 and M2 macrophages. Hep3B cells co-cultured with Mϕ and M2 macrophages had reduced PP2A activity compared to basal activity in Hep3B cells. Altered PP2A function has been linked to an increase in cell proliferation in cancerous cells [562, 563]. Similarly, in chapter 3 we found that altering PP2A activity with okadaic acid increases Hep3B cell proliferation. Loss of PP2A activity in cells changes the phosphorylation status of numerous signalling pathways implicating PP2A in macrophage mediated Hep3B proliferation [564]. Macrophage co-culture did not alter PP2Ac (catalytic subunit) protein expression in Hep3B cells suggesting the alteration in activity was due to differential PP2A regulation rather than loss of catalytic subunit.

As PP2Ac expression remained unchanged, we next investigated whether CIP2A expression was altered following macrophage co-culture. Co-culture with M2 macrophages resulted in an increase in CIP2A mRNA and protein expression in Hep3B cells. However, Mϕ and M1 macrophage co-culture did not alter the mRNA expression of CIP2A in Hep3B cells, yet protein expression was altered, albeit to a lesser degree than when co-cultured with M2 macrophages. These discrepancies may be as a result of reduced protein turnover or CIP2A accumulation in Hep3B cells resulting from release of macrophage derived paracrine-signalling factors, which may influence post-translational regulation of CIP2A. However, this warrants further investigation [565].

The increase in CIP2A protein expression however, as a result of M2 macrophage co-culture is very interesting. M2 macrophages promote an immuno-supressed microenvironment, conducive for cell proliferation

[566]. Parallel to this are the similarities in conditions between M2 macrophages-linked increase in Hep3B CIP2A expression and *H. pylori* mediated increase; persistent *H. pylori* infection and exposure to oncogenic CagA protein have been found to increase CIP2A expression in gastric cancer [249, 567]. *H. pylori* evades immune detection through alterations in its microenvironment, by competing with pro-inflammatory macrophage-derived NOS2 production through the up-regulation of arginase-1. This modulates the immune response by depleting the macrophages of L-arginine, which is a substrate for NOS2 [568]. As M2 macrophages produce high levels of arginase-1, we can speculate that the immunosuppressive environment provided to Hep3B cells promotes CIP2A expression, through an as yet underdetermined mechanism.

We next sought to identify whether the decrease in Hep3B PP2A activity and increased CIP2A protein corresponded with an increase in MMP-9. MMP-9 was increased in Hep3B cells compared non-cultured cells. Though we have found in previous studies that PP2A activity suppression increases MMP-9 activity, MMP-9 can also be increased trough cytokine-dependent mechanisms and thus the increase in MMP-9 is proposed as hybrid of both direct cytokine stimulation from macrophages and loss of Hep3B PP2A activity [559, 569]. This is also the hypothesis for the resulting increase in Hep3B cells IL-8, IL-10 and VEGF expression found following M2 macrophage co-culture. In previous reports, M2 co-culture with tumour cells promoted expression of metastatic genes through the secretion of soluble growth factors such as TNF-α [557, 570, 571]. Whether these increases are solely PP2A-dependent or as a consequence of paracrine secretion of soluble growth factors remains to be deduced further.

Co-culture of THP-1 cells with Hep3Bs resulted in the creation of an acidic environment through the induction of glycolytic gene expression. We have tentatively linked this with the increase in CIP2A in Hep3B cells. As well the identification of phosphorylation of LDHA, PKM2 and

ARG1 in Hep3B cells following exposure to okadaic acid and CIP2A overexpression also increased lactate levels to concentrations similar to that found in M2 co-cultures. High lactate secretion from tumour cells inhibits disturbs T-cell metabolism and their anti-tumour function [572]. An acidic extracellular pH not only activates secreted soluble factors that aid in pro-metastatic activities, it also activates intracellular pro-survival signalling cascade through the up-regulation of HIF1-α [573, 574]. Hypoxic microenvironments have been found with decreased PP2A activity [349]. Whether acidosis of the tumour microenvironment positively upregulates CIP2A remains to be investigated.

A dichotomy has been proposed for macrophage activation: classic vs. alternative, in which TAM exist as a hybrid of both. M1 cells are high proliferating cells that produce high-levels of pro-inflammatory cytokines, while M2 cells are anti-inflammatory and low proliferative [575]. M1 macrophages have been found to have highly activated signalling pathways such as NF-κB, ERK and JNK pathways [576, 577]. PP2A has been found to have a role to play in counter acting the inflammatory responsive: α1-antitrypsin has been found to activate PP2A in human alveolar macrophages and limit the inflammatory and proteolytic responses induced by TNF signalling in the lung [578]. Our study builds on these findings while highlighting that M2 macrophages express higher levels of PP2A activity compared to pro-inflammatory M1 cells. However, co-cultured with Hep3B cells, these M2 macrophages have significantly reduced PP2A activity compared to classical M2s and resembled a M1 PP2A activity phenotype. TAMs isolated from established tumours have an immunomodulatory M2-like phenotype consistent with the smouldering nature of cancer-related inflammation [579]. However, TAM exists as mixed cell populations, expressing both pro-inflammatory and anti-inflammatory markers [580]. Our study postulates the idea that within these subset populations, PP2A activity may be reduced in both types of

macrophages, fostering conditions conducive for tumour growth and proliferation.

As well as altered PP2A activity, TAMs have increased c-Myc expression compared to both M1 and M2 macrophages. c-Myc has been found to play a role in macrophage polarisation with c-Myc reported to control up to 45% of M2 macrophage gene expression [581]. Increased c-Myc expression has also been reported in TAMs and has been linked with to an increase in expression of MMP-9 and VEGF production [582]. Resting cells normally express low levels of c-Myc, which as an immediate early-response gene is dramatically increased upon exposure to growth factors or changes in the cell microenvironment [583]. Our study highlights for the first time an increase in CIP2A expression associated with co-culturing M2 with Hep3B cells. Previous studies have found that in lung cancer that IL-10 promotes CIP2A expression via PI3K and CREB signalling [251], while high levels of IL-10 expressing monocytes and macrophages have been found in patients with HCC [584]. CIP2A has also been found to play a role in monocyte macrophage differentiation, potentially as a transcription factor modulator in the lipid related differentiation process[585]. Deregulation of monocyte-macrophage differentiation is a hallmark of cancer and metabolic diseases and associated with persistent low-grade inflammation. From this, we can postulate that the increase in CIP2A may have a role to play in the reduction of PP2A activity in TAMs, altering their phenotype to one conducive of tumourigenesis. Reduced PP2A activity in TAMs also promoted the expression of MMP-9, VEGF and IL-8 from the macrophages. This builds on previous work from our lab that links loss of PP2A activity results in an increase in MMP-9 expression [485].

TAMs also were found to higher expression of lactate dehydrogenase 1 and arginase 1 compared to M2 macrophages. Increase in LDHA expression promotes lactate formation and the acidification of the tumour microenvironment. High levels of ARG1 expression in TAMs

results in depletion of, L-arginine from the extracellular environment [586]. Reduction in L-arginine reduces nitric oxide production weakens the anti-tumour profiles of immune cells, resulting in a reduction of cytotoxic T-cell infiltration into the microenvironment [587, 588]. M2 macrophage co-culture and exposure to 15 mM lactate promotes Hep3B cell invasion through a Matrigel membrane. Together, we can tentatively postulate a potential role for the up-regulation of CIP2A and subsequent stabilisation of c-Myc in TAMs: foster their pro-tumour activities in the microenvironment. Whether this is as a result of alterations in microenvironment conditions or tumour soluble factors is as yet undermined.

In conclusion, anti-inflammatory macrophages promote CIP2A expression in Hep3B cells through the modulation of the tumour microenvironment. The increased Hep3B cell proliferation was found mediated through a PP2A/CIP2A axis. CIP2A promotes an anti-inflammatory niche through the increase in tumour lactate, acidifying the microenvironment, depleting substrates essential for anti-tumour immune cells [589]. This study highlights a novel role for loss of PP2A function and increase in CIP2A expression in promoting HCC tumourigenesis.

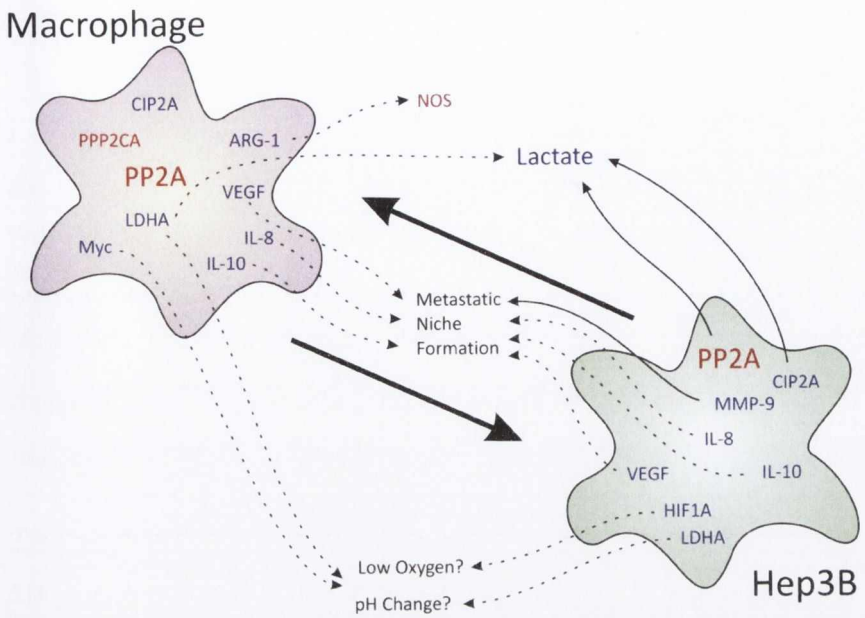


Figure 6.14: Schematic diagram presenting the key findings in THP-1 macrophages and Hep3B cells

Hep3B and THP-1 derived M2 macrophages both have reduced PP2A activity following co-culture. Co-culture increases Hep3B derived MMP-9 activity and also the gene expression of pro-metastatic factors. Macrophage co-culture increases Hep3B CIP2A expression that in turn increases tumour production of lactate.

Chapter 7.

Conclusions and Future Direction

7.1 Conclusions

PP2A is an indispensable enzyme which transverses cellular signalling pathways and regulates a diverse array of proteins. This thesis assesses the functional consequences of PP2A inhibition on factors associated with the formation of the metastatic niche. Subsequent inhibition of PP2A results in a deregulation of PP2A signalling, which alters MMP-9 expression, TIMP-1 regulation and has implications on the smouldering inflammation phenomenon in a model of the tumour microenvironment.

The first study characterises the proliferative effects of okadaic acid in Hep3B cells and highlights the potential implications of PP2A inhibition in an *in vitro* model. Previous studies have found okadaic acid to mediate cell proliferation through the structural rearrangement of cytoskeletal microfilaments and the up-regulation of intracellular protein phosphorylation [421, 590]. Using a proteomic approach, we identified that exposure to okadaic acid and subsequent inhibition of PP2A stimulates the phosphorylation of 180 proteins affiliated with cell migration, gene regulation and cell proliferation at a 24 h time point. Proven and putative phosphoproteins were identified following our enrichment method.

The identification of Casein kinase 2 (CK2) as a common phosphorylation motif is intriguing. CK2 is reported to phosphorylate over 300 different proteins, though many believe this to be a conservative estimate [591]. CK2 phosphorylation can trigger activation of AKT prompting downstream signalling cascades resulting in an increase in cell proliferation. Many of the proteins that were identified in our screen are among those phosphorylated by CK2. Interestingly, the catalytic subunit of PP2A itself is phosphorylated by CK2 [592] along with the oncogene c-Myc [593].

Exposure of Hep3B cells to okadaic acid also promoted the phosphorylation of members of the EIF2 signalling family, which are

involved in the initiation phase of eukaryotic translation. We identified eif3H, eif4G1 and eif4A3 as phosphorylated following okadaic acid exposure. Previous studies have indicated a role for PP2A in the dephosphorylation of another member of the eIF family, eif4E [401, 402]. Our study extends this to show that okadaic acid results in phosphorylation of other members of the eukaryotic translation initiation factor family and thus promotes protein synthesis necessary for high proliferative cells. Although multiple cellular signalling components were identified through bioinformatics, Myc was identified as the top upstream regulator within our data set. Okadaic acid was found to increase the protein expression of c-Myc in Hep3B.

These results indicate a loss of PP2A activity resulting from exposure to okadaic acid as facilitating the increase in c-Myc expression, aggregating its cell proliferator effects in Hep3B cells [364]. However, our data set found no single signalling pathway overrepresented. This could be due to a potential limitation of the present study as phosphorylation is a transient modification but only one time point was investigated. As a result, our data is only a snapshot of the potential regulatory effects resulting from PP2A inhibition. Also, whether okadaic acid is a true representative of PP2A inhibition within this context remains to be determined.

Arginase 1 and lactate dehydrogenase A were also identified in our experiments as proteins phosphorylated following the exposure to okadaic acid. LDHA converts pyruvate to lactate, acidifying the tumour microenvironment and promoting conditions conducive for metastasis. ARG-1 depletes the milieu of L-arginine, a key metabolite needed for the pro-inflammatory response against tumours. This observation was a key factor in directing our investigation into the role of okadaic acid and PP2A in the regulation of soluble secreted proteins involved in the metastatic niche formation, MMPs.

Okadaic acid increases MMP-9 abundance and mRNA expression in hepatic epithelial cells and hepatic stellate cells through the loss of PP2A activity. Although okadaic acid altered cell migration, it did not alter any other member of MMP family studied, suggesting a role for MMP-9 in okadaic acid induced cell invasion of Hep3B cells. Knockdown of the catalytic subunit of PP2A mimicked the effects seen with okadaic acid in cells, while pharmacological inhibition or knockdown of PP1 or PP2B had no effect on MMP-9, suggesting the increase in MMP-9 is PP2A-mediated. The finding that the cancerous inhibitor of PP2A (CIP2A) also increased MMP-9 abundance and cellular invasion of Hep3B cells strengthens our findings that the loss of PP2A function promotes tumour cell invasion through the up-regulation of MMP-9. For okadaic acid to increase MMP-9 expression there was an absolute dependence on the proximal AP-1 motif at position -79 on the MMP-9 promoter. While loss of the distal AP-1 site at -533 obstructed the response, it did not obliterate it. Interestingly, the inflammatory transcription factor NF-κB is redundant in PP2A mediated increase in MMP-9 expression. These data illuminate a new prospect for PP2A in regulating the extracellular matrix and tumour remodelling.

Intriguingly in chapter 4, okadaic acid increased the expression of TIMP-1 in epithelial cells, but decrease it in hepatic stellate cells. The disparity of okadaic acid in the regulation of TIMP-1 has been reported previously; okadaic acid both increases [342] and decreases TIMP-1 expression [474]. The difficulty in understanding TIMP-1 regulation is that it differs depending upon the stimuli and cell type studied; therefore, we compared the effect of okadaic acid on TIMP-1 regulation Hep3B, Huh-7 and LX-2 hepatic cell lines.

Okadaic acid (40 nM) increased TIMP-1 expression in Hep3B and Huh-7 cells, but decreased it in LX-2 cells. Okadaic acid elicited a dose dependent response in Hep3B cells, and increase in Huh-7 cells, while decreasing TIMP-1 expression in LX-3 cells. Silencing of the PPP2CA gene in all three-cell lines reduced TIMP-1 expression, while

overexpression of both $I2^{PP2A}$/SET and CIP2A decreased TIMP-1 expression. However, cells overexpressing SET and CIP2A exposed to high levels of okadaic acid had increased TIMP-1 expression. From this, we sought to deduce the signalling pathways mediating the increase in TIMP-1 resulting from the okadaic acid. Inhibition of NF-κB signalling lowered basal TIMP-1 abundance, but had no effect on the OA-mediated TIMP-1 expression. Inhibition of either PI3K by LY-294002 or MEK by PD-0325901 negated the OA effect. In the presence of SP 600125, a c-Jun inhibitor, OA (40 nM) reduced TIMP-1 expression well below basal expression levels. This study potentially links PP2A both as a positive and negative regulator of TIMP-1 expression in hepatocytes.

Transcriptional regulation of TIMP-1 was found to be one of two stories. First, we identified suppressor element regions involved in TIMP-1 basal promoter activity both upstream and downstream of transcription start site. The -1718 to -890 region upstream and the +96 to +988 region downstream from the transcription start site of TIMP-1 both contained as yet unidentified inhibitory promoter elements. Okadaic acid-mediated TIMP-1 expression required functional AP-1 sites, with the proximal AP-1 binding motif at -102 being an essential for okadaic acid's effect. This study also identified a putative CREB binding motif at -231, as being essential for basal TIMP-1 expression and CIP2A-mediated inhibition of TIMP-1. Collectively, this study highlights the complexity of TIMP-1 regulation with regards PP2A and basal TIMP-1 regulation, and identifies differences between pharmacological and molecular inhibition of PP2A.

Based upon our previous findings that PP2A inhibition modulates intracellular signalling which is manifest as alterations in extracellular protein expression we investigate if the cell microenvironment may have in fact contributed to PP2A's loss of function in tumours. As tumours are persistently barraged and influenced by their environment, we postulated that persistent inflammation resulting from paracrine interactions or "smouldering inflammation" would decrease PP2A

function. Indeed, Hep3B cells co-cultured with anti-inflammatory macrophages were found to have reduced PP2A activity compared to those cultured with pro-inflammatory macrophages co-culture.

With regard to PP2A activity in Hep3B cells, co-culturing with anti-inflammatory macrophage cells increased CIP2A expression without loss of PP2Ac protein expression. The increase in CIP2A expression coincided with an increase in MMP-9 expression and activity in Hep3B cells and increased expression in THP-1 macrophages. HIF1A mRNA was altered in Hep3B cells co-cultured with anti-inflammatory macrophages. Complementary to this, okadaic acid and CIP2A over expression increased the concentration of lactate in the tumour supernatant conducive to that of the TAM/Hep3B co-culture. The increase in CIP2A in Hep3B cells by macrophages is through an unidentified mechanism; however, it may be caused by a metabolic shift within the cells resulting from acidification of the microenvironment. This study provides a potential mechanism for the up-regulation of CIP2A in tumours and also delineates further the importance of PP2A activity in macrophage function.

Parallel to this, M1 pro-inflammatory macrophages had reduced PP2A activity compared to M2 macrophages and activated monocytes. Conversely, M2 macrophages cultured with Hep3B cells had reduced PP2A activity yet increased mRNA expression of both CIP2A and c-Myc. M2 macrophages cultured with Hep3B cells had higher mRNA expression of IL-10 and IL-8 in comparison to classical M2 macrophages, consistent with the phenotype of tumour promoting TAM macrophages [594]. As Hep3B cells seemingly re-programme M2 macrophages through reduction in PP2A activity possibility exists that PP2A/CIP2A are important in development of the tumour-associated macrophage (TAM) phenotype in cancer. However, TAM exists as mixed cell populations, expressing both pro-inflammatory and anti-inflammatory markers [580]. A limitation to our findings is that we used macrophages with pre-defined cellular phenotypes that are also an

immortalised cell line. Whether true TAM cells decrease PP2A activity within tumours remains to be determined.

In conclusion, intracellular PP2A inhibition leads to alterations in tumour cell signalling, which alters the tumour's ability to produce extracellular proteins, such as MMP-9 and TIMP-1, and promotes a microenvironment that fosters reduced PP2A activity. Together these findings highlight a central role for PP2A in altering tumour homeostasis, conducive of metastasis, proliferation, immune evasion and metabolic reprogramming.

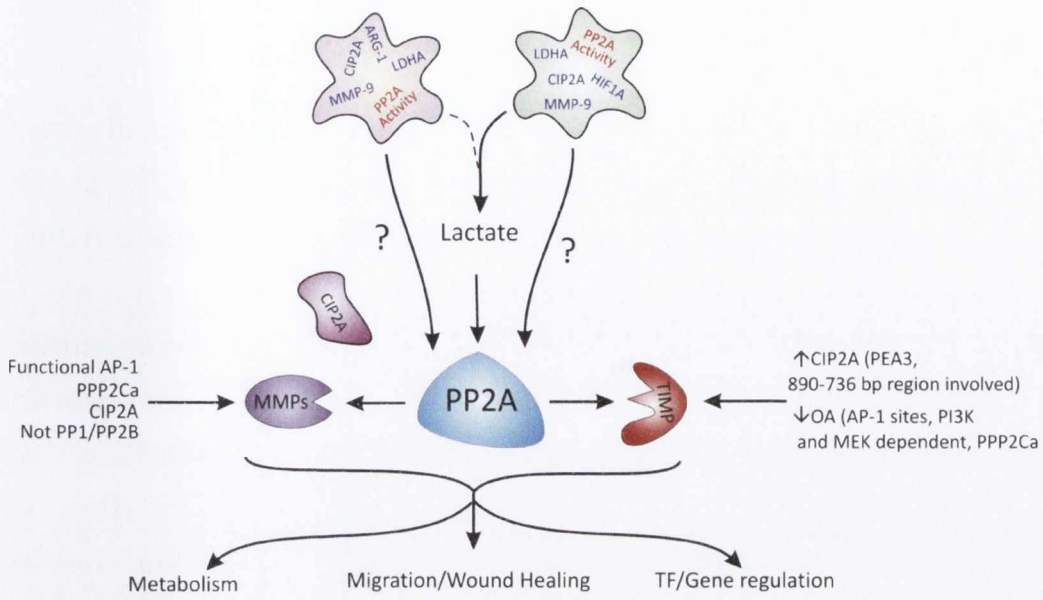


Figure 7.1: Summary of the role of PP2A in modulating the formation of the metastatic niche in HCC

PP2A inhibition is central for the formation of the metastatic niche. Inhibition of PP2A promotes cell migration, alters cellular metabolism and promotes the up-regulation of genes conducive for metastasis. PP2A regulates MMP-9 expression through functional AP-1 sites with overexpression of CIP2A mimicking pharmacological inhibition. Molecular inhibition of PP2A reduces TIMP-1 expression while pharmacological inhibition increases TIMP-1 trough functional AP-1 and CREB sites. Bidirectional paracrine communication between anti-inflammatory macrophages reduces PP2A activity while increasing tumour cell CIP2A expression. Loss of PP2A activity promotes tumour cell lactate production, acidifying the tumour milieu further, promoting the formation of the metastatic niche.

7.2 Future Directions

The potential for further experiments within the context of this study is pronounced. Though our study investigates a novel role for PP2A in the regulation of the MMP system and formation of the metastatic niche, many important questions remain unanswered.

With regards okadaic acid regulation of the phosphoproteome within cell, a more detailed approach is required. Mapping of all protein phosphorylation status changes through ITRAQ labelling of phosphorylation sites and a quantification of phosphorylation is needed. Also a comparison of pharmacological inhibition versus molecular inhibition of PP2A would shed light upon the potential issue of whether OA inhibition and proliferative effects are mimicked by either subunit deletion of endogenous protein inhibitor overexpression.

Further signalling work is needed to expand our understanding of PP2A regulation of both MMP-9 and TIMP-1. Though we identified the importance of the AP-1 complex

Chapter 8.

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