The role of Protein Kinase D signalling in the induction of collagenase gene expression in human articular chondrocytes

Jonathan Baker BSc (Hons)

A thesis submitted to the Institute of Cellular Medicine
Newcastle University
For the Degree of
Doctor of Philosophy

Musculoskeletal Research Group
Institute of Cellular Medicine
Newcastle University, UK
March 2014

Abstract

The destruction of articular cartilage is a central feature of arthritis. The activity of collagenase enzymes, induced by pro-inflammatory cytokines, is a key step in this process. Collagenases have been targeted therapeutically but trials have not proven beneficial due to off-target effects. Understanding the signalling consequences which drive the expression of these proteolytic enzymes is therefore a major area of research. The aim of the present study was to elucidate the signalling events that regulate collagenase expression, focusing on the role of a small family of serine/threonine kinases termed protein kinase D (PKD).

To understand the role of the individual PKD isoforms in the modulation of collagenase gene expression, each isoform was selectively silenced. Using a model of the pro-inflammatory milieu prevalent in arthritic disease, the consequences of isoform specific gene silencing on the expression of MMP-1 and MMP-13 was studied in primary chondrocytes stimulated with IL-1 in combination with OSM. Data suggest PKD1 to be 'anabolic', with gene silencing leading to increased collagenase gene expression. PKD2 was shown not to significantly modulate the collagenase expression, whereas, PKD3 silencing markedly reduced the collagenase gene expression.

To understand the signalling consequences orchestrated by each isoform of PKD, the role each isoform in the regulation of signalling pathways known to modulate collagenase gene expression was examined. PKD1 and PKD3 silencing both abrogated the phosphorylation of the MAPK signalling pathways (ERK, JNK and p38). PKD3 silencing also led to decreased STAT-1 and STAT-3 serine phosphorylation. This contrasted with the effects of PKD1 silencing, in which STAT-1 serine and tyrosine phosphorylation increased, as well as increased Akt and p65 phosphorylation being observed. These opposing roles may explain the differences in the regulation of collagenase gene expression between each isoform. To further understand this modulation the expression of the AP-1 components, Fos and Jun were examined, along with other recently identified post-AP-1 factors (ATF3, EGR2 NFATc1, and BMP-2). Data showed PKD3 silencing to reduce their expression, suggesting a potential mechanism by which PKD3 signalling can modulate MMP expression. In conclusion, this work identifies the distinct individual roles of PKD isoforms in the modulation of collagenase gene expression, illustrating the need to assess individual kinases within a family.

Acknowledgements

I would like to thank Professor Drew Rowan for all of his help and encouragement throughout my PhD, especially during the writing of my thesis. I would like to also thank Dr Gary Litherland for all his help in experimental design and answering of all my incessant questions of signalling mechanisms and also which new ales to try.

I'd like to thank all members of the MRG who have helped me throughout my PhD, especially all fellow tunnel dwellers. My PhD would not have been the same without you all. A special mention must go to Andrew Butcher for all his help with the lentiviral experiment and also to Matt Barter for all his answering of questions and general advice. I would also like to thank Martina Elias for all her help at the beginning of my PhD, as well as the kind gift of the lentiviral packaging and envelope plasmids.

A special thank you to my parents and friends for all their support and interest into my research, I could not have got through this without you all. The biggest thank you of all goes to my girlfriend Victoria, thank you for all you have done to help me over the past three years and for proof reading this thesis, even if most of it was gibberish to you.

Finally I would like to thank Arthritis Research UK for funding of this project and giving me the opportunity to partake in this work.

Contents

List of Figures

List of Tables

Abbreviations

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AK	Adenylate kinase
AP-1	Activator Protein-1
aPKC	atypical PKC
APS	Ammonium peroxodisulphate
ATCC	American Type Culture Collection
AXUD1	Axin-1 induced gene
BCP	Basic Calcium Phosphate
BMP2	Bone morphogenic protein 2
Bp	Base pair
BSA	Bovine serum albumin
C/EBP	CCAAT enhancer-binding protein
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CAMK	calcium/calmodulin dependent Kinase
CEB	Cytoplasmic Extraction Buffer
CER	Cytoplasmic Extraction Reagent
cPKC	conventional PKC
Cyt	Cytoplasmic tail
DAG	Diacylglycerol
DMARDs	Disease-modifying antirheumatic drugs
DMEM	Dulbecco's modified Eagle's medium
DMEM F12	Dulbecco's modified Eagle's medium: Hams F12 medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotides
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGR2	Early growth response 2
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-regulated kinase

Ets	Erythroblastosis twenty-six
FBS	Foetal bovine serum
FSB	First strand buffer
Fu	Furin recognition motif
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GPI	Glycosylphosphotidylinositol
GRB2	Growth factor receptor-bound protein 2
H_2O	Water
H_2O_2	Hydrogen peroxide
HA	Haemagglutinin
HAC	Human articular chondrocytes
HBBS	Hank's balanced salt solution
HRP	Horseradish peroxidase
IC_{50}	half maximal inhibitory concentration
ICE	IL-1 converting enzyme
IFN	Interferon
Ig-like	Immunoglobulin-like domain
IKB	Inhibitor of NFκB
IKK	IκB kinase
IL	Interleukin
IL-1R	IL-1 receptor
IL-1RI AcP	IL-1 receptor accessory protein
IP_3	Inositol trisphosphate
IRAK	IL-1 receptor-associated kinase
JAK	Janus kinase
JIP	JNK-interacting protein
JNK	Jun N-terminal kinase
K_{cat}	First-order rate constant
LGB	Lower gel buffer
LIFR	Leukemia inhibitory factor receptor
MAPK	Mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MATra	Magnet assisted transfection
MEB	Membrane extraction buffer
miRNA	microRNA

MMLV	Moloney murine leukamiea virus
MMP	Matrix metalloproteinase
MOI	Multiplicity of Infection
MSC	Mesenchymal stem cell
MT-MMPs	Membrane type MMPs
MyD88	Cytosolic myeloid differentiation 88
NEB	Nuclear extraction buffer
NEMO	NFκB essential modifier
NER	Nuclear extraction reagent
NFATc1	Nuclear factor of activate T-cells cytoplasmic 1
NF-E1	Erythroid-specific transcription factor
NFκB	Nuclear factor kappa B
NIK	NFκB-inducing kinase
NOF	Neck of femur
nPKC	novel PKC
OA	Osteoarthritis
OSM	Oncostatin M
OSMR	OSM Receptor
PBS	Phosphate buffered saline
PCR	Polymerisation chain reaction
$pd(N)_6$	Random hexamers
PDK1	3-Phosphoinositide-dependent protein kinase-1
PEB	Pellet extraction buffer
PI3K	Phosphatidylinositol-3 kinase
PIAS-3	Protein inhibitor of activated STAT-3
PIP_2	Phosphatidylinositol 3, 4 bisphosphate
PIP_3	Phosphatidylinositol 3, 4, 5 trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
Pol III	RNA polymerase III
Polybrene	Hexadimethrine bromide
PtdIns	Phosphatidylinositol
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
Rac1	Ras-related C3 botulinum toxin substrate 1
RCLs	Replication competent lentiviruses
RFP	Red fluorescent protein

RIN-1	Ras and Rab interactor-1
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA Interference
RNase	Ribonuclease
ROS	Reactive oxygen species
RRE	Rev response element
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-PCR	Real Time PCR
rtTA3	Reverse tet-transactivator
RUNX2	Runt-related transcription factor 2
SBE	STAT binding element
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH2	Src homology 2 domain
SHP-2	Src homology phosphotyrosyl phosphatase
shRNA	short hairpin RNA
SIE	Sis-inducible element
SILAC	Stable isotope labelling of amino acids
siRNA	small inhibitory RNA
SOCS	Suppressors of cytokine signalling
SOS	Son of sevenless
SP-1	Specificity Protein-1
SRE	Serum response element
STAT	Signal transducers and activators of transcription
TAB2	TGF-beta activated kinase 1/MAP3K7 binding protein 2
TAK1	Transforming growth factor-beta-activated kinase 1
TBS-T	Tris buffered saline- tween
TEMED	N,N,N’N’-tetramethylenediamine
TGN	Trans-Golgi network
TIMPs	Tissue inhibitor of metalloproteinase
TM	Transmembrane
TNF	Tumour necrosis factor
TPA	Tissue-type plasminogen activator
TRAF	TNF receptor associated factor
TRE	Tetracycline response element
Tween 20	Polyoxyethylenesorbitan monolaurate

UGB	Upper gel buffer
VSV-G	Glycoprotein of the vesicular stomatitis virus
Zn	Zinc

1 Chapter 1. Introduction

1.1 The joint

Arthritis is a progressive disease of load bearing joints. The main type of joint found within the body are synovial joints which are composed of a number of tissue types and fluids. These include articular cartilage, the capsule, the synovium, synovial fluid, tendon, ligament and subchondral bone. In addition, in joints such as the knee, the fat pad, menisci and patella are present. Joint homeostasis is crucial, with damage, changes in load bearing and the infiltration of inflammatory mediators all lead to changes in joint pathology. Imbalance in this homeostasis leads to arthritic disease which is seen to be detrimental to the joint (see *Figure 1.3.* for differences between diseased and healthy joints).

1.2 Human articular cartilage

Articular cartilage is a smooth friction-less surface that coats the articulated surfaces of bones. This tissue is unique in the fact that it is avascular, alymphatic and anerual. Embedded within this tissue is the sole cell type, the chondrocyte. These cells derive their nutrients and remove their waste products by diffusion through the extracellular matrix (ECM) to the synovial capsid and fluid (Rowan and Young, 2007). Articular cartilage derives its mechanical properties from its ECM (Buckwalter et al., 2005). Water contributes up to 70% of the wet weight of articular cartilage. Interactions between water and the many macromolecules which cartilage comprises of, influences the mechanical properties of the tissue. Collagens, proteoglycans, non-collagenous proteins and glycoproteins make up 20-40% of the wet weight (Pearle et al., 2005). The composition of these macromolecules gives the cartilage the ability to absorb and distribute high compressive forces.

Collagen, the most widely expressed protein within the body, provides a fibrillar meshwork that gives form and tensile strength to the cartilage. Proteoglycans and non-collagenous proteins can bind or become mechanically entrapped within this mesh, while water fills the interstitial space. Type II collagen is the primary collagen within articular cartilage, accounting for around 90% of all collagen molecules (Pearle et al., 2005). Type II collagen is composed of 3

identical αI polypeptide chains that intertwine to form a triple helix. The triple helix align to form collagen fibrils; these fibrils provide the framework for the collagen meshwork (Shoulders and Raines, 2009). This is stabilised by internal crosslinking between molecules. Type XI collagen, another cartilage specific fibrillar collagen, covalently binds to type II collagen, aiding in the stability of the meshwork (Buckwalter et al., 2005). Type IX collagen is another cartilage specific collagen which does not form fibrils but binds to the surface of the type II/type XI fibril (Kuettner, 1992). Type X collagen is found in the zone of calcified cartilage and is synthesised only by hypertrophic cells. Type X collagen has been described as an indicator of chondrocyte differentiation to bone (Aigner et al., 1993).

Proteoglycans resist compression and generate turgidity due to their affinity for water. Aggrecan is the predominant proteoglycan found within articular cartilage and is non-covalently bound to hyaluronic acid via a link protein. This provides the osmotic properties that are needed for articular cartilage to resist compressive loads (Martel-Pelletier et al., 2008). It has a high sulphated glycosaminoglycan (GAG) content, which is negatively charged, drawing water into the tissue. The osmotic properties of aggrecan, along with the meshwork of collagen fibrils, form the structure and provide the tensile properties of cartilage. Other molecules such as decorin, biglycan, laminin, tenascin, Cartilage oligomeric matrix protein (COMP) and fibromodulin assist in maintaining tissue structure through the spatial organisation of the ECM (Temenoff and Mikos, 2000). *Figure 1.1* is a representative diagram of the extracellular matrix of cartilage.

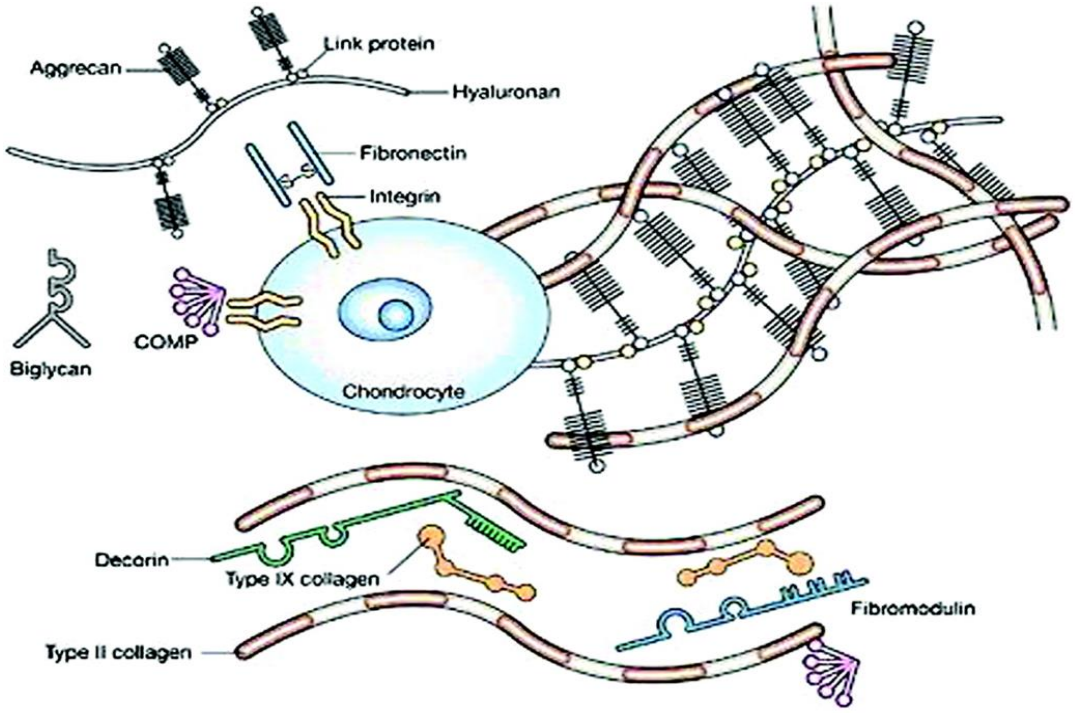


Figure 1.1. The extracellular matrix of cartilage. The extracellular matrix of cartilage consists of two major load-bearing macromolecules: collagen and proteoglycan. Of these; type II collagen and aggrecan are the major components. Smaller classes of macromolecules are present and aid in the tensile strength of cartilage. Embedded within the ECM is the sole cell type the chondrocyte, which aids in the synthesis and degradation of these macromolecules. Taken from (Chen et al., 2006).

1.2.1 The layers of human articular cartilage

Articular cartilage can be divided into five distinct zones (superficial, transitional, middle, deep and calcified), as depicted in *Figure 1.2*. Each zone has different morphological features, with the boundaries between each zone not being easily distinguishable. The superficial zone is in contact with the synovial fluid and provides a smooth surface for articulation. This zone represents 10-20% of the articular cartilage volume and can be divided into two layers. The first is an acellular sheet of fibrils, covering the joint surface. Below this is a second layer composed of flattened chondrocytes with a high collagen and water content, but low proteoglycan composition compared to the other zones (also known as the transitional zone) (Buckwalter et al., 2005). The middle zone consists of around 40-60% of articular cartilage volume. This zone is less organised than the upper superficial zone due to the unorganised orientation of thicker collagen fibrils. The chondrocytes within this zone are more rounded, larger and less compacted. The deep zone (representing 30% volume) contains large diameter fibrils which orientate themselves in a perpendicular fashion to the articular

surface. This layer has the greatest proteoglycan content, but lowest water content, allowing for the greatest compression of any other zone. The chondrocytes within this zone align themselves parallel to the fibrils in long columns. The tidemark separates the deep cartilage zone from the calcified cartilage which lies directly on the subchondral bone (Pearle et al., 2005). This thin zone of calcified cartilage links the middle zone to the subchondral bone, with collagen fibrils extending from the calcified cartilage into the articular cartilage.

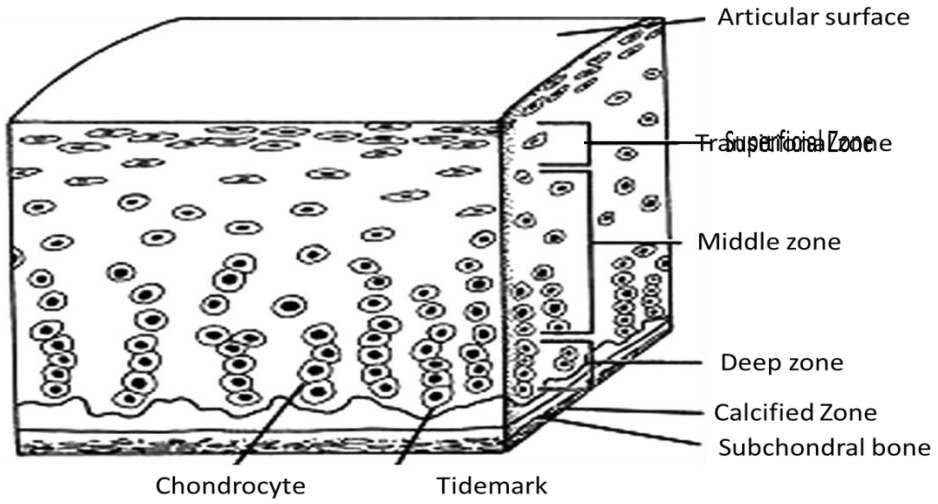


Figure 1.2. The zones of articular cartilage. There are 5 distinct zones of the articular cartilage. These zones are known as superficial, transitional, middle, deep and calcified, with all have distinct characteristics. Changes in collagen, aggrecan and water content are seen between each zone, aiding in their distinct properties. See *section 1.2.1* for full details. Adapted from (Buckwalter et al., 1994).

1.3 Human articular chondrocytes (HAC)

Human articular chondrocytes represent around 0.4-2% of the total volume of articular cartilage (Martel-Pelletier et al., 2008). Chondrocytes are vital in cartilage homeostasis as they are involved in both the synthesis and degradation of the cartilage matrix. Chondrocytes originate from mesenchymal stem cells (MSCs) which undergo chondrogenesis to produce mature chondrocytes (Goldring, 2012). During early development, MSCs undergo recruitment to correct loci, migration and proliferation. Then through cell-cell and cell-matrix interactions, as well as the secretion of differentiation factors, MSCs are differentiated into mature chondrocytes (Goldring et al., 2006). Differentiation begins with the synthesis of the ECM, rich in hyaluronan and type I collagen, aggregation of cells and finally the expression of chondrocyte

specific macromolecules such as type II collagen and aggrecan (Goldring et al., 2006). These molecules are synthesised into an ordered and organised framework, providing structure for the articular cartilage.

Proliferation of chondrocytes in normal conditions is limited with maintenance of the ECM regulated by low turnover of the ECM, and replacement of matrix proteins. If the synthesis and degradation of the ECM is disrupted, as in ageing and joint disease, then an imbalance in these processes occurs. Here the rate of collagen and proteoglycan degradation, by matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), exceeds synthesis, leading to destruction of the ECM (Cawston and Wilson, 2006). Cartilage was originally believed to be an inert tissue, with chondrocytes unable to respond to external stimuli. This has been disproved with cartilage being shown to be able to respond to external stimuli and extrinsic factors, leading changes in gene expression and protein synthesis (Sandy et al., 1984, Muir, 1995).

Two mechanisms of damage to cartilage have been described: the first of these is known as abnormal load on normal cartilage. This mechanism describes how chondrocytes can respond to changes in loading. Chondrocytes respond to these mechanical stresses, such as excessive load, inducing changes in the synthesis of cartilage matrix proteins (Fitzgerald et al., 2004). Injury to tissue can also lead to changes in load, leading to global gene expression changes (Kurz et al., 2005). The second of these mechanisms is described as normal load on abnormal chondrocytes. This describes changes in chondrocytes due to age or a genetic pre-disposition, leading to the imbalance of synthesis and degradation of ECM macromolecules. Chondrocytes can also respond to cytokines and growth factors leading to changes in gene expression which can alter the ECM of the cartilage.

1.4 Arthritis

Joint disease such as rheumatoid arthritis (RA) and osteoarthritis (OA) affect millions of people within the United Kingdom. Changes to the external factors surrounding the cartilage as well a genetic disposition can lead to alterations in the synthesis and degradation of the ECM; leading to disease. Deregulation of the anabolic and catabolic activity of the chondrocyte can lead to a diseased

tissue in which irreversible damage occurs. Arthritis is a disease which causes painful inflammation and stiffness of the joints. This disease comprises of many different arthritides in which different clinical markers and phenotypes are seen. OA and RA are the two major forms of the disease and although have different clinical phenotypes both have the same end result, albeit with different initiating factors. These are two of the major diseases of the 21st century, which are becoming increasingly more relevant due to an aging population. It is estimated that 8 million people within the UK have OA. With around 1 million of these seeking medical treatment (ARUK, 2011a). In 2010 an estimated £852 million was spent on total hip and knee joint replacements alone within the UK and is therefore a massive cost both socially and economically (Chen et al., 2012). RA on the other hand affects around 1.5% of all adult women and 0.5% of all adult men in the UK,(data from 2006) (ARUK, 2011b). It is estimated that approximately 690,000 adults have rheumatoid arthritis in the UK (NRAS, 2013). Data has shown that arthritis and rheumatoid arthritis care led to a medical cost of around £689 million a year in the UK (NRAS, 2010). Together, the total cost of musculoskeletal conditions, due to loss of working hours and treatments, was believed to be around £5.7 billion in 2008 (ARUK, 2008), showing both the social and economic costs of the disease. *Figure 1.3* represents some of the pathological changes which occur to knee joints during the progression of RA and OA.

Arthritis can be characterised by the progressive loss of the articular cartilage which surrounds articulated joints (Buckwalter et al., 2005). The removal of this cartilage leads to the loss of friction-less movement of the joint. Degradation of collagen is a key phenotype of cartilage destruction, as unlike other components of the ECM, collagen cannot be readily replaced (Goldring et al., 2008). The turnover of collagen within the ECM is slow, with the collagen laid down during development having the ability to last for the duration of an individual's life (Goldring and Marcu, 2009). Collagen cleavage is therefore known as the rate limiting step within cartilage breakdown. The chondrocyte is therefore crucial in this process, acting as a sensor within in the cartilage. The secretion of pro-inflammatory cytokines into the synovial fluid can lead to an increase in catabolic mediators leading to the production of cartilage lesions as well as cartilage remodelling (Yasuda, 2006). The induction of catabolic

mediators and the down regulation of anabolic mediators, leads to the progression of cartilage damage and disease. Healthy ECM is therefore maintained in a non-disease state by the balance between synthesis and degradation of the ECM macromolecules; in a disease state such as in RA and OA this balance is lost. Degradation exceeds synthesis and the irreversible loss of cartilage occurs.

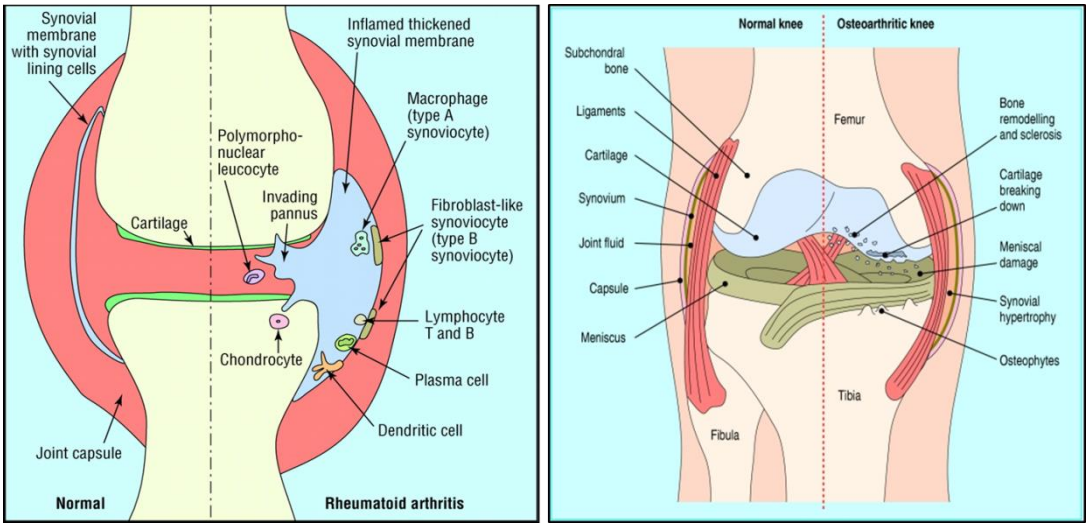


Figure 1.3. The pathological changes which occur in a knee joint due to rheumatoid arthritis and osteoarthritis. The cellular components and sites of destruction in rheumatoid and osteoarthritic knee joints. During rheumatoid arthritis an infiltration of proinflammatory cytokines enter the joint, these include dendritic cells, plasma cells, lymphocyte T and B cells and macrophages. This can then lead to an inflamed and thickened synovial membrane, the formation of a pannus and cartilage destruction. In an osteoarthritic joint, bone remodelling, meniscal damage, cartilage destruction, synovial hypertrophy and osteophytes can all be observed. Taken from (Buckley, 1997) and (Hunter and Felson, 2006).

1.4.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune disease in which the body's own immune system attacks the synovial joints. RA affects around 1% of the population, with a female to male ratio of around 3:1 (ARUK, 2011b). RA can be characterised by chronic inflammation of multiple joints causing synovitis. The aetiology of RA is not fully understood, but it is known to have an autoimmune element involving interplay between T and B cells, as well as cytokine networks (Choi and Brahn, 2010). Macrophages, neutrophils and synovial fibroblasts are also important in the mechanism of disease. Other contributory factors include: the environment, infection, hormonal factors as well as genetic pre-disposition.

RA is believed to be initiated by the infiltration and activation of immune cells such as T cells, B cells, macrophages and dendritic cells into the synovial lining of the joint. These activated immune cells secrete pro-inflammatory cytokines leading to further inflammation through self-activation and the stimulation of surrounding cells within the joint. B cells are responsible for antigen recognition and antibody production. The role of B cells in RA is poorly defined but B cell depletion treatments, such as Rituximab and Epratuzumab have highlighted the importance of B cells in RA (Edwards et al., 2004). B cells have been linked with the production of autoantibodies such as rheumatoid factor and other antibodies which target citrullinated proteins (Schellekens et al., 1998). The use of B cell depletion therapies has therefore been shown to be positive in improving patient health in people with RA.

B cells have also been shown to present antigens to T cells, leading to their activation and further exacerbation of disease. T cell activation, as well as their migration is seen to occur as an early consequence of RA, adopting a proinflammatory phenotype (Firestein, 2003). T cells have been linked to RA due to the high numbers of T cells found within the synovium, a genetic link with MHC class II alleles and with the lymphoid-specific protein tyrosine phosphatase, non-receptor type 22 (PTPN22) mutation (Panayi, 2006). T helper 1 cells are the T cells believed to drive RA, as they have the capabilities to produce the pro-inflammatory cytokines interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (McInnes and Schett, 2007), which can potentiate the progression of the disease. As well as Th1 cells being important in RA, Th17 cells have recently been linked with RA (Nakae et al., 2003) and are found to be elevated in the peripheral blood of patients with early onset RA (Leipe et al., 2010). Th17 are thought to be important in RA as they produce then cytokine IL-17. IL-17 plays is seen to have synergistic effects when induced with tumor necrosis factor (TNF) and IL-1, which can induce cartilage degradation (Lubberts et al., 2005). However, Th17 cells are not widely found within the synovium of RA patients (Hueber et al., 2010), with this work suggesting mast cells to be the prominent source of IL-17 in RA synovium. Macrophages have also been shown to play a key role in RA as they produce pro-inflammatory cytokines which exacerbate disease.

Inflammation caused by the infiltration of immune cells occurs within the synovium of the joint. The synovium lines the joint cavity and is the site of production of synovial fluid. Normal synovium consists of macrophages and fibroblast like cells known as synoviocytes. In RA the lining of the synovium swells, with increased numbers of macrophages and fibroblasts. The synovium proliferates and formation of a pannus (an abnormal layer of thickened synovial tissue which grows over the joint surface) occurs, extending over the articular cartilage. Currently, it is unclear as to whether inflammation and joint destruction are linked. Degradation of the ECM is believed to be driven by the infiltration of pro-inflammatory cytokines into the joint cavity, leading to the stimulation of the sole cell type, the chondrocyte (van den Berg and van Riel, 2005). Cartilage destruction is then mediated through the increased expression and activity of proteolytic enzymes, resulting in cartilage degradation and erosion of the subchondral bone (Otero and Goldring, 2007, Sweeney and Firestein, 2004).

1.4.2 Osteoarthritis

Osteoarthritis (OA) is a disease of articular joints, and is affecting a third of people over the age of 45 in the UK (ARUK, 2011a). A total of 76,759 primary total hip replacements and 81,979 total knee replacements were performed I 2010, showing the large numbers of patients with which treatment of the symptoms of disease is no longer a viable option (Registry, 2011). OA is one of the leading causes of chronic disability and is the most prevalent forms of arthritis. Symptoms include joint pain, stiffness, loss of mobility and non-systemic inflammation. Both joint pain and stiffness are used for diagnosis, but clinically the use of X-rays, evidence of swelling and fluid in and around the joint is used. The uncertainty in diagnosis is due to there being no clear clinical markers for the disease, hindering early diagnosis. The aetiology of OA has not fully been resolved with further research required into the causes and development of the disease. It is believed that multiple factors can lead to the disease with numerous risk factors being flagged as contributors to the disease. These factors include genetics, age, weight, gender and other joint abnormalities.

OA can be characterised by the softening, fibrillation and loss of articular cartilage, synovial inflammation as well as the formation of osteophytes and

subchondral cysts (Goldring and Goldring, 2007). Age is the major risk factor of OA, with normal quiescent chondrocytes changing phenotypically compared to healthy or normal cells (Price et al., 2002). OA can also develop due to damage to the cartilage, leading to further degradation of the cartilage surrounding the joint. OA has been seen as a 'wear and tear' disease compared to the inflammatory disease of RA. This view is becoming outdated as OA is now beginning to be shown to have an inflammatory phenotype. Patients with OA have been shown to have increased vascularity and inflammatory-cell infiltration of their joints (Smith et al., 1997), albeit these tend to be of lower grade than those observed in RA (Pessler et al., 2008). Inflammatory mediators, such as cytokines and chemokines are seen to be elevated within the synovial fluid of patients with OA. Interleukin-1β (IL-1β) and TNFα (tumor nercrosis factor α) have been shown to be elevated in OA, and these cytokines stimulate the down regulation of matrix synthesising genes as well as promoting cartilage catabolism (Goldring and Marcu, 2009).

1.4.3 Chondrocytes and arthritis

During the initial stages of OA, chondrocytes begin to divide leading to the formation of multicellular clusters. These chondrocytes begin to express hypertrophic markers such as type X collagen, Runt-related transcription factor 2 (RUNX2), osteocalcin and alkaline phosphatase (van der Kraan and van den Berg, 2012). These markers are usually only found in terminally differentiated chondrocytes found within the growth plate (Aigner et al., 1993). Ossification can begin to occur with osteophyte boney growths protruding from the cartilage being observed. These hypertrophic-like chondrocytes also begin to express increased levels of the MMPs, specifically MMP-13 (Goldring and Goldring, 2007). The increased expression of MMP-13 leads to the irreversible degradation of the main ECM component type II collagen, leading to the breakdown of the ECM.

1.4.4 Treatment for arthritis

Treatment for OA is limited with no preventative or progressive treatments available. Currently, only pain relief is offered, and in severe cases, total joint replacement is the only viable clinical option. Joint replacements have their limitations, with the duration of the replacement being around 10-15 years (Lucas, 2004). As well as their short life span, the surface of these

replacements does not provide the same frictionless surface that articular cartilage provides. RA on the other hand has many more viable treatment options. These include the disease-modifying anti-rheumatic drugs (DMARDs) which are designed to slow down the progression of the disease. DMARDs include Rituximab, the B cell targeting drug, as well as anti TNF-α therapy. These drugs though useful, do not work in all patients and only treat the symptoms of inflammation, they do not target the disease process itself directly. Further research is therefore needed into treatments which target the disease directly rather than only treating the symptoms. A key research aim is therefore to discover the mechanisms behind cartilage degradation, looking at the roles of both inflammation and the signalling consequences leading to disease.

1.5 Proteinases and cartilage degradation

The breakdown of the ECM of articular cartilage is one of the major processes involved in the progression of arthritis. This process is catalysed by proteinases, proteolytic enzymes which are up-regulated and activated in many arthritides. Proteinases cleave covalent peptide bonds within protein structures; this can lead to the inactivation and degradation of the target protein. Proteinases are synthesised and secreted from a number of cells found within the joint, including chondrocytes and synovial fibroblasts. The expression and activation of these enzymes is regulated by cytokines and growth factors secreted in the synovial fluid (Goldring et al., 2008). As mentioned previously, one of the key processes in arthritis is the irreversible cleavage of type II collagen found within the ECM of cartilage. This process is catalysed by these proteinases. The cleavage of type II collagen is preceded by further cleavage of other ECM components. The cleaved collagen cannot be readily replaced by the cells, due to its low turnover rate. The estimated half-life of type II collagen in healthy cartilage is over 100 years (Verzijl et al., 2000). In the disease state the degradation of collagen therefore exceeds its synthesis.

Proteinases can be classified into 5 main classes; this is dependent on the chemical group which participates in the hydrolysis of the peptide bond. Proteinases therefore belong to either the cysteine, aspartate, threonine, serine or metallo-proteinase groups. These 5 classes are shown in *Figure 1.4*. These proteinases work within different subcellular and extracellular locations, dictated by the pH at which the enzymes are active. Cysteine, aspartate and threonine

proteinases are active at acidic pH and therefore act intracellularly. This contrasts with the serine and metalloproteinases that act extracellularly at neutral pH (Rengel et al., 2007). All classes of proteinases are involved in the catalytic breakdown of connective tissues, with members of each family having the potential to activate themselves or other family members. The matrix metalloproteinase class of metzincin enzymes are strongly linked with the degradation of the ECM of cartilage and are believed to be the major collagenolytic enzymes (Ellis et al., 1994).

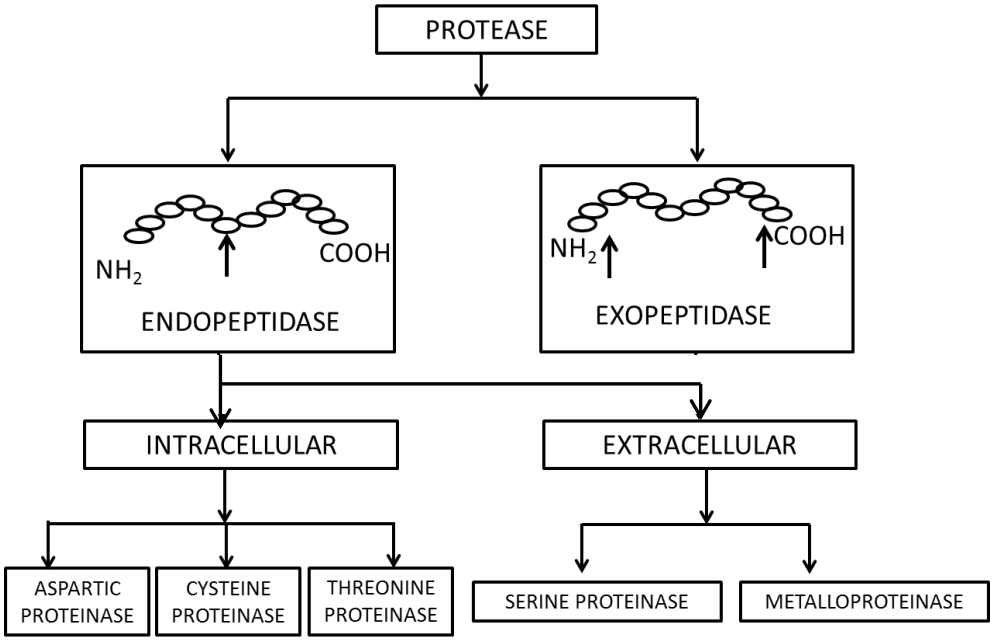


Figure 1.4. The 5 main sub-divisions of the Proteinase family. The 5 main groups include; the collagenases, gelatinases, matrilysins, stromelysin and the membrane type MMPs, all of which work extracellularly at neutral pH. Further detail on family members and role in ECM degradation can be found in *section 1.5***.** Adapted from (Rengel et al., 2007).

1.5.1 Matrix metalloproteinases

MMPs have been shown to cleave key components of the ECM, with the levels of MMPs being significantly raised in patients with RA and OA (Hembry et al., 1995, Yoshihara et al., 2000, Davidson et al., 2006). Collagenase-1 (MMP-1), the first member of the MMP family to be identified (Gross and Lapiere, 1962), has the capability to specifically cleave triple helical collagen. MMPs can be either cell surface bound or secreted. MMPs require the neutral pH of the extracellular environment to become fully active. These family members have a similar amino acid sequence and all share a common structure and function, but

differ in their target sites. MMP-1, -8 and -13 form the ‘classic’ collagenases and degrade collagen, whilst MMP-2 (Aimes and Quigley, 1995) and MMP-14 (Ohuchi et al., 1997) also have this capability *in vitro*.

Currently, 23 members of the MMP family have been identified. All are divided into 5 traditional groups, dependent on their known catalytic target, sequence homology and cellular location (Clark et al., 2008). The 5 main groups include; the collagenases, gelatinases, matrilysins, stromelysin and the membrane type MMPs. In normal homeostasis of the cell, within healthy individuals, MMP levels and ECM degradation is low. In arthritis this balance changes and MMP levels rise, especially the collagenases (Kevorkian et al., 2004).

1.5.1.1 MMP structure

All MMPs consist of a propeptide of around 80 amino acids, a catalytic metalloproteinase domain of around 170 amino acids, a hinge region of variable length and a haemopexin domain of 200 amino acids (Murphy and Nagase, 2008) as depicted in *Figure 1.5*. The propeptide domain contains a highly conserved PRCGXPD cysteine switch motif; this can ligate to a zinc ion within the active site, maintaining latency (Clark et al., 2008). The zinc ions bind to the HEXGHXXGXXH zinc-binding sequence within the catalytic domain (Clark et al., 2008). The catalytic domain also contains a conserved methionine, which forms a ‘Met-turn’, eight residues after the zinc binding motif. This forms a support structure around the catalytic zinc (Bode et al., 1993). The hinge region links together the C- and N- terminal domains. The hemopexin domain has various roles depending on the MMP. It plays a crucial role in substrate specificity, the binding of TIMP-2 (tissue inhibitor of matrix metalloproteinase 2) to proMMP2, as well aiding in the cleavage of collagen by MMP-1 (Lauer-Fields et al., 2009).

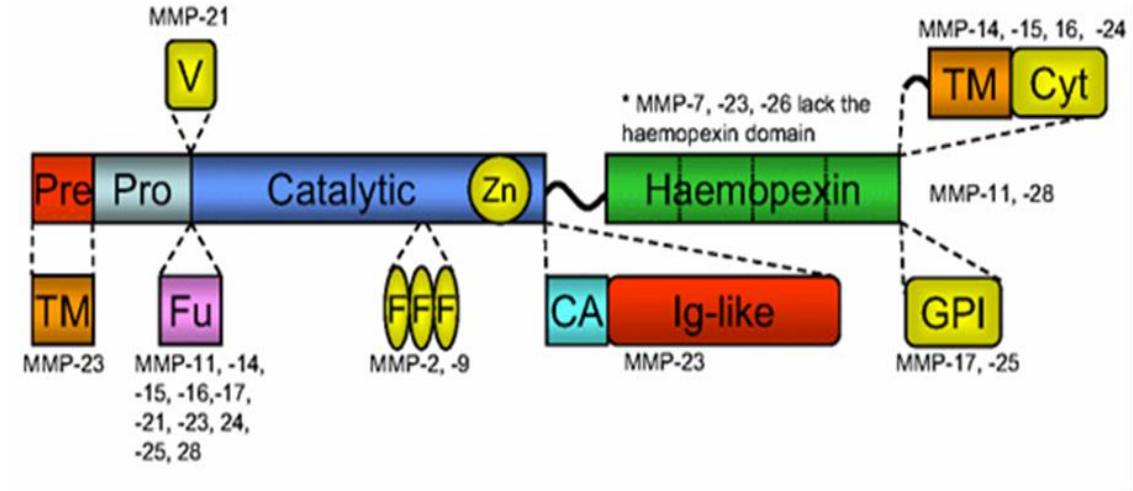


Figure 1.5. The domain structures of the MMPs. All MMPs have a catalytic domain containing the active site zinc (Zn). Some MMPs contain a furin recognition motif (Fu), allowing intracellular activation by furin-like proteinases. All MMPs (apart from MMP-7, -26 and -23) contain a haemopexin domain, which can determine substrate specificity. Other domains found include; the fibronectin-like domains (F), found in MMP-2 and -9 and the vitronectin-like domain (V) in MMP-21. Some MMPs contain a trans-membrane domain (TM) with cytoplasmic tail (Cyt) or via a glycosylphosphatidyl inositol (GPI) anchor which anchors them to the cell surface. MMP-23 is structurally unique compared to the others MMPs. MMP-23 contains an N-terminal TM domain, acysteine array (CA) and an immunoglobulin-like domain (Ig-like). Taken from Cawston and Young, (Cawston and Young, 2010).

1.5.1.1.1 The collagenases

The collagenases play a vital role in the turnover of collagen. This ability therefore makes the collagenases of great importance in the progression of arthritis. There are three classical collagenases; MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3). MMP-13 preferentially cleaves type II collagen (Knauper et al., 1996a) whereas MMP-1 preferentially cleaves type III and MMP-8 type I; they all have the capability to cleave type II collagen. Of the collagenases MMP-1 and MMP-13 are thought to be of greatest importance in relation to arthritis, due to their cleavage affinities and expression. MMP-13 is restricted in expression and in normal cartilage believed only to be expressed during development, fulfilling its role of removing type II collagen from the growth plate during bone formation (Johansson et al., 1997). MMP-1 is more abundant and expressed within more cell types (Vincenti and Brinckerhoff, 2002). MMP-1 is described as the key collagenase in RA, whereas MMP-13 is thought to be the important collagenase in OA. MMP-8 is not well expressed in human articular chondrocytes (Stremme et al., 2003), and therefore is not thought to be as prominently involved in cartilage destruction in OA and RA. Both MMP-1 and -13 are shown to have a similar activity against type I collagen

but MMP-13 appears to have a 10-fold higher activity against type II (Knauper et al., 1996a). This inefficiency of MMP-1 is believed to be counteracted by increased expression of MMP-1 compared to MMP-13 (Vincenti and Brinckerhoff, 2002).

Type II collagen is a major component of the ECM and its degradation is one of the major factors in both OA and RA. Collagen is resistant to degradation by most proteinases until digestion by MMPs (Visse and Nagase, 2003). MMPs cleave collagen fibrils at a single glycine-isoleucine/leucine bond (Visse and Nagase, 2003). This cuts the helical domain of the collagen into ¾ and ¼ fragments (Birkedal-Hansen et al., 1985), causing them to unwind and be susceptible to further proteolysis. The mechanism by which MMPs degrade collagen is not well established but data imply MMPs must first unwind the collagen chains before cleaving the chains individually (Chung et al., 2004). The loss of type II collagen leads to the degradation of the ECM as it exposes further cleavage sites for further proteolytic enzymes.

1.5.1.1.2 Gelatinases

The subgroup of MMPs known as the gelatinases consists of the MMP-2 (gelantinase A) and MMP-9 (gelatinase B). The two gelatinases cleave denatured collagen, type IV and V collagen as well as elastin (Aimes and Quigley, 1995). MMP-2 and MMP-9 differ from other MMPs as they contain fibronectin-like inserts in their catalytic domain, enabling them to bind to denature collagen (gelatin). MMP-2 is the most widely expressed gelatinase and has the ability to activate proMMP-9 (Fridman et al., 1995) and -13 (Knauper et al., 1996c). MMP-9 is expressed within transformed and tumour driven cells as well as in chondrocytes, neutrophils and monocytes (Matrisian, 1992). Both gelatinases are seen to be elevated in RA (Ahrens et al., 1996).

1.5.1.1.3 Stromelysins

MMP-3 (stromelysin 1) and MMP-10 (stromelysin 2) both form the subgroup of MMPs known as the stromelysins. MMP-11 is called stromelysin 3 but because of sequence and substrate differences is not always placed within this group. Both MMP-3 and MMP-10 gene and protein expression can be induced by IL-1 in both chondrocytes (Murphy et al., 1986), as well as in synovial fibroblasts (MacNaul et al., 1990). MMP-3 and MMP-10 have similar substrate specificities; MMP-3 however has a slightly greater proteolytic efficiency compared to MMP-

10. Besides ECM degradation, MMP-3 can also activate growth factors, chemokines, cytokines and various receptors. MMP-3 is also able to activate proMMP-1, -3, -7, -8, -9 and -13 (Ito and Nagase, 1988). MMP-3 is also involved in a self-activation loop in which MMP-3 cleaves and induces its upstream activators plasminogen and urokinase-type plasminogen activator (Ogata et al., 1992, McCawley and Matrisian, 2001). MMP-10 also has the capability to activate various MMPs, including MMP-1 and -8 (Knauper et al., 1996b). Both of these enzymes are therefore important in the degradation of the ECM and the activation of other MMPs needed to initiate degradation.

1.5.1.1.4 Matrilysins

MMP-7 and MMP-26 form the matrilysins. These differ from other MMPs as they contain no haemopexin domain. MMP-7 is synthesised by epithelial cells whereas MMP-26 is expressed in many different cell types such as those found in the endometrium, they are also found in some carcinomas (Murphy and Nagase, 2008). MMP-26 can digest a number of ECM products whilst MMP-7 also has the capability to process cell surface molecules such as E-cadherin and pro-Tκ-α (Visse and Nagase, 2003).

1.5.1.1.5 Membrane-type MMPs (MT-MMPs)

There are 6 MT-MMPs: four are type I transmembrane proteins (MMP-14, MMP-15, MMP-16 and MMP-24), the two other MMPs are glycosylphosphotidylinositol (GPI) anchored proteins (MMP-17 and MMP-25) (Visse and Nagase, 2003). All MT-MMPs contain a furin-like pro-protein convertase recognition sequence and are activated intracellularly. All MT-MMPs except MMP-17 have the capability to activate proMMP2. They can all degrade ECM components, with MMP-14 having collagenolytic activity on type I, II and III collagen (Ohuchi et al., 1997). MMP-14 can cleave and activate MMP-13 on the cell surface and is thus important in arthritis due to its ability to activate this collagen degrading enzyme.

MMP	Class	Name	Substrate
MMP-1	Collagenases	Collagenase-1	Collagens (i–iii, vii, viii, and X), gelatin, aggrecan, MMP-2, MMP-9 and IL-1β
MMP-8	Collagenases	Collagenase-2/ neutrophil collagenase	Collagens (i–iii, v, vii, viii, and X), gelatin, aggrecan, fibronectin
MMP-13	Collagenases	Collagenase-3	Collagens (i–iv, iX, X, and Xiv), gelatin, plasminogen, aggrecan, fibronectin, osteonectin, MMP-9
MMP-2	Gelatinases	Gelatinase-A	Gelatin, collagen iv–vi, X, elastin, fibronectin
MMP-9	Gelatinases	Gelatinase-A	Collagens (iv, v, vii, X, and Xiv), gelatin, aggrecan, elastin, fibronectin, plasminogen, IL-1β
MMP-3	Stromelysins	Stromelysin-1	Collagens (iii–v, and iX), gelatin, aggrecan, , laminin, elastin, plasminogen, MBP, IL-1β, MMP-2/TiMP-2, MMP-7, MMP-8, MMP-9, MMP-13
MMP-10	Stromelysins	Stromelysin-2	Collagens (iii–v), gelatin, casein, aggrecan, elastin, MMP-1, MMP-8
MMP-11	Stromelysins	Stromelysin-3	Unknown (casein)
MMP-7	Matrilysins	Matrilysin-1 (PUMP)	Collagens (iv, X), gelatin, aggrecan, decorin, fibronectin, laminin, elastin, casein, transferrin, plasminogen, MMP-1, MMP-2, MMP-9, TiMP-1
MMP-26	Matrilysins	Matrilysin-2	Collagen IV, fibronectin, fibrinogen, gelatin, α (1)-proteinase inhibitor
MMP-14	MT-MMP	MT1-MMP (membrane type)	Collagens (i–iii), gelatin, casein, fibronectin, laminin, proteoglycans, MMP-2, MMP-13
MMP-15	MT-MMP	MT2-MMP	Fibronectin, entactin, laminin, aggrecan, MMP-2
MMP-16	MT-MMP	MT3-MMP	Collagen iii, gelatin, casein, fibronectin, MMP-2
MMP-17	MT-MMP	MT4-MMP	Unknown
MMP-24	MT-MMP	MT5-MMP	Fibronectin, but not collagen type i or laminin
MMP-25	MT-MMP	MT6-MMP	Progelatinase A

Figure 1.6 MMPs and their substrates. A table containing the names and substrates of the 4 major classes of MMPs. All MMPs contain a similar structure, with greatest homology beingn found within the catalytic domain. Differences in extracellular distribution, processing and substrate specificity are all found. For further detail see *section 1.5.1.1.* Adapted from (Sekhon, 2010)

1.5.2 Regulation of matrix metalloproteinases

Due to their catabolic properties, MMPs are tightly regulated within healthy cells, at both the level of transcription and activation (Burrage et al., 2006). MMP activity is therefore regulated at 3 key stages; synthesis, proenzyme activation and inhibition. In diseases such as arthritis, this regulation is disrupted leading to increased MMP catalytic activity.

1.5.2.1 Transcriptional regulation of MMPs within chondrocytes

The transcription of MMPs is tightly regulated, with low expression of MMPs found within normal resting chondrocytes (Vincenti and Brinckerhoff, 2002). In disease, this regulation is disrupted, with increased MMP expression and secretion being observed (Hembry et al., 1995, Yoshihara et al., 2000, Davidson et al., 2006).

Expression of MMPs is dependent on the binding of transcription factors to the promoter of each MMP gene at numerous DNA binding regions known as *cis*-elements. These elements vary between each MMP, allowing for gene specific regulation of each MMP, even under the same stimulus. The MMP-1 and MMP-13 promoters both contain a TATA box approximately 30 base pairs (bp) upstream of the transcription start site as well as a proximal activator protein-1 (AP-1) binding site approximately 40bp upstream of the TATA box (Goldring et al., 2008). Variation occurs between MMP-1 and MMP-13 through the binding of other transcription factors. As *Figure 1.7* shows, MMP-1 and MMP-13 have varying *cis*-elements within their promoters, giving rise to different expression patterns. The key *cis*-elements found within the promoters of MMPs include nuclear factor kappa B (NFκB), signal transducer and activator of transcription (STAT), RUNX2 and specificity protein-1 (SP-1).

In disease, an infiltration of pro-inflammatory cytokines into the joint activates signalling events which in turn activate numerous transcription factors. These cytokines include IL-1β, TNF-α and oncostatin M (OSM). The activation of cell signalling pathways by IL-1 in combination with OSM therefore leads to the phosphorylation and increased expression of numerous transcription factors involved in collagenase expression.

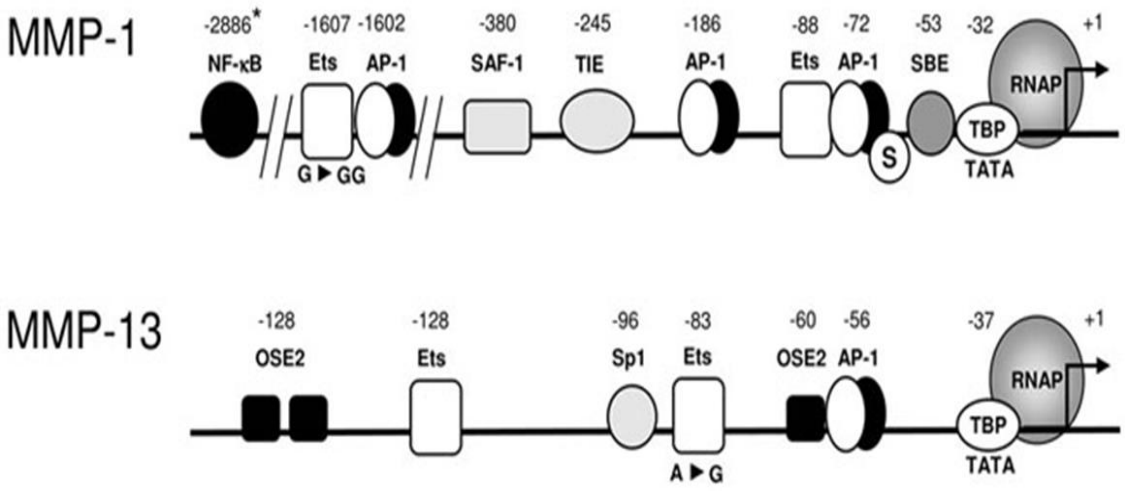


Figure 1.7. Schematic of MMP-1 and MMP-13 promoter region. The consensus transcription factor binding sites and their approximate nucleotide position relative to the transcription start site of MMP-1 or -13. These binding sites include; SBE, the STAT-binding element; TIE, TGF-beta inhibitory element; S, Smad-binding site; SAF-1, serum amyloid A-activating factor-1; OSE2, osteoblast-specific element 2 (Cbfa1/Runt binding site); TBP, TATA binding protein; RNAP, RNA polymerase II complex. Taken from (Rowan and Young, 2007)

1.5.2.2 Activation of proMMPs

MMPs are first synthesised as pre-proenzymes. During translation the signal peptide is first removed forming the proenzyme. The zymogen must then undergo further cleavage and processing to become active. This latency is maintained until the pro-peptide sequence is cleaved, removing a bound cysteine residue from the catalytic zinc ion (Nagase et al., 2006).

Thirteen MMPs are secreted from the cell as proMMPs. These contain a proteinase susceptible 'bait' region in the pro-peptide, which can be cleaved by multiple proteinases (Nagase et al., 2006). These MMPs can be activated by the proteolytic cleavage of their pro-domain via the action of serine proteinases such plasmin (Eeckhout and Vaes, 1977) or matriptase (Milner et al., 2010). This method is referred to as the 'step wise method' (Nagase et al., 1990). MMPs themselves also have the capability to cleave other MMP pro-domains, leading to further MMP activation. These MMPs may themselves be activated by serine proteinases in an activation cascade of pro-collagenases (Milner et al., 2001).

Other MMPs are cleaved by the catalytic action of furin, a known pro-protein convertase (Cawston and Young, 2010). These MMPs contain a furin recognition site between the pro-peptide and catalytic domain. Once cleaved the inhibitory binding of the cysteine residue is removed. This can occur within the trans-Golgi Network (TGN) leading to the secretion of active proteinase capable of degrading the components of the ECM.

1.5.3 Tissue inhibitors of metalloproteinases (TIMPs)

Once synthesised and activated the proteolytic activity of MMPs can be regulated by the endogenous MMP inhibitors, TIMPs. TIMPs specifically inhibit MMP activity by binding to the MMP active site. The regulation of ECM turnover is dependent on a fine balance between MMPs and TIMPs, with alterations in the levels contributing to diseases such OA and RA (Bokarewa et al., 2005) (Cawston and Young, 2010). As well as active site inhibition, TIMPs can bind to the proMMPs inhibiting their activation (Bigg et al., 2001).

There are 4 TIMPs; TIMP-1, TIMP-2, TIMP-3 and TIMP-4. All consist of around 184-189 amino acids, sub-divided into N-terminal and C-terminal subdomains (Nagase et al., 2006). The N-terminal domain has the capability to fold as an

independent unit, inhibiting MMPs (Murphy et al., 1991). TIMPs are able to inhibit all MMPs, except for TIMP-1 which cannot inhibit MMP-14 (Will et al., 1996). TIMPs bind to MMPs in a 1:1 stoichiometry; their wedge-like structures inserts itself into the active site of an MMP, in a similar manner to that of a substrate. They bind non-covalently via a cysteine within the N-terminal domain to the zinc ion within the active site; this dispels the water molecule bound to the zinc ion, rendering the enzyme inactive (Gomis-Ruth et al., 1997). Due to non-covalent binding this process is reversible. Although all TIMPs are described as broad spectrum MMP inhibitors, their specificity for individual MMPs differs.

TNF-α and IL-1 are both seen to induce MMP synthesis, but inhibit TIMP-1 expression in chondrocytes. Conversely, IL-6 can promote TIMP-1 expression but not MMP expression (Lotz and Guerne, 1991) in synoviocytes. Since IL-6 is expressed under the control of IL-1 or TNF-α (Radtke et al., 2010), this could be a potential regulatory feedback loop which may try to counteract the catabolic effects of the MMPs under such pro-inflammatory conditions.

Several other proteins are reported to inhibit MMPs. These include α-macroglobulins, which are general endopeptidase inhibitors, which inhibit by entrapping the MMP within the macroglobulin (Cawston and Mercer, 1986). Tissue factor pathway inhibitor-2 is a serine proteinase inhibitor also capable of inhibiting MMPs. MMP homeostasis within the joint is a key process with inhibition of MMPs a tightly controlled process; changes within this balance can lead to degradation of the ECM and diseases such as OA and RA.

1.5.4 Synthetic MMP inhibitors

The use of broad spectrum MMP inhibitors in the treatment of both RA and OA has been tried as a potential therapeutic target. Early MMP inhibitors were broad specificity inhibitors, they were ineffective with many drugs causing musculoskeletal side-effects (Clark and Parker, 2003). Once available, the crystal structures of each MMP gave insight into the lack of specificity due to the catalytic domains of each MMP being structurally similar. Inhibition of MMPs for the treatment for arthritides has many challenges, such as; deciding on whether to use broad spectrum or MMP specific inhibitors as well as deciding on whether proteoglycan or collagen release should be the main focus. Also to be considered is the deleterious effects of inhibition of MMPs which are essential and beneficial for tissue integrity (Cawston and Young, 2010). Understanding

the signalling events that lead to the induction of individual MMPs may therefore lead to therapeutics capable of inhibiting the expression and synthesis of individual MMPs. This may avoid some of the side effects observed and lead to specificity against individual MMPs.

1.6 Pro-inflammatory cytokines in arthritis

Cytokines are small soluble proteins that regulate a variety of biological processes; most common of these is the regulation of immunomodulatory functions. Cytokines are produced in response to cellular stress, which includes infection, inflammation and injury. Their release leads to modulation of a target cell, leading to changes within transcription and protein expression. The complex milieu of cytokines released within inflammation includes both pro- and anti-inflammatory cytokines, which are designed to control cellular stress and minimise damage. However, disease can occur when this tightly controlled inflammatory response becomes deregulated. The dysregulation of cytokine networks leads to the pathology of multiple diseases and chronic inflammation.

RA and OA differ in their pathology, but both are driven by the production of catabolic factors in response to a complex milieu of elevated pro-inflammatory cytokines. In RA, chronic inflammation is seen, with the infiltration of cytokine producing immune cells, including macrophages, T-cells and neutrophils into the synovium being observed (Smeets et al., 2003). This leads to the dysregulation of cytokine networks leading to chronic inflammation of the joint. OA, though not historically thought to be an inflammatory disease, due to low numbers of immune cells found within the synovial fluid of OA patients, is beginning to be re-evaluated in this context. The role of inflammatory cytokines in the pathogenesis of OA is now thought to be a key process in disease progression (Abramson and Attur, 2009).

Inflammation and the release of pro-inflammatory cytokines stimulates the production of enzymes involved in the degradation of the ECM of cartilage, whilst also impairing the ability of the chondrocyte to repair the damage. Cytokines released by the synovium and the chondrocytes themselves lead to the activation of the normally quiescent chondrocyte. The chondrocytes, which usually promote low matrix turnover, become active and undergo phenotypic modulation due to the action of both anabolic and inflammatory cytokines

(Goldring and Otero, 2011). Two key pro-inflammatory cytokines implicated in the destruction of articular cartilage are IL-1β and TNF-α. Both cytokines have been found to be elevated in RA synovium and are detected in the synovial fluid of RA patients (Lubberts et al., 2000). The production of both IL-1 and TNF-α is also seen to be elevated in osteoarthritic articular chondrocytes (Pelletier et al., 2001). IL-1 and TNF-α target the chondrocyte, leading to the expression of catabolic and anabolic genes, resulting in an imbalance in cartilage homeostasis. In addition to the up-regulation of catabolic and anabolic genes, both cytokines can suppress the expression of COL2A1, the collagen II gene, *in vitro* (Tyler and Benton, 1988, Goldring et al., 1988, Lefebvre et al., 1990). These cytokines can therefore increase degradation of the ECM as well as inhibit the synthesis of its key component.

Other pro-inflammatory cytokines have been shown to be elevated within RA and OA joints, leading to the induction of catabolic factors capable of degrading cartilage. A key characteristic of these cytokines is their ability to synergise, leading to a greater than additive induction of gene expression than when used individually. One of these cytokines is OSM, a member of the IL-6 cytokine family. This cytokine has the capability to synergise with IL-1 and TNF-α leading to MMP induction (Cawston et al., 1998a, Rowan et al., 2001, Cawston et al., 1998c, Cawston et al., 1998b).

1.6.1 Interleukin-1

The IL-1 family is a group of 11 pleiotropic cytokines which can regulate immune and inflammatory responses. The IL-1 family consist of; IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33 andL-36, α, β and γ. IL-1 activates multiple signalling pathways, acting as a mediator in the pathology of many diseases. IL-1α and IL-1β are the two major inflammatory cytokines within the family, while IL-1Ra exhibits anti-inflammatory effects (Dinarello, 1996, Dinarello, 2011). IL-1α and IL-1β have the same biological activities as both form the same complex with the IL-1 receptor. IL-1α and IL-1β are both synthesised as a 31kDa pro-IL-1 precursors (Dinarello, 1996, Dinarello, 2011). Pro-IL-1α is processed by the cleavage of the N-terminal domain, leaving an 18kDa mature protein. Both pro- and mature IL-1α are biologically active. In contrast, pro-IL-1β is processed into a 17.5kDa (Black et al., 1988). The pro-IL-1β is immature and therefore cleavage of the prodomain is needed for the

cytokine to be biologically active. IL-1α associates with the membrane and therefore acts locally whereas IL-1β is secreted and acts systematically.

The IL-1 receptor (IL-R) consists of two major forms (IL-1RI and IL-1RII), of which there are 9 members (Daun and Fenton, 2000). All isoforms contain immunoglobulin-like domains within their own extracellular domain. IL-1RI is an 80 kDa receptor which binds IL-1, aiding in inflammation. IL-1RII is a 60 kDa protein with considerable homology to that of IL-1RI (Daun and Fenton, 2000). IL-1RII binds IL-1 with the same affinity as IL-1RI but has no signal transduction capabilities, therefore acting as a signal transduction suppressor. IL-1RII is also enzymatically cleaved from the surface and acts a soluble IL-1 binding protein. The IL-1RII can therefore bind to circulating IL-1α and –β. The receptor is therefore known as a decoy receptor, competing with IL-1RI for IL-1.

The IL-1RI does not work alone, for greater affinity binding of IL-1α and IL-1β to the receptor a an accessory protein is also needed, this is known as IL-1 receptor accessory protein (IL-1R AcP) (Wesche et al., 1997). IL-1R AcP aids in the internalisation of the active IL-1R complex inducing intracellular signalling. Further recruitment of IL-1 receptor- associated kinase (IRAK) and TNF receptor associated factor (TRAF) accessory proteins aid in initiating downstream signalling (O'Neill and Greene, 1998). Active IL-1RI can therefore activate mitogen activated protein kinase (MAPK) signalling pathways, the NFκB pathway, as well as the tyrosine phosphorylation of STATs.

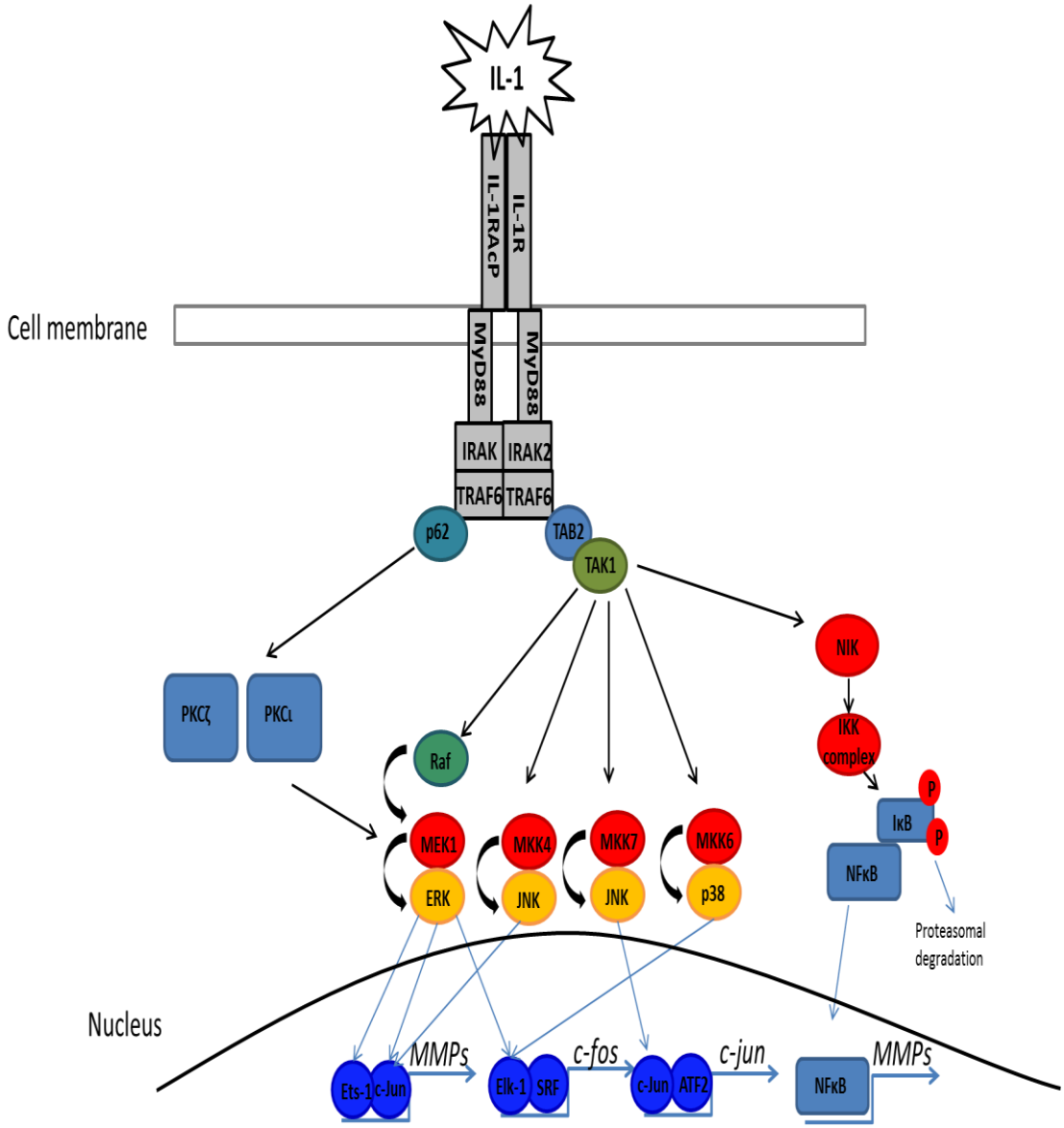


Figure 1.8. IL-1 signalling pathway. IL-1 binds to its receptor (IL-1R) and accessory protein IL-1RAcP. This leads to the association and conformational changes in the further accessory proteins (cytosolic myeloid differentiation protein (MyD88), IRAK and TRAF proteins). Hyperphosphorylation of IRAK leads to its dissociation from the receptor, leading to its association with TARF6 (Takaesu et al., 2000).This complex binds TGF-beta activated kinase 1/MAP3K7 binding protein 2 (TAB2) and Transforming growth factor-beta-activated kinase 1 (TAK1). Phosphorylation and activation of TAK1 leads to further signal transduction, TAK1 activates both the MAPK and NFκB pathways (Ninomiya-Tsuji et al., 1999). TAK1 activates the NFκB pathway via the phosphorylation of NFκB-inducing kinase (NIK). NIK phosphorylates the inhibitor of κB kinase (IKK) complex, leading to the phosphorylation of inhibitor of NFκB (IκB). IκB is then polyubiquinated and targeted for proteasomal degradation. This releases the transcription factor NFκB from its inhibitory state, allowing the translocation of the protein to the nucleus, in which it can initiate gene transcription. TAK1 can also activate all members of the MAPK signalling family. TAK1, also known as a member of the MAP3K family, phosphorylates its downstream substrates (members of the MAP2Ks), leading to activation of the MAPKs. Activation of these pathways leads to the induction and phosphorylation of numerous transcription factors involved in the induction of the MMPs. As well as the activation of TAB2 and TAK1, TRAF6 can also associate with p62, this leads to the binding of atypical Protein Kinase Cs (aPKC) to TARF6. TARF6 then activates aPKC leading to their downstream signalling and activation of the ERK pathway. p62/aPKC have been shown to activate the NFκB pathway (Geetha and Wooten, 2002), but silencing of the aPKC had no effect on NFκb signalling (Litherland et al., 2010). Adapted from (Vincenti and Brinckerhoff, 2002) and (Litherland et al., 2010).

1.6.1.1 IL-1 and arthritis

IL-1 was first identified within the synovial fluid of patients with RA in the early 1980s (Fontana et al., 1982). IL-1 was later also seen to be elevated within the blood plasma (Eastgate et al., 1988), the cartilage (Goldring and Marcu, 2009, Towle et al., 1997) and the synovial membranes of RA patients (Rowan et al., 2003). Raised levels of IL-1 within the synovial fluid and the sera are associated with disease activity and cartilage breakdown in RA. Chondrocytes from patients with OA display high levels of both IL-1α and IL-1β and have elevated membrane bound IL-1R1 (Martel-Pelletier et al., 1992). The levels of IL-RII are also down-regulated (Martel-Pelletier et al., 1992), indicating not only increased levels of IL-1 but also increased sensitivity.

IL-1 stimulates cartilage degradation by inducing the expression and activation of proteolytic enzymes capable of cleaving ECM components, such as the MMPs and aggrecanases. Intra-articular injection of recombinant or highly purified IL-1 into the knee joints of adult rabbits led to infiltration of leukocytes into the synovial cavity as well as the loss of proteoglycan from the articular cartilage (Pettipher et al., 1986). This loss of proteoglycan was not due to the infiltration of leukocytes but instead, due to the activation of proteolytic enzymes. IL-1 had already been implicated in the induction of the collagenases within sites of inflammation when released by macrophages (Postlethwaite et al., 1983). As well as increasing the levels of catabolic enzymes, decreases in the synthesis of type II collagen were observed (Goldring et al., 1988). IL-1 has therefore a clear role in the progression of arthritis, with elevated levels being identified. This leads to the activation of cell signalling events, which cause the degradation of the ECM by proteolytic enzymes.

1.6.2 Oncostatin M

OSM is a secreted glycoprotein monomer of 28 kDa which belongs to the IL-6 type cytokine family. OSM is synthesised and secreted by T cells and monocytic cells (Cawston et al., 1998b). This family of cytokines signal through a common gp130 receptor but differ in their second receptor component. OSM signals primarily through the OSM Receptor (OSMR), but also has the unique ability to signal through the leukemia inhibitory factor Receptor (LIFR) (Mosley et al., 1996). However, in chondrocytes, data suggests that in the chondrocytic cell line T/C28a4 (Catterall et al., 2001) and in primary HAC (Litherland et al., 2008)

LIFR is not expressed, suggesting that OSM signals only through the OSMR/gp130 heterodimer in chondrocytes.

Signalling through the receptor is initiated by the binding of OSM to the OSMR leading to the recruitment and hetero-dimerisation of the OSMR with gp130. gp130 receptors do not have an endogenous kinase activity within their cytoplasmic region. The receptor therefore has to recruit complementary factors to transduce their signal. On binding, gp130 interacts with members of the Janus kinase (JAK) family, maintaining a constitutive association with the receptor. JAK1, JAK2 and Tyk2 are all activated on OSM binding (Lutticken et al., 1994, Wang and Fuller, 1994). Following binding, JAKs are activated by autophosphorylation, leading to JAK mediated tyrosine phosphorylation of the gp130 receptor. These tyrosine residues act as docking sites for multiple accessory proteins which can aid in the activation of signalling cascades. Proteins containing a Src homology 2 (SH2) domains have the capabilities to bind to these phosphorylated tyrosine residues, leading to downstream signalling activation.

The signal transducers and activators of transcription (STATs) are one such signalling proteins that can bind these phosphorylated tyrosine residues. JAKs phosphorylate 4 tyrosine residues, Y767, Y814, Y905 and Y915, within the cytoplasmic tail of the receptor (Lehmann et al., 2006). Binding of STATs to the receptor leads to JAK-mediated tyrosine phosphorylation in their SH2 domain. This causes the formation of STAT dimers. These active STAT dimers can then translocate to the nucleus to drive gene expression.

OSM binding to the OSMR/gp130 heterodimer also has the capability to activate signalling cascades other than just the JAK/STAT pathway. Phosphorylated tyrosine 757 also has the capability to act as a phosphorylation-dependent docking site for Src homology phosphotyrosyl phosphatase (SHP-2). SHP-2 is a ubiquitously expressed cytoplasmic tyrosine phosphatase (Hof et al., 1998). Once active, SHP-2 phosphorylates and activates phosphatidyl inositol-3 kinase (PI3K) signalling, leading to the activation of Akt, NFκB and other downstream signalling events.

OSM signalling can also activate the MAPK pathways. As well as SHP-2, numerous other SH2 domain containing proteins can bind to the OSMR/gp130

receptor. One such example is Ras, a monomeric GTPase, which is recruited to the receptor by growth factor receptor-bound protein 2 (GRB2), SHC and the GTP exchange factor, son of sevenless homolog (SOS), which in turn activates downstream MAPKKKs and thus MAPK (Kolch, 2000). OSM therefore activates multiple signalling cascades which are known to be important in the transcription of MMPs. *Figure 1.9* shows the potential signalling cascades which OSM induces, leading to collagenase gene expression in HAC.

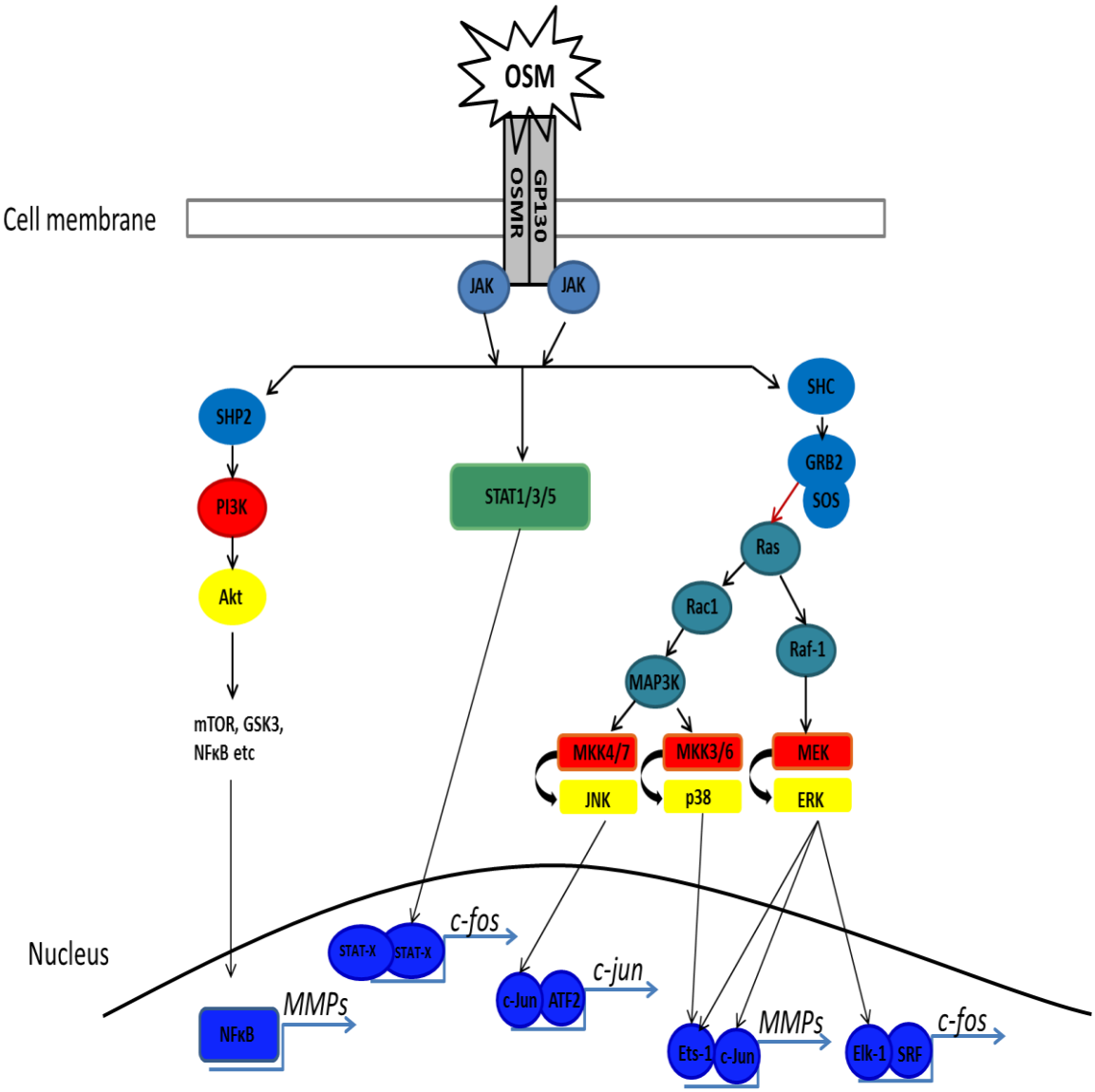


Figure 1.9. **OSM signalling cascade**. The binding of OSM to its receptor subunits, OSM receptor (OSMR) and gp130, activates several signalling pathways. These pathways include the PI3K/Akt pathway, the JAK/STAT pathway and the MAPK pathway. The IL-6 family of receptors do not possess intrinsic kinase activity, therefore for signal transduction the recruitment and activation of the JAKs is required. JAKs phosphorylated numerous downstream targets leading to signal transduction. JAKs are seen to phosphorylate and activate SHP2, leading to PI3K signalling pathway, which results in the production of the signalling molecule phosphatidyl-3, 4, 5-trisphosphate (PIP_3). PIP_3 can then go onto to activate Akt leading further downstream phosphorylation. This includes the activation of GSK-3, which has recently been implicated in arthritis under OSM simulation(Litherland et al., 2014), and NFκB, a well-established MMP transcription factor. As well as the activation of Akt, PIP_3 can activate phosphoinositide dependent protein kinase-1 (PDK1). PDK1 can then activate PKC isoforms. OSM signalling through the receptor also induces the JAK/STAT signalling pathway. Activated JAKs recruit and activate STAT proteins. The STATs then dimerise, leading to their translocation to the nucleus. These STATs can further be phosphorylated by the MAPKs priming the STATs for transcription. The STATs then bind to regulatory elements in the promoter of OSM-responsive genes and leading to gene expression. SHC can also be activated by OSM, this signalling molecule initiates MAPK signalling via the phosphorylation of GRB2. Activate GRB2 binds the GTP-exchange factor SOS. SOS interacts with Ras, recruiting Ras-related C3 botulinum toxin substrate 1 (Rac1) and Raf-1. Rac1 activates the MAP3K/MKK cascades resulting in the activation of p38 and JNK via. Raf-1 activates the MEK/ERK1/2 cascade. The activation of all these downstream cascades leads to alterations in gene expression. Adapted from Heinrich et al., (Heinrich et al., 2003)

1.6.2.1 Oncostatin M and arthritis

OSM is seen to be elevated in the synovial fluid of patients with RA, but not patients with OA (Hui et al., 1997). The role of OSM in both osteo- and rheumatoid arthritis is not as clear as that of IL-1, with contradictory evidence suggesting OSM to have both pro- and anti-catabolic functions. Stimulation of HACs with OSM increases the levels of TIMPs, suggesting OSM to promote MMP inhibition and protect against cartilage degradation (Nemoto et al., 1996). In contrast, murine models have shown OSM to be pro-inflammatory and catabolic, leading to synovial hyperplasia, ECM remodelling and cartilage loss (Langdon et al., 2000). OSM also increases the levels of MMP-1 within RA synovial cells and HACs, leading to increased proteoglycan degradation and GAG release, whilst an increase in TIMP-1 is also observed (Fearon et al., 2006). OSM has been shown to synergistically induce MMP-13 gene expression as well as cartilage degradation of cartilage in both bovine and human models, suggesting OSM works in combination with other pro-inflammatory cytokines (Hui et al., 2003b, Rowan et al., 2001, Koshy et al., 2002a).

1.6.3 The synergistic properties of IL-1 in combination with OSM

Numerous cytokines and growth factors are found within inflamed joints (van den Berg, 1999). These cytokines have the capability to interact synergistically to promote marked collagenolyisis by inducing collagenase gene expression. One of the most marked inductions of collagenase gene expression is observed when cells are stimulated with both IL-1 in combination with OSM (Rowan et al., 2001). The first observations of the synergistic effect of IL-1 in combination with OSM on cartilage degradation were observed in bovine nasal cartilage. Here, dramatic collagen release from bovine explant cultures was seen, when compared to stimulation with IL-1 alone (Cawston et al., 1995a, Cawston et al., 1995b). This suggested that IL-1 in combination with OSM further stimulated chondrocytes than when used individually. Further studies in bovine, porcine and human cartilage showed IL-1 in combination with OSM to increase MMP-1 expression, suggesting this collagen release was collagenase-dependent (Cawston et al., 1998b). A decrease in the levels of TIMP production was also seen compared to that of OSM alone. The adenoviral delivery of IL-1 and OSM into murine joints confirmed these findings *in vivo* (Rowan et al., 2003). Collagenase gene expression has been shown to be synergistically induced in

HAC grown in monolayer (Barksby et al., 2006), indicating a potential mechanism by which collagen release is being induced in cartilage models. Other IL-6 family members have also been studied to see if they share these synergistic properties. Apart from IL-6 in combination with its soluble receptor, no other IL-6 family members synergise with IL-1 (Rowan et al., 2001). IL-11 also synergises with IL-1 to induce MMP gene expression (Rowan, personal communication).

All of these data suggest that IL-1 in combination with OSM stimulates collagen release and the breakdown of the ECM of cartilage. However, there is little knowledge of the signalling mechanism by which IL-1 and OSM synergise to induce collagenase gene expression. Recent work on the signalling cascades which are activated by IL-1 and OSM, leading to collagenase gene induction are beginning to emerge {Litherland, 2008 #65;Catterall, 2001 #13;Litherland, 2010 #66}. Understanding of the signalling pathways induced by both cytokines individually may therefore aid in the understanding of disease progression. Finding drug targets which inhibits chondrocyte production of MMPs under a complex stimulus is more likely to inhibit disease progression compared to targeting one factor. Further understanding is therefore needed to understand how IL-1 and OSM together regulate collagenase expression and the pathways at which this synergistic cross-talk occurs.

IL-1 in combination with OSM has been shown to be the most prolific inducer of MMP gene expression in HAC grown in monolayer (Cawston et al., 1998b), with this synergistic stimulus also leading to the degradation of bovine nasal cartilage and the release of proteoglycan (Cawston et al., 1995b). For these reasons, this stimulus is often used as model of the cytokines elevated during arthritic disease. Many studies use one cytokine as a stimulus to examine cell signalling. However, multiple cytokines are released during disease, activating many pathways which exhibit cross-talk. Therefore understanding the effects of cell signalling in a complex stimulus gives a greater understanding of the pathways activated during a disease. This stimulus helps us to understand the signalling cascades activated during the progression of both OA and RA.

1.7 Signalling pathways regulated by IL-1 and OSM

For pro-inflammatory cytokines to relay their inflammatory signal, inducing transcriptional changes, the cytokine must activate the internal signalling pathways of the cell. Binding of the cytokine to the receptor can lead to autophosphorylation events and signal transduction. IL-1 and OSM, on binding to their receptor, are known to activate multiple signalling pathways within chondrocytes. These pathways include the MAPKs, PI3K, STATs and the NFκB pathways.

Signalling in inflammation is complex, with multiple signalling cascades being activated on cytokine stimulation within different cell contexts. Further complexity is due to interaction between different signalling pathways, leading to further induction of these cascades. This 'crosstalk' has a possible role in the synergy seen between various cytokines (Koshy et al., 2002a, Cawston et al., 2003, Hui et al., 2003a). IL-1 and OSM can activate independent pathways, but in some cases both cytokines activate the same pathway. Cross-talk between signalling pathways activated by either cytokine can also occur. This cross-talk leads to further signalling activation and therefore the synergistic induction of MMP gene expression seen with these stimuli.

Little is known about the signalling events which regulate collagenase gene expression within chondrocytes stimulated with IL-1 in combination with OSM. Some of this is due to the paradigm that inflammatory disease is synovium-driven (Rowan and Young, 2007). However, this concept is changing, with more research being performed to understand inflammation-driven activation of proteinases within chondrocytes. Therefore, elucidating and understanding the signalling events which both cytokines activate is crucial in understanding how IL-1 and OSM regulate collagenase expression through these pathways within chondrocytes with an inflammatory context.

1.7.1 The MAPK pathway

The Mitogen-activated protein kinase cascade consists of multiple serine/threonine kinases. These kinases have the capability to transduce the cytokine signal from receptor into transcriptional changes within the cell. The signal transduction occurs by a series of phosphorylation cascades that culminate in the activation of effector proteins and as transcription factors. This leads to transcriptional changes within the cell, dictated by the cytokine. The

MAPK pathways consists of three distinct signalling cascades; extracellular signal-regulated kinase (ERK), Jun N-terminal Kinase (JNK) and p38 kinase (SAPK). MAPK signalling is a three tiered process in which upstream activation of a MAP kinase kinase kinase (MAPKKK) initiates the downstream signalling cascade. MAPKKK activation leads to MAP kinase kinase (MAPKK) phosphorylation which can then phosphorylate the MAPK itself. MAPKs share many upstream activating factors, therefore to ensure signal specificity, scaffold proteins are used. These scaffolds bind to components of the signal cascade in specific modules, forming complexes, ensuring correct signal transduction. These scaffolds include, β-arrestin, MEK partner-1, kinase suppressor of Ras as well as the tribbles (Brown and Sacks, 2009) (Kiss-Toth et al., 2004). All of these measures ensure that the signal is transduced from receptor into transcriptional changes. IL-1 and OSM individually and in combination have been shown to activate all three MAPK pathways within chondrocytes (Liacini et al., 2002, Li et al., 2001, Geng et al., 1996).

1.7.1.1 ERK pathway

The ERK pathway is well established in regulating collagenase gene expression within chondrocytes. Under the stimulation of IL-1 and OSM, ERK can be activated by the phosphorylation of the MAPKKK RAF, which in turn phosphorylates the MAP2K MEK, leading to the phosphorylation and activation of ERK (Vincenti and Brinckerhoff, 2002). Once active, ERK can play numerous roles in regulating collagenase expression. The inhibition of ERK using siRNA and ERK inhibitors shows a clear loss of collagenase gene expression within chondrocytes and collagen release from explant cultures (Litherland et al., 2010). The mechanism by which ERK induces collagenase expression has not been fully elucidated. It is however believed that ERK can induce MMP-1 gene expression via the phosphorylation of a CCAAT enhancer-binding protein (C/EBP) (Raymond et al., 2006). IL-1 stimulation of ERK is seen to phosphorylate C/EBP at threonine 235 within chondrocytes, leading to activation. This transcription factor is then seen to bind to the C/EBP binding site found within the MMP-1 promoter, leading to transcription. ERK may also work indirectly, activating Elk-1, leading to the transcription of Fos, a known member of the AP-1 transcription factors (Gille et al., 1995).

1.7.1.2 JNK pathway

JNK consists of 3 individual isoforms (JNK1, JNK2 and JNK3), which are all expressed within chondrocytes. JNK is regulated by several MAPKKK, which phosphorylate the MAP2Ks, MAPKK4 or MAPKK7 leading to the phosphorylation and activation of JNK. Jun is a downstream target of JNK. This key member of the AP-1 transcription factor is seen to be directly phosphorylated at serine residues 63 and 73 (Pulverer et al., 1991), increasing the transcriptional activity of Jun. JNK also plays a role in the priming of STAT-1 and STAT-3 by phosphorylating these at the serine residue 727, leading to greater transcriptional activity (Lim and Cao, 1999). Through inhibitor and siRNA mediated gene silencing, JNK has been shown to regulate collagenase gene expression within chondrocytes and other tissues of the joint (Mengshol et al., 2000, Han et al., 2001).

JNK inhibition in the human chondrosarcoma cell line SW1353, by the overexpression of the inhibitory scaffold protein JNK-interacting protein (JIP), led to reduced MMP-13 gene expression by 60%. The levels of phosphorylated Jun were also seen to be reduced (Mengshol et al., 2000). Isoform specific gene silencing of each isoform of JNK in HAC showed down-regulation of MMP-1 and MMP-13 gene expression when stimulated with IL-1 in combination with OSM (Litherland and Rowan, unpublished data). JNK inhibition within synovial fibroblasts also causes a decrease in the phosphorylation of Jun and the transcription of collagenase genes (Han et al., 2001). Adjuvant-induced arthritic rats, when treated with the JNK inhibitor SP600125, had reduced swelling as well as a decrease in bone and cartilage damage (Han et al., 2001). JNK has therefore a clear involvement in the regulation of collagenase gene expression, and inhibition within the joint leads to a reduction in the progression of disease.

1.7.1.3 p38 pathway

p38, named so after its size of 38 kDa, consists of 4 distinct isoforms; p38α, p38β, p38γ and p38δ. p38 can be activated under stress and inflammatory conditions. p38 again follows the three-tiered kinase cascade of the MAPK pathway. In general, p38 is phosphorylated and activated by several MAP2Ks including MAPKK3, 4 and 6, these are in turn activated by MAP3K, such as MEKK1-4 (Cuadrado and Nebreda, 2010). The p38 Kinases contribute to MMP-1 (Mengshol et al., 2000, Ridley et al., 1997) and MMP-13 (Mengshol et al.,

2001, Mengshol et al., 2000) gene expression in direct response to inflammatory mediators. In chondrocytes, p38 is seen to recruit the AP-1 complex and the chondrocyte/osteoblast-specific transcription factor RUNX2 to the promoter of MMP-13, leading to increased transcription (Mengshol et al., 2001). p38 also increases the stability of MMP-1 mRNA, although the mechanism by which this occurs has not been elucidated (Reunanen et al., 2002). Treatment of rat and mouse models with the p38 inhibitor SB 203580 led to decreased bone resorption and paw inflammation, implicating p38 in regulating the arthritic phenotype (Badger et al., 1996).

1.7.2 The PI3K pathway

PI3Ks are a family of enzymes that catalyse the phosphorylation of the 3-position of the inositol ring of phosphoinositides (Hawkins et al., 2006). Phosphorylation of phosphatidylinositol (PtdIns) by PI3K leads to the production of phosphatidylinositol 3, 4 bisphosphate (PtdIns 3, 4, P_2) and phosphatidylinositol 3, 4, 5 trisphosphate (PtdIns 3, 4, 5, P_3), two important signalling molecules. These two signalling molecules can be further processed into diacylglycerol (DAG) and inositol trisphosphate (IP3). PI3K have the capability to bind to receptors which bind growth factors, hormones, antigens and inflammatory stimuli, leading to the activation of signalling pathways which regulate cell growth, survival, proliferation and movement. (Rodriguez-Viciana et al., 2004). Three major classes of PI3Ks have been described: Classes I, II and III. PI3K is divided into these groups based on structure and functional homologies (Hawkins et al., 2006).

In chondrocytes class IA PI3Ks have been implicated in the regulation of collagenase gene expression (Litherland et al., 2008). Class IA consist of dimers of either p110α, p110β or p110δ catalytic subunits and either p50-55/p85 regulatory subunits. Class IA PI3Ks are activated by binding to phosphotyrosine receptors such as the OSMR. Phosphorylated tyrosine residues within the receptor act as binding ligands for SH2 domains found within the regulatory subunits of PI3Ks. The mechanism by which activation occurs is still unclear; it may be down to conformation changes within the regulatory domain. PI3K signalling was first shown to regulate collagenase expression through OSM stimulation of chondrocytes. PI3K activation led to increased MMP-13 expression. Blocking PI3K using the PI3K inhibitor

LY294002 lead to a decrease in MMP-13 mRNA and protein levels within HACs (El Mabrouk et al., 2007). Litherland et al., (2008) further showed that the PI3K subunit p110α was required for MMP-1 and MMP-13 gene expression, whereas p110δ was required for MMP-13 gene expression (Litherland et al., 2008). This, for the first time, specifically implicated the PI3K IA class of PI3Ks as the regulators of collagenase expression; this activation occurred via the known PI3K substrate Akt.

1.7.2.1 AKT

One of the major kinases activated by PI3K is Akt (otherwise known as protein kinase B). This is a serine/threonine kinase known to be phosphorylated and activated by OSM stimulation (Litherland et al., 2008). Activation of the OSM receptor leads to tyrosine autophosphorylation and PI3K activation. PtdIns 3, 4, P_2 is then phosphorylated and converted to PtdIns 3, 4, 5, P_3, which binds Akt leading to a change in confirmation. This exposes the amino acid threonine 308 within the activation loop of Akt. This is a known phosphorylation site of PDK1. Phosphorylation leads to Akt activation. Within chondrocytes, isoform specific roles of Akt were observed (Litherland et al., 2008). Here, Akt1 was required for MMP-1 and MMP-13 mRNA expression, whereas PDK-1 and Akt3 induced MMP-13 collagenase expression. The mechanism by which Akt regulates collagenase expression has not been fully elucidated. It is though proposed that Akt regulates collagenase expression through regulation of the NFκB pathway (Chen et al., 2013), a known downstream substrate of PI3K-PDK1-Akt.

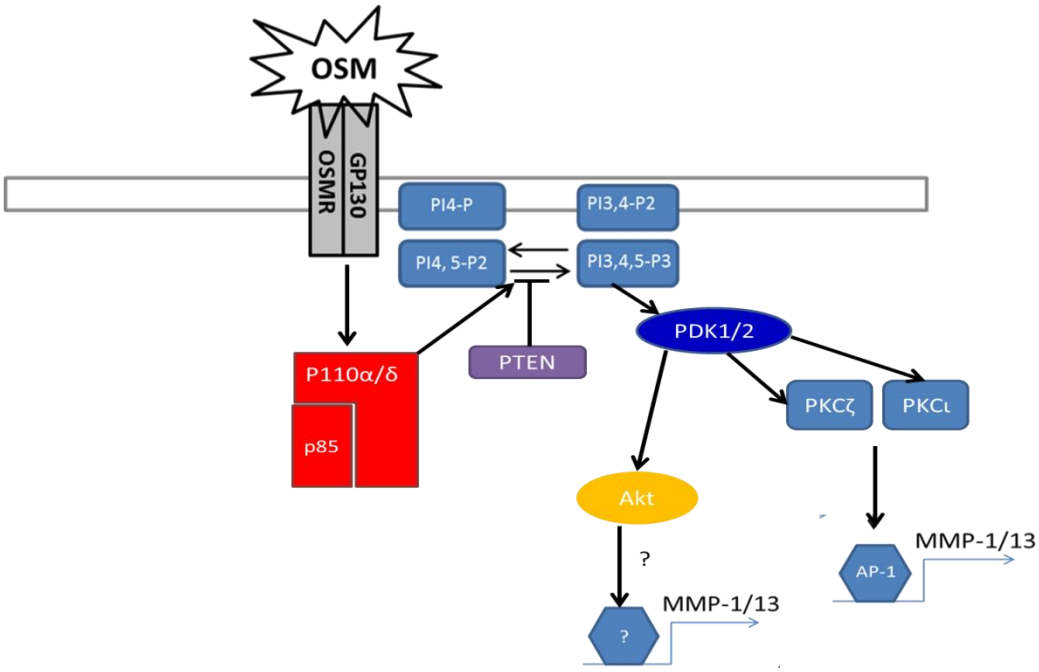


Figure 1.10 PI3K signalling. OSM stimulation leads to the phosphorylation and activation of the PI3K signalling pathway. This leads to the induction of PKC isoforms that contain an AGC loop, as well as Akt activation, this is meditated by PDK1. PTEN dephosphorylated PIP_3, reducing the amounts of this secondary messenger. Induction of both pathways in chondrocytes has been shown to induce collagenase gene expression in HAC (Litherland et al., 2008, Litherland et al., 2010). However, the precise mechanism by which Akt regulates MMP gene expression in chondrocytes is currently unknown; this may be via GSK-3 modification as suggested by (Litherland et al., 2014) or NFκB regulation.

1.7.3 Transcription factors induced by IL-1 and OSM

1.7.3.1 AP-1

One of the major transcription factors involved in MMP gene expression is the activator protein-1 (AP-1). This transcription factor complex consists of homo/heterodimers made up of multiple proteins from major proto-oncogene families; these include the major family members, Jun (c-Jun, Jun B and Jun D) and Fos (c-Fos, Fos B). AP-1 members can also bind to Fos-related antigen (Fra)-1 and Fra-2), as well as activating transcription factor (ATF), ATF1, ATF2, ATF3, ATF4, ATF5, ATF6, and ATF7 to form the AP-1 transcription factor. The composition of these factors determines their transcriptional activity.

AP-1 binding elements found within the promoters of MMP-1 and MMP-13 are well established as important transcription factor binding sites involved in collagenase gene expression within chondrocytes. The main AP-1 site or TPA responsive element (TRE) (5'-TGAG/CTCA-3') sits approximately -70bp upstream of the transcription start site (Benbow and Brinckerhoff, 1997) and

binds dimers of Fos and Jun. Several other AP-1 sites are found throughout the MMP promoter and may aid in transcription. Induction of Fos and Jun protein expression is therefore crucial in the expression of the MMPs. Signalling pathways known to activate MMP gene expression are seen to induce the expression of these two family members. IL-1 stimulation of numerous cell types, leads to the AP-1 dependent transactivation of many MMPs via the activation of the MAPK signalling pathways. JNK and ERK are of particular relevance to MMP transcription as they phosphorylate and activate Jun (Karin, 1995, Leppa et al., 1998). The ERK pathway also regulates the activity of erythroblastosis twenty-six (Ets) which can cooperate with AP-1 proteins in the promoter of multiple MMP promoters (Vincenti and Brinckerhoff, 2002). p38 signalling is seen to increase the protein expression of Fos and Jun, contributing to collagenase gene expression indirectly through AP-1 regulation (Vincenti and Brinckerhoff, 2002). Protein Kinase C (PKC) has been implicated in regulating Fos mRNA and protein levels within chondrocytes stimulated with IL-1 in combination with OSM (Litherland et al., 2010), and the silencing of Fos led to a decrease in mRNA expression of MMP-1 and MMP-13 under the stimulation of IL-1 in combination with OSM (Litherland et al., 2010). All of these signalling cascades have the potential to increase the transcription and stability of Fos and Jun protein, leading to their binding of the AP-1 binding site in the promoters of MMPs, leading to increased transcription. Increased Fos expression is seen in the mid- and calcified zones of cartilage from inflammatory arthritis (Tsuji et al., 2000). All of these data indicate signalling pathway induced transcription and activation of Fos and Jun leading to increased expression of MMPs within chondrocytes.

1.7.3.2 STATs

As previously mentioned, the JAK/STAT pathways can be activated by OSM binding to the OSMR, leading to STAT phosphorylation and transcriptional activation. Currently seven STATs have been identified: STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6. STATs are transcription factors which must form homo- or hetero-dimers to become transcriptionally active. JAK-dependent phosphorylation leads to dimer formation and translocation to the nucleus. Further modification occurs by serine phosphorylation within the transactivation domain, increasing their transcription

potential. Phosphorylation at this site is dependent on the MAPK signalling pathways. STAT signalling has been implicated in both RA and OA, with STAT3 being shown to regulate MMP-1 gene expression under the stimulation of IL-1 in combination with OSM in HAC. STAT3 was shown to not directly bind to the promoter of MMP-1 but instead bind to a hSIE (sis-inducible element) binding site (Catterall et al., 2001). It was therefore proposed that STAT3 was binding to the SIE in the c-*fos* promoter leading to increased transcription, increasing the levels of this AP-1 protein. This work showed STAT3 to indirectly led to increased transcription of MMP-1 via c-*fos* gene induction (Catterall et al., 2001). The role of STAT3 was later confirmed by Litherland *et al.,* 2010 in which STAT3 gene silencing led to reduced MMP-1 and MMP-13 gene expression when under the stimulation of IL-1 in combination with OSM in chondrocytes. Silencing also led to a decrease in Fos protein levels which was shown to down regulate collagenase expression (Litherland et al., 2010). Electrophoretic mobility shift assay (EMSA) data also confirm that STAT-1, -3 and -5 all have the potential to bind to the c-*fos* promoter under synergistic conditions in HAC (Rowan, unpublished data).

The importance of STAT signalling in arthritis is further implicated by the development of JAK inhibitors which are in circulation and undergoing clinical trials. Tofacitinib, is a JAK3 inhibitor which is clinically available to treat patients with moderate to severe RA (Rakieh and Conaghan, 2013). It is currently available in the United States, Japan and Russia. The activation of STAT signalling is therefore crucial in the progression of both RA and OA and further understanding of this pathway has the potential to lead arthritic drugs.

1.7.3.3 Nuclear factor κB

NFκB is a family of transcriptional factors which regulate the expression of multiple genes involved in controlling both innate and adaptive immunity. Canonical NFκB signalling and transcription occurs through tightly regulated protein complexes. NFκB transcriptional control arises from the assembly of homo- and heterodimers of 5 different NFκB proteins (RelA/p65, RelB, c-Rel, NFκB1/p105, NFκB2/p100) (Marcu et al., 2010). Inhibitory proteins known as IκB (IκBα, IκBβ, IκBε) sequester these homo/heterodimers within the cytoplasm, inhibiting their transcriptional activities. Upon stimulation, dimers are activated by the amino-terminal phosphorylation of IκB by the IκB kinase (IKK)

signalosome complex (IKKα, IKKβ and NEMO) (Scheidereit, 2006). Phosphorylation of IκB within conserved destruction boxes targets the protein for polyubiquitination and proteasomal degradation. NFκB is freed from its inhibitory protein exposing a nuclear localization sequence, this targets the dimer to the nucleus; binding to the specific NFκB binding site then occurs, leading to active transcription. The binding and activation of NFκB binding sites also stimulates IκB to bind NFκB and translocate to the cytoplasm acting as a negative feedback loop.

IL-1 signalling is well established as an activator of NFκB, with IL-1 receptor activation stimulating the canonical pathway (Bonizzi and Karin, 2004, Wickremasinghe et al., 1999, Fan et al., 2006, O'Neill and Greene, 1998). IL-1 activation of NFκB signalling has been shown to lead to MMP gene induction within chondrocytes (Fan et al., 2006). NFκB has therefore been implicated in the progression of OA, with a clear role in the regulation of collagenase gene expression. NFκB binding sites are found with the proximal promoters of many MMPs (Clark et al., 2008); both MMP-1 and MMP-13 contain NFκB binding sites. MMP-1 is shown to contain a non-canonical NFκB binding site (Vincenti et al., 1998), whereas MMP-13 is shown to contain a canonical site. Studies in mice with experimental arthritis immunised with collagen have also showed NFκB expression to correlate with MMP-13 and MMP-3 levels, indicating NFκB to regulate the expression of both MMPs (Han et al., 1998).

1.8 Protein Kinase C

The PKC family consists of 10 serine/threonine kinases. These kinases are divided into three distinct groups, dependent on their domain structure and activation. These groups consist of; the conventional (cPKC), atypical (aPKC) and novel PKCs (nPKC). The PKC family belongs to the AGC family of protein kinases, which includes over 70 proteins including PKA, PKC and PKG; all are grouped together as share a similar structure (Roffey et al., 2009). *Figure 1.11* shows the domain structures for each of the three PKC groups and the isoforms which belong to these groups, defining their individual differences. Phosphatidylserine acts as an essential co-factor for all 3 PKC groups aiding in PKCs activation, with each individual group of PKCs needing additional allosteric factors. The cPKCs comprise of α, βI, βII and γ, with all requiring both DAG and calcium (Ca^{2+}) for activation. nPKCs consist of δ, θ, ε and η, all lack

the Ca^{2+} binding domain but still require DAG for activation. The aPKC consist of both ζ and ι isoforms. These isoforms lack both the ability to bind DAG and Ca^{2+} as they contain no C2 domain and have an atypical C1 domain; they still require PIP_3 and/or ceramide (Steinberg, 2008). PKC structural and cellular diversity allows the family to differentially regulate various cellular signalling pathways.

All PKC isoforms contain a conserved catalytic or kinase domain, as well as an N-terminus or regulatory domain. The regulatory domain in the inactive form acts as a pseudosubstrate, binding the active site and inhibiting the catalytic domain (Steinberg, 2008). The pseudosubstrate positions itself within the catalytic domain of the PKC and positions an alanine in the serine/threonine phospho-acceptor site preventing activation of the protein (Steinberg, 2008). For PKC activation, PKC must translocate and bind to the cell membrane. Phosphorylation of PKC by phosphatidylserine, DAG or Ca^{2+}, causes PKC to translocate to the cell membrane. Further phosphorylation of PKC, via upstream allosteric factors, leads to a conformational change within the protein, causing the expulsion of the N-terminal pseudosubstrate from the catalytic region, activating the protein (Roffey et al., 2009). PKC is then primed for activity via phosphorylation initiated by PDK-1 (Steinberg, 2008). This protects PKC from degradation, stabilising it, for further phosphorylation by allosteric factors. Once activated the catalytic domain of PKC can then bind ATP, hydrolyse and transfer one phosphate group to either a serine or threonine of its downstream substrate.

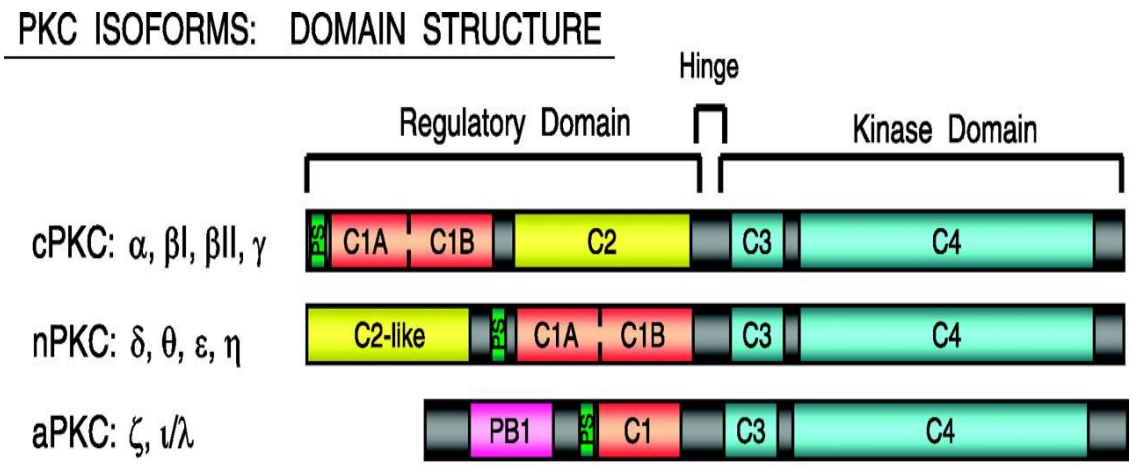


Figure 1.11. Diagram showing the domain structures of each individual PKC group. PKC has 2 major conserved domains, the kinase domain and less conserved regulatory domain. The C2 domain acts as a calcium dependent phospholipid binding domain, except in the novel isoforms which are calcium independent. The C1 domain acts to bind PMA and DAG in all isoforms except in the atypical isoforms, which bind PIP_3 or ceramide. Taken from Steinberg et al (Steinberg, 2008)

1.8.1 Protein kinase C in arthritis

PKCs can regulate multiple cellular processes including proliferation, apoptosis and migration (Griner and Kazanietz, 2007). PKC has mainly been studied in relation to cancer, with PKC signalling being implicated in tumor invasion and metastasis, with the regulation of the MMPs being a major area of focus (Beliveau et al., 2010). Altered gene expressions of PKC isoforms are observed within tumour cells, suggesting PKC to play an important role in tumor progression and growth. Because of the downstream consequences of PKC signalling, PKC has been implicated in the progression of arthritis. PKC is known to be activated by numerous pro-inflammatory cytokines leading to the induction of multiple downstream signalling events which can lead to disease progression.

In relation to arthritis and cartilage biology, PKC has been implicated in cartilage homeostasis. PKC is seen to regulate the differentiation of mesenchymal cells into chondrocytes, implicating PKC in producing the foundations of cartilage (Chang et al., 1998). PKC can also maintain the phenotype of the chondrocyte, with the dedifferentiation of chondrocytes in monolayer being linked with PKC regulation (Yoon et al., 2002). The regulation of this pathway is therefore important in the maintenance of healthy chondrocytes.

PKC is also shown to regulate the remodelling of the ECM of cartilage, specifically via the regulation of MMP gene expression. Research into the role of PKC in the regulation of MMP gene expression began with broad spectrum inhibition. PMA (a DAG homologue known to activate PKC) stimulation of chondrocytes was the first link between MMP gene induction and PKC. Tardif *et al*., showed chondrocytes stimulated with PMA led to increased MMP-13 production (Tardif et al., 1999). Inhibitor studies were then performed to further understand PKC role in chondrocyte homeostasis and MMP gene induction. Broad spectrum PKC inhibition by the PKC inhibitor bisindolylmaleimide, led to a decrease in MMP-13 expression, indicating PKC involvement in MMP gene regulation. The PKCδ isoform was then implicated in MMP-13 regulation with inhibition using Rotterlin (now seen as a poor, unselective PKC inhibitor (Davies et al., 2000) leading to decreased expression. PKC is also implicated in collagen breakdown, with bovine nasal cartilage explant cultures stimulated with IL-1 and OSM showing reduced collagen breakdown when PKC was inhibited using Gö6983, Gö6976 and Cal C (all three of these inhibitors are pan-PKC inhibitors) (Litherland et al., 2010). Individual PKC isoform gene silencing led to the atypical PKCs being implicated in the regulation of MMP-1 and MMP-13 gene expression. Silencing of PKCζ led to decreased MMP-1 and MMP-13 gene expression, whereas PKCι silencing led to reduced MMP-13 gene expression only, in HAC stimulated with IL-1 in combination with OSM (Litherland et al., 2010). PKC was seen to regulate STAT and ERK signalling leading to increased gene expression of *c-fos*. Isoform specific roles for the PKCs where therefore shown in chondrocytes, illustrating the importance of isoform specific study.

PKC signalling is involved in the transduction of the inflammatory signals observed within RA. PKC regulates T-cell production of IL-2 (Isakov and Altman, 2002), implicating PKC in the regulation of the inflammation seen within RA. T-cells deficient of PKC are seen to have reduced proliferation, differentiation and survival (Chand et al., 2012). PKC in inflammatory arthritis is implied through gene KO studies in antigen-induced arthritis. Here PKCθ KO mice are protected from Th1-dependent antigen induced arthritis (Healy et al., 2006).

Studies have therefore shown PKC to regulate chondrocyte and cartilage homeostasis as well as the induction of inflammatory mediators associated with arthritis. PKC therefore has the potential as a therapeutic target, with the understanding of further downstream signalling via this kinase aiding in the understanding of the signalling events activated during disease.

1.9 Protein kinase D

PKD is a family of three closely related serine/threonine kinase isoforms. PKD is a downstream substrate of PKC, a known modulator of MMP gene expression within chondrocytes stimulated with pro-inflammatory cytokines (*section 1.8*). PKD has not previously been studied in the context of arthritis, but has been shown to be a potential therapeutic target in other diseases such as breast and prostate cancer (Biswas et al., 2010, Eiseler et al., 2009). PKD has already been shown to modulate multiple signalling pathways and shown to be involved in cell survival/proliferation, apoptosis, plasma membrane-directed transport, metastasis and inflammation (Kim et al., 2010, McEneaney et al., 2010, Eiseler et al., 2009). The isoforms of PKD therefore have the potential to be signalling kinases involved in the induction of MMP gene expression within HAC in a pro-inflammatory context.

Most of the current work studying PKD has been referred to PKD as a kinase 'entity', with no consideration of isoform specificity. This situation is beginning to change, with data now emerging in which each individual isoforms of PKD has been studied (Bernhart et al., 2013, Biswas et al., 2010). In this thesis when work refers to 'PKD' no specific isoform is being referred to.

1.9.1 The structure of PKD

PKD comprises of 3 isoforms (PKD1, 2 and 3), all of which share a similar domain structure, with greatest homology found within the catalytic domains. PKD was first described as a novel member of the aPKC family, and named PKCμ, in humans (Johannes et al., 1994). A murine form of PKCμ had previously been described and was known as PKD (Valverde et al., 1994). The two other isoforms of PKD were described later; PKD2 described by Sturany et al in 2001 and PKD3/PKCv by Hayashi et al in 1999.

PKD was first classified as belonging to the AGC family of serine/threonine kinases, with a unique molecular architecture distinct to other members (Van

Lint et al., 2002). Once further studied, PKCμ was re-classified as a novel serine/threonine kinase and renamed PKCμ/PKD, a new family of proteins. This was due to differences in domain structure compared to that of PKC. The isoforms of PKD were then re-classified as members of the calcium/calmodulin (CAMK) family based on sequence similarity in the kinase domain and substrate specificity.

Each isoform of PKD has a molecular weight of around 115 kDa (Johannes et al., 1994) and range from 875-918 amino acids in length, depending on isoform (Avkiran et al., 2008). These proteins consist of an N-terminal regulatory domain consisting of two cysteine-rich zinc finger-like motifs and a pleckstrin homology (PH) domain. This domain is then linked to a C-terminal catalytic domain (Avkiran et al., 2008). The N-terminus of PKD comprises of many apolar amino acids, consisting mainly of alanines and prolines (Lint et al., 2002). This is linked by the tandem repeats of cysteine rich zinc finger-like motifs named Cys1 and Cys2. Deletion studies of both the zinc fingers showed activation of the protein suggesting these to have inhibitory affects (Iglesias and Rozengurt, 1999). These are linked by long Zn finger-linker region ranging from 64-76 amino acids in length. This linker region is much longer than the PKC isoform linkers which also link two Zn finger-like motifs. Both of the cysteine-rich domains allow PKD to bind to DAG and phorbol esters with great affinity, aiding in the regulation of the protein (Van Lint et al., 1995). *Figure 1.12* shows the structure of each PKD isoform.

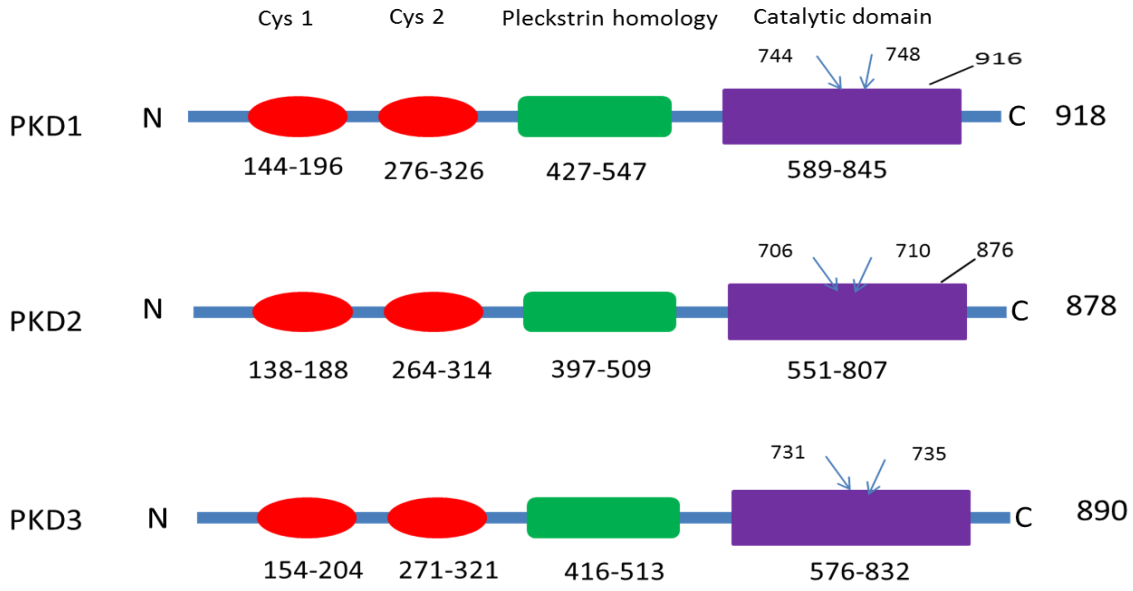


Figure 1.12. PKD isoform domain structures. Each PKD isoform has a molecular weight of close to 115 kDa and around 875-918 amino acids in length. Each isoform contains two cysteine rich domains (cys1 and cys2) which are known to bind to DAG. These are joined by a long linker region. Next is a pleckstrin homology domain which acts as an autoinhibitory domain, as well as being able to bind PKC and other phospholipids. This is followed by the catalytic domain of the protein which contains key PKC phosphorylation sites which vary between each isoform. Also found within PKD1 and PKD2 is an autophosphorylation domain which is known to represent PKD activity when phosphorylated.

Following this region is a PH domain. The PH domain within PKD has also an autoinhibitory function, with deletion of the PH domain causing the protein to be constitutively active (Iglesias and Rozengurt, 1998). The PH domain therefore keeps the protein in a state of low basal activity due to its intramolecular autoinhibitory interactions (Rybin et al., 2009). The PH domain also aids in the binding of PKD to phosphatidylinositol as well as other components of signalling pathways: PKC binds to PKD via the PH domain of PKD, which aids in signal transduction (Brandlin et al., 2002).

Following the PH domain is the catalytic domain of the protein. The catalytic region of the protein is regulated by phosphorylation of specific amino acids within and around the catalytic domain. These specific phosphorylation sites vary between isoform but are within a short distance of each other. Within the catalytic domain is the PKC phosphorylation site, which once phosphorylated can activate the protein.

PKC phosphorylates PKD at serine residues found at 744/748 in PKD1, 706/710 in PKD2 and 731/735 in PKD3. These amino acids are found within the activation loop of the protein. Phosphorylation leads to the stabilisation of the activation loop such that it is in the optimal conformation for catalysis (Rybin et al., 2009). In addition to the 744/748 phosphorylation site PKD contains an autophosphorylation site, found at ser916 in the carboxy-terminal of PKD1, and found at ser876 in PKD2. This site has not been identified in PKD3. Phosphorylation modifies the conformation of the protein and is an important marker of activity (Vertommen et al., 2000). The phosphorylation site itself is not required for activity *in vivo* or *in vitro* (Vertommen et al., 2000). Ser203 is also another known autophosphorylation site for PKD1 and is located within the regulatory domain. Phosphorylation is also seen at Ser255 which is also located in the regulatory domain of the protein; this protein is shown to also be phosphorylated by members of the PKC family. Ser255 mutations showed a decrease in the K_{cat} of the protein suggesting that this phosphorylation site regulates protein kinase activity (Vertommen et al., 2000).

PKD and its isoforms though structurally similar, are though to have different roles within individual cell types. Each isoform may therefore interact within multiple signalling pathways in a cell-specific manner.

1.9.2 Protein Kinase D activation

PKD is activated in a multi-step process. Three major events can cause this activation; the direct binding of DAG, phosphorylation and caspase-mediated proteolytic cleavage, all of which lead to activation of the catalytic domain of PKD.

1.9.2.1 Protein kinase C phosphorylation

PKD is a well-known substrate of PKC, with PKC directly phosphorylating PKD within its activation loop. Phosphorylation occurs at Ser744 leading to the autophoshorylation of Ser748, activating the protein. Upon Ser744/Ser748 phosphorylation, in PKD1, Ser916 autophosphorylates, this is a known indicator of PKD activation. PKC inhibitors block the activation and phosphorylation of these sites, showing PKC to be playing a crucial role in the activation of PKD (Waldron et al., 2004). In relation to arthritis, PKC delta (Im et al., 2007) and the atypical PKCs are the only isoforms to be shown to regulate MMP gene expression (Litherland et al., 2010) and therefore these isoforms may initiate

PKD activation in HAC. This contrasts to many findings within numerous other cells types in which the phosphorylation of PKD is initiated by the novel PKCs. PKD phosphorylation at the serine744/748 site was first identified by the Rozengurt group (Iglesias et al., 1998). The Rozengurt group showed that PKCε and η were both capable of stimulating PKD to comparable levels to that of stimulation with phorbol esters (Zugaza et al., 1996). However, they showed that PKD could not be phosphorylated by atypical PKCζ. This suggested that the novel PKC had specific roles in the stimulation of PKD. This may be cell specific, with different isoforms of PKC activating PKD within different cell types.

1.9.2.2 Diacyl glycerol activation

DAG is generated by the hydrolysis of phosphatidlyinositol 4, 5-bisphosphate by Phospholipase C (PLC). PLC breaks down PIP_2 to produce two secondary messengers; IP_3 and DAG (Fukami et al., 2010). IP_3 triggers the release of calcium from intracellular stores (Fukami et al., 2010), while DAG can activate PKC and PKD directly (Rozengurt, 2011). DAG can regulate PKD in two ways; firstly it can directly bind to PKD by binding the C1 domain in PKD, activating the protein, secondly DAG can bind and activate PKC which can then activate PKD by phosphorylation. DAG can also recruit PKC and PKD to the plasma membrane, ensuring both are in close enough proximity, enabling PKC to phosphorylate PKD (Rozengurt, 2011).

1.9.2.3 Caspase mediated activation in oxidative stress

PKD can also be activated through caspase-mediated proteolytic cleavage in cells induced by reactive oxygen species (Vantus et al., 2004). PKD cleavage by caspase-3 leads to increased kinase activity. Cleavage leads to the production of two PKD catalytic fragments which are thought to be produced via two capase-3 cleavage sites found within PKD. Cleavage occurs between the catalytic and regulatory domain of the protein, suggesting the loss of the regulatory zinc finger motifs can cause activation of the protein (Vantus et al., 2004).

1.9.2.4 Activation by tyrosine phosphorylation

PKD can also be activated by phosphorylation events other than PKC mediated phosphorylation. PKD can be activated by the phosphorylation of tyrosine residues found within the PH domain, when the cell is under oxidative stress. PKD has been shown to be phosphorylated at Tyr432, Tyr463, and Tyr502

leading to PKD activation (Vertommen et al., 2000). Further study showed that Tyr463 phosphorylation is a crucial event in the activation of PKD. Mutation and siRNA silencing studies showed that, via the Src–Abl pathway, PKD could be activated, independent of PKC (Storz et al., 2003). Tyrosine phosphorylation may allow the binding of PKC isoforms to the PH domain causing further PKD phosphorylation and activation PKD (Storz et al., 2003, Waldron et al., 1999).

1.9.3 PKD activation via IL-1 and OSM

The potential mechanism by which PKD is activated and phosphorylated by IL-1 and OSM in HAC is unclear. However, data from the literature has shown PKD to be downstream of the IL-1 receptor, with direct and indirect activation via IL-1 stimulation (Song et al., 2007). PKD1 has been shown to be phosphorylated by PKC in a IL-1 dependent mechanism, suggesting PKD to be phosphorylated and activated by this mechanism. PKD has also been shown to bind to the TLR9/MyD88 receptor complex via an interaction with IRAK4, IRAK1 and TRAF6 (Park et al., 2009). This suggests a mechanism by which PKD could bind to the IL-1 receptor through IRAK and TRAF6 binding. OSM, on the other hand, has not been shown to previously phosphorylate and activate PKD. OSM has, however, been shown to activate PKC signalling (Litherland et al., 2010), a known upstream activator, as well as stimulating the PI3K signalling cascade, a further upstream activator of PKC and also PKD (Lynch et al., 2013).

1.9.4 PKD cellular distribution

PKD is known to shuttle between numerous compartments within the cell in response to cellular stimulation. The localization of PKD is dependent on isoform and stimuli. PKD can translocate from the cytosol to DAG containing environments upon stimulation by G-protein couple receptor (GPCR) (Rey et al., 2001). PKC stimulation on the other hand has shown to cause PKD3 to translocate from the cytosol to the nucleus (Rey et al., 2006). PKD can also be seen to accumulate and localise at the Golgi apparatus, as well as the mitochondria. Not only does this show the diverse compartments within the cell that PKD can be found but also suggests how different stimuli can cause different responses of the protein, aiding in its multiple regulatory processes.

1.9.5 Protein kinase D signalling

Protein kinase D has numerous roles within multiple cellul types, with the 3 different isoforms having more predominant roles in distinct cell types. PKD has

a consensus substrate sequence of LXR(Q/K/E/M)(M/L/K/E/Q/A)S*XXXX with greatest specificity found for a leucine residue found at the 5 position (Wang, 2006, Doppler et al., 2005). PKD activation by cytokine or ligand binding to a receptor complex leads to upstream signalling activation, which drives PKD phosphorylation and activation. PKD lies at a crucial crossroad between DAG and PKC signalling, with both regulating PKD activity. PKD has not been studied in relation to arthritis; but as a downstream substrate of PKC and known activator of MMP gene expression, PKD has the potential to be of great interest. PKD has been shown to be involved in the activation of the ERK pathway, modulation of the JNK pathway and stimulation of NFκB (Storz and Toker, 2003, Sinnett-Smith et al., 2007b, Hurd et al., 2002). *Figure 1.13* illustrates the signalling pathways which PKC and PKD are believed to modulate.

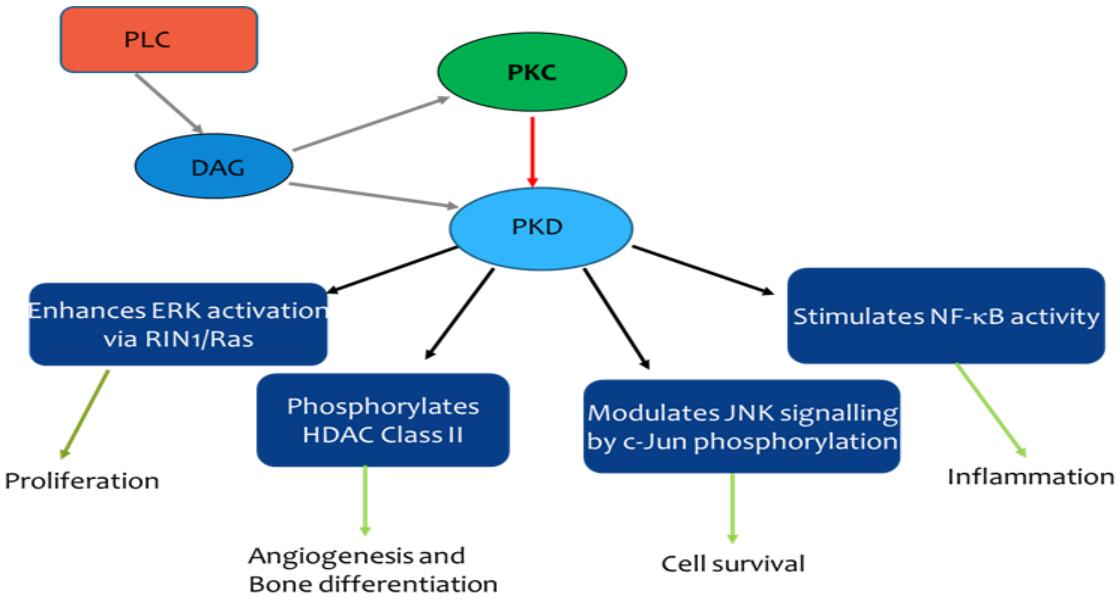


Figure 1.13. PKC/PKD signalling cascade. PKD can be directly activated by DAG generated by Phospholipase C (PLC). GPCRs are known activators of PLC. DAG can also activate PKC which leads to the phosphorylation of PKD on key serine residues leading to PKD activation. PKD can then via its catalytic domain activate multiple signal pathways and phosphorylate key proteins. These include members of the ERK pathway, JNK pathway, phosphorylation of HDACs and stimulate NFκB activity. Adapted from (Rozengurt, 2011)

1.9.5.1 ERK pathway

The ERK pathway is well established in the regulation of collagenase expression. As mentioned earlier, ERK can regulate collagenase expression via the regulation of the gene transcription and phosphorylation of the AP-1 transcription factor component Fos. Litherland *et al*., have shown ERK dependent transcription of c-*fos* in chondrocytes stimulated with IL-1 in combination with OSM (Litherland et al., 2010). Activation of ERK via PKD has the potential to be either PKC dependent or independent. PKC mediated activation of ERK through PKD occurs via the trans-phosphorylation of Ser744/748 leading to PKD activation and signal transduction (Sinnett-Smith et al., 2009). PKD1 has already been implicated in the increased activation of the ERK pathway leading to MMP-3 and MMP-9 expression in prostate cancer cells (Eiseler et al., 2009). This suggests that a similar process could occur within chondrocytes. The PKD-ERK-Fos signalling pathway has the potential to have a crucial role in the progression of arthritis, due to the regulation of AP-1 activity.

PKD2 has recently been implicated in the prolonged activation of the ERK pathways, leading to increased Fos accumulation via GPCR activation. Bombesin, a potent GPCR activator, stimulates GPCR signalling leading to the activation of PLC. This breaks down PIP_2, producing DAG. DAG can then activate PKD directly via interaction with the protein or indirectly through PKC phosphorylation. PKD2 overexpression was seen to increase the duration and activation of ERK signalling, as well as increasing Fos accumulation. This was seen in the presence of Bombesin, suggesting that PKD2 regulated ERK signalling when activated by GPCR (Sinnett-Smith et al., 2007a). The activation of PKD by Bombesin appears to lead to phosphorylation and activation of the MEK-ERK-RSK signalling pathway leading to Fos phosphorylation, stabilising Fos (Sinnett-Smith et al., 2007a).

PKD also regulates the ERK pathway through the phosphorylation of Ras and Rab interactor 1 (RIN1), a protein that when unphosphorylated inhibits Ras/Raf interaction, preventing ERK activation (Wang et al., 2002). RIN1, in its unphosphorylated state binds to RAS and is shown to bind in a way which is competitive with RAF. PKD can directly phosphorylate RIN1 at Ser351 inducing the binding of the *14-3-3* protein. This restricts RIN1 to the cytosol, preventing its ability of blocking Ras/Raf-1 interaction at the plasma membrane (Wang et

al., 2002). *14-3-3* is a known negative regulator of RIN1 membrane localization and its association with Ras (Wang et al., 2002). This leads to the activation of the ERK signalling cascade allowing signal transduction. All this data implies PKD may directly phosphorylate RIN1 leading to the Ras-Raf-MEK-ERK signalling. ERK is therefore shown to be a major downstream substrate of PKD, and has the potential to be a mechanism by which PKD regulated collagenase gene expression in chondrocytes.

1.9.5.2 JNK and Oxidative stress

Oxidative stress is a process in which an imbalance between the production and removal of reactive oxygen species (ROS) occurs; the cell is not able to detoxify or resolve this imbalance leading to cellular damage. ROS and H_2O_2 are known activators of gene expression via the JNK pathway (Zhang et al., 2005). H_2O_2 is shown to cause the activation of PKD leading to activation of the JNK pathway (Zhang et al., 2005). This is believed to occur via apoptosis signal-regulating kinase 1 (ASK1). In H_2O_2 stimulated cells PKD is activated leading to its translocation from the plasma membrane to the cytoplasm (Zhang et al., 2005). This then leads to the dephosphorylation of ASK1 by PKD via an unknown mechanism (it is suggested a phosphatase 2A family-like phosphatase may be involved (Zhang et al., 2005)). This causes the dissociation of 14-3-3 enabling ASK1 to activate JNK (Zhang et al., 2005). PKD has also been shown to regulate AP-1 signalling via JNK in response to ROS. Increased toxicity is found when PKD is inhibited using Gö6976, leading to sustained activation of JNK/AP-1 signalling. The activation of the JNK pathway leading to AP-1 accumulation could therefore potentially be an area of interest in regards to PKD signalling and arthritis.

1.9.5.3 p38

p38 has not been widely studied in relation to PKD signalling. Inhibition of PKD by the pan-PKD inhibitor CRT0066101 (Bernhart et al., 2013) and the PKC/PKD inhibitor Gö6976, in two different cell lines, led to reduced p38 phosphorylation (Lemonnier et al., 2004), suggesting PKD to be an upstream activator of p38. Overexpression of PKD1 has shown conflicting results, with a decrease in phosphorylation of p38 being seen (Song et al., 2009). p38 has also been shown to directly phosphorylate PKD leading to inhibition (Sumara et al., 2009).

These data suggest that the role of PKD in p38 signalling is still unclear, but this discord may have a stimulus and cell-specificity.

1.9.5.4 Nuclear factor κB

Numerous studies have indicated that PKD activates the NFκB pathway when cells are exposed to GPCR agonists or oxidative stress (Chiu et al., 2007, Storz and Toker, 2003, Mihailovic et al., 2004). NFκB has also been shown to be involved in IL-1-dependent MMP regulation in chondrocytes (Fan et al., 2006). Cells stimulated alone with IL-1 are seen to activate NFκB signalling through phosphorylation and degradation of the inhibitory protein IκB (O'Neill and Greene, 1998). IκB is bound to NFκB transcription factor components and upon phosphorylation IκB is released, exposing the nuclear localisation signal, causing the transcription factor to translocate to the nucleus, allowing NFκB to bind to its consensus sequence on target genes (O'Neill and Greene, 1998). Two kinases have been identified as the kinases responsible for phosphorylation of IκB, named IκB kinase 1 and 2 (DiDonato et al., 1997, Woronicz et al., 1997). PKD therefore may be acting upstream of these proteins leading to their activation, inducing MMP expression in chondrocytes.

1.9.6 The regulation of AP-1 via PKD signalling

Many of the signalling pathways activated by PKD induce the transcription and stabilisation of the transcription factor AP-1. As previously mentioned, AP-1 plays a key role in the induction of the MMPs. It has been shown that under the stimulation of IL-1 in combination with OSM, Jun and Fos form the major AP-1 heterodimers in chondrocytes (Litherland et al., 2010, Richards et al., 2001, Catterall et al., 2001). Through the regulation of the signalling pathways mentioned above, PKD has been shown to regulate these major components of AP-1.

Within the literature there is much conflicting data in relation to how PKD regulates Jun at both the transcriptional and protein level. Phosphorylation of Jun is known to increase the stability and transcriptional activity of the transcription factor. PKD has been shown to directly bind to JNK, leading to a loss of JNK dependent phosphorylation of Jun, indicating PKD to decrease the stability and activity of Jun (Hurd et al., 2002). However, conflicting data indicate Jun to be phosphorylated by PKD in a JNK independent manner (Yamashita et al., 2010), suggesting a different mechanism of Jun phosphorylation. PKD is

also shown to directly phosphorylate Jun at a PKD specific phosphorylation site, Ser58 (Waldron et al., 2007). The phosphorylation of Jun in a PKD dependent manner is shown to increase nuclear Jun levels, indicating increased transcriptional activity (Bernhart et al., 2013). Although conflicting, all of these data implicate PKD in the regulation of the stability of Jun. The manner in which regulating Jun is still under debate but it appears to play a crucial role. The role of PKD in the phosphorylation of Jun could be isoform, cell and stimuli dependent.

Fos, the other main component of the AP-1 transcription factor in chondrocytes, is also regulated by PKD signalling. Overexpression of PKD2 led to increased duration of MEK-ERK phosphorylation. ERK then phosphorylates its downstream substrate RSK, which together, phosphorylated Fos stabilising the protein and promoting its cellular accumulation (Sinnett-Smith et al., 2007a, Sinnett-Smith et al., 2007b)**.** PKD1 is also shown to regulate the protein levels of Fos, with silencing leading to decreased levels (Sinnett-Smith et al., 2011).

PKD acts as an upstream regulator of multiple signalling cascades which are seen to regulate AP-1 components. Manipulation of the cellular levels of PKD leads to dysregulation of these pathways and impaired Fos and Jun levels. As AP-1 is a key transcription factor in the regulation of the collagenases, PKD may regulate their expression through these transcription factors.

1.9.7 The regulation of MMPs via PKD signalling

PKD has not previously been studied within the context of arthritis or within chondrocytes. PKD has however been studied in relation to cancer. Here, PKD has been shown to be a regulator of metastasis in invasive cancers, via the regulation of MMPs (Biswas et al., 2010, Eiseler et al., 2009). In initial studies the majority of this work focussed on the role of PKD1. This was because, at the time, PKD1 was the most characterised isoform of the family. PKD1 was also found to be epigenetically suppressed in many cancer tissues and cell lines (Eiseler et al., 2009, Jaggi et al., 2003, Kim et al., 2008). Hypermethylation of the PKD1 promoter in these cells compared to healthy tissue led to reduced expression. These same changes were not observed for PKD2 and PKD3 (Eiseler et al., 2009). The decreased expression of PKD1 in these tumour cells was shown to benefit tumour progression. Active PKD1 in breast cancer cell lines was shown to down regulate the expression of MMP-2, -7, -9, -10, 11, -13,

-14 and 15, leading to a reduction in metastasis (Eiseler et al., 2009). Similar results are found in osteosarcoma cells, with PKD1 being down regulated and PKD1 overexpression leading to decreased MMP-2 expression (Onishi et al., 2012). These data therefore support the idea that PKD1 is a negative regulator of MMPs in cancer tissues.

Conflicting data in the regulation of MMP gene expression via the regulation of PKD1 are observed within different cellular contexts. In prostate cancer cells, pan-PKD inhibition prevented JunD recruitment to the AP-1 binding site within the MMP-1 promoter, preventing gene expression. This appeared to be modulated by PKD activation of the JNK pathway (Yamamoto et al., 2010). Also in prostate cancer, overexpression of constitutively active PKD1 led to increased secretion and activation of MMP-2 and MMP-9. This was shown to be mediated by the MEK-ERK signalling (Biswas et al., 2010). PKD1 is also shown to regulate the subcellular localisation of HDAC7 relieving its inhibitory effects upon MEF2 mediated MMP-10 and MMP-14 gene expression (Ha et al., 2008). Although not a cancer cell, osteoblasts stimulated with basic calcium phosphate (BCP) crystals, which are known to occur in patients with OA, activate PKD leading to ERK activation, which induces MMP-1 and MMP-3 gene induction (Reuben et al., 2004). All these data implicate that the regulation of MMP gene expression by PKD is cell specific and dependent upon cell stimulation.

Although most of the initial data focused upon PKD1 signalling and regulation of MMPs, data are now emerging on how PKD2 and PKD3 regulate gene expression. Zou *et al*., showed PKD2 and PKD3 silencing lead to decreased expression of MMP-9 and MMP-14 through regulation of the NFκB pathway (Zou et al., 2012), whilst Bernhart *et al.,* showed PKD2 silencing lead to impaired ERK, JNK and p38 signalling, which inhibited Jun protein expression and phosphorylation, reducing subsequent MMP-1 gene expression (Bernhart et al., 2013).

PKD therefore needs to be studied as the three individual isoforms it comprises to fully understand the role of each in regulating collagenase expression within chondrocytes. Studying PKD as an entity or only one of the three isoforms is insufficient. Therefore, understanding the roles of each isoform of PKD in the regulation of collagenase gene expression stimulated by IL-1 in combination

with OSM will give greater insight into the signalling mechanisms involved in collagenase induction in HAC within an inflammatory context.

1.9.8 Protein kinase D in skeletal development

Currently there has been no research into the roles of PKD signalling within chondrocytes, but data are emerging on the role of PKD is skeletal development. All isoforms of PKD are identified in limb buds during early embryogenesis (Oster et al., 2006). Recently, the development of mouse models has begun to further implicate PKD in the regulation of skeletal development. PKD1 knockout mice are shown to be embryonic lethal (Fielitz et al., 2008), whereas PKD2 and PKD3 knockout mice are seen to be viable (Matthews et al., 2010, Rogers et al., 2005). Currently no data are available on the role of PKD1 and PKD2 in skeletal development is available as only tissue specific roles have been identified. PKD3 however has been shown to be expressed during skeletogenesis in mice, most notably in the cartilage primordia of bones (Ellwanger et al., 2008) which are areas of active ECM degradation during cartilage remodelling prior to mineralization. A mutant mouse strain of PKD3, harbouring a gene-trap deletion of PKD3, revealed a mild skeletal abnormality, which included a decrease in the mean trabecular bone volume and thickness (Rogers et al., 2005). These data implicate the PKD family in the regulation of skeletal development and thus may play a role in the arthritic phenotype.

1.10 Aims

The aims of this thesis are to examine the individual roles of each PKD isoform in the regulation of collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. Experiments will focus on the role of each isoform in the regulation of MMP-1 and MMP-13 gene expression, as well as the signalling pathways which are known to regulate their expression. Work will also focus on the AP-1 transcription factor family members and how each isoform of PKD regulates their expression and stabilisation. Therefore the main aims of this thesis are to:-

- Establish the individual role of each PKD isoform in the regulation of MMP-1 and MMP-13 gene expression in HAC
- Determine the signalling pathways which each isoform of PKD regulates under the stimulation of IL-1 and OSM alone as well as IL-1 in combination with OSM.
- Understand whether the individual isoforms of PKD regulate the expression of AP-1 family members.
- Examine the effects of PKD on novel post-AP-1 collagenase regulating cytokines and transcription factors.

2 Chapter 2. Material and methods

2.1 Materials

2.1.1 Antibodies

All antibodies used were rabbit polyclonal and diluted 1:1000 in bovine serum albumin (BSA) in TBS-T (Sigma-Aldrich, Poole, UK), unless otherwise stated. Antibodies, PKCζ (#9372), PKCι (#2998), PKD3 (#5655), PKD*^{S916} (#2051), PKD*$^{S744/748}$ (#2054), ERK1/2*$^{T202/Y204}$ (#9101), JNK (#9252), JNK*$^{T183/Y185}$ (#9251), p38*$^{T180/Y182}$ (#9211), Akt*^{S473} (#4060), c-Fos (#4384), STAT-1*^{S727} (#9177), STAT-1*^{Y701} (#9171), STAT-3*^{S727} (#9134), STAT-3*^{Y705} (#9131), caveolin-1 (#3238) p65*^{S536} (#3033) and lamin A/C (#2032) were purchased from Cell Signalling Technology (Danvers, MA, USA). MEK2 (#04-377) monoclonal antibody, anti-HA tag (ab18181), PKD2 (#1969-1) and β-tubulin polyclonal antibodies (#1799-1) were purchased from Epitomics (Insight Biotech, Wembley). PKD1 (L905) antibody was purchased from Bio-world (Dublin, USA). GAPDH (mouse) (MAB374) was purchased from MerckMillipore (Watford, UK) (Diluted 1:4000). Jun H-79 (sc-1694) and c-Jun*^{T93} (sc-101722) (both diluted 1:500) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal goat, anti-rabbit immunoglobulin HRP and polyclonal goat, anti-mouse immunoglobulin HRP secondary antibodies were purchase from DAKO (Cambridge, UK). All secondary antibodies were diluted 1:2000, except when used for GAPDH when diluted 1:10,000 in 5% (w/v) non-fat dry milk in TBS-T.

2.1.2 Cell lines

Two different cell lines were used in this thesis. The human chondrosarcoma cell line, SW1353, generated from cells taken from the humerus of a 72 year old female Caucasian, and the Human Embryonic Kidney cell line HEK293T. Both were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were routinely passaged. SW1353 cells were used up to a passage of 20 to ensure they maintained their responsiveness.

2.1.3 Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified Eagle's medium: Hams F12 medium (DMEM-F12), Hank's balanced salt solution (HBSS), and foetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK). Phosphate buffered saline (PBS) was from Lonza (Wokingham, UK). Penicillin-streptomycin solution (10 000 U/ml and 10 mg/ml, respectively), L-glutamine solution (200 mM), nystatin suspension (10,000 U/ml), trypsin-EDTA (ethylenediaminetetraacetic acid) solution (0.5 g porcine trypsin and 0.2 g/L EDTA), hyaluronidase (from bovine testes, 439 U/mg), trypsin (from porcine pancreas, 1020 U/mg), collagenase (from *Clostridium histolyticum* type II) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich. Centrifuge tubes, 50 ml and 15 ml were obtained from Corning/Costar UK Ltd. (High Wycome, UK). 50 ml syringes were obtained from Scientific Laboratory Supplies Ltd. (Nottingham, UK). Syringe filters (0.2 μm) were purchased from Pall Life Sciences (Portsmouth, UK). Syringe filters made of polyethersulfone (PES) (0.45 μm) were purchased from Alpha Laboratories (Hampshire, UK). Cell strainers (100 μm) were from BD Biosciences, (Oxford, UK).

2.1.4 Cytokines

All cytokines were recombinant human. IL-1α was a generous gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, UK) and was stored at -20°C until use. OSM was prepared in-house using expression vectors kindly provided by Prof. J. Heath (University of Birmingham, UK) and methods described in (Staunton et al., 1998). OSM was prepared at a concentration of 60 μg/ml in Dulbecco's phosphate buffered saline (DPBS) (with 0.1% bovine serum albumin (BSA)) and stored at -80°C. Immediately prior to use all cytokines were dilute to working concentrations in cell culture medium.

2.1.5 Commercially available kits

Qiaprep Spin Maxi Kit was purchased from Qiagen (Crawley, UK). Pierce Cytoplasmic and Nuclear Fractionation and the Subcellular Protein Fractionation Kit were purchased from Thermo Fisher Scientific (Loughborough, UK). Lenti-X™ Concentrator and Lenti-X™ qRT-PCR Titration Kit were both purchased from Takara Bio Europe/Clontech (Paris, France).

2.1.6 Immunoblotting reagents

BSA (2 mg /ml) standards, Ammonium peroxodisulphate (APS), b-mercaptoethanol, N,N,N'N'-tetramethylenediamine (TEMED), 1,10-phenanthroline and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich. 40% (w/v) acrylamide/bis-acrylamide (37.5:1) solution was obtained from Amresco (OH, USA). Bradford ULTRA was purchased from Novexin (Invitrogen). PageRulerTM prestained protein ladder was purchased from Thermo Fisher Scientific. Enhanced chemiluminescence (ECL), ECL prime or Advanced Western Blotting Detection systems were obtained from Amersham ™ GE Healthcare UK Ltd, (Buckinghamshire, UK). ECL plus was obtained for MerckMillipore. Reagents for the iBlot gel Transfer System were all purchased from Invitrogen.

2.1.7 Molecular Biology reagents

Cells to cDNA lysis buffer was purchased from Ambion (Huntington, UK). SidestepTM Lysis and Stabilization Buffer was purchased from Agilent Technologies, Stratagene Product Division (CA, USA). Polybrene (hexadimethrine bromide), doxycycline hyclate (D9891), RNase- and DNase-free H2O, real-time reverse transcriptase polymerase chain reaction primers and probes were purchased from Sigma-Aldrich. Moloney Murine Leukamiea Virus (M-MLV) reverse transcriptase and RNaseOut recombinant ribonuclease inhibitor were purchased from Invitrogen. Random hexamers were purchased from IDT (Coralville, IA, USA). RQ1 RNase-free DNase was purchased from Promega (Southampton, UK). DNTPs were purchased from Bioline (London, UK). Taqman Fast Universal PCR Master MIX (2x) and Taqman Universal Master Mix II were purchased from Applied Biosystems ((Life techonlogies) Foster City, CA, USA). Dharmafect^{TM} 1 lipid reagent, ON-TARGETplus Non-targeting siRNA #2 (catalogue number 001210-02) and PKCζ and PKCι SMARTpool® small interfering RNA siRNAs were purchased from Dharmacon (a Thermo Fisher Scientific company) (Cramlington, UK). PKD1, PKD2, PKD3, JNK1 and JNK2 siRNAs were all purchased from Sigma-Aldrich and were originally identified from the Sigma-Aldrich MISSION™ siRNA Human Kinase Panel. GIPZ and TRIPZ short hairpin RNAs (shRNAs) for PKD1 were purchased from Open Biosystems (a Thermo Fisher scientific company) (Pittsburgh, PA, USA). The expression vector pcDNA-PKD1 was purchased

from Addgene (plasmid; 10808) (Storz et al., 2003). pcDNA3.1 was used as the empty vector control. The packaging plasmid pCMV-dR8.91 and the envelope plasmid pMD2.G were a kind gift for Martina Elias (Newcastle Univeristy). For plasmid maps of all expression plasmids see *section 7.9* in the appendices

2.1.8 Inhibitors

The PKC inhibitor Gö6983 and the PKD inhibitor kb NB 142-70 were both purchased from Tocris Bioscience (Bristol, UK). The STAT3 inhibitor S3I-201 was purchased from Stratech Scientific Ltd (Suffolk, UK). All inhibitors were reconstituted in sterile DMSO at appropriate concentrations. All inhibitors were screened for cytotoxicity in all cell types used, using the ToxiLight^{TM} Bioassay Kit (Lonza). 100x protease inhibitors were made from dissolving one complete miccrococcal protease inhibitor mini tablet (Roche products limited, Welwyn Garden City, UK) in RNase- and DNase-free H_2O.

All other biological chemicals were purchased from Sigma-Aldrich (unless otherwise stated) and were of the highest purity availability.

2.2 Methods

2.2.1 Isolation of Human articular chondrocytes (HACs)

Background

Human articular cartilage samples were acquired from osteoarthritis patients undergoing total knee or hip replacement surgery at the Freeman hospital, Newcastle upon Tyne. Ethical approval and informed consent were obtained prior to surgery. All subjects gave informed consent and the study was approved by the NRES Committee North East - Newcastle & North Tyneside 1; REC reference 09/H00906/72. Post-surgery joint were kept at 4^{o}C in HBBS supplemented with penicillin, streptomycin and nyastatin. All joints were assessed for tissue viability within 24 hours post-surgery.

The extracellular matrix of human articular cartilage was digested to release embedded chondrocytes. These chondrocytes were used for *in vitro* experiments. Sequential enzyme digestion of the ECM using hyaluronidase (for hyaluronan), trypsin (for aggrecan) and collagenase (for collagen) released chondrocytes for monolayer culture.

Reagents

- HBSS containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 IU/ml nystatin
- DMEM medium containing 10% (v/v) FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 IU/ml nystatin.
- PBS containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 IU/ml nystatin.
- Hyaluronidase (1 mg/ml in PBS)
- Trypsin (2.5 mg/ml in PBS)
- Collagenase (2.0 mg/ml in DMEM)

Method

HAC were extracted from the cartilage by enzymatic digestion of macroscopically normal articular cartilage from OA patients. Cartilage was dissected from the joint and cut into small pieces to increase the surface area. The cartilage was then enzymatically digested using hyaluronidase (bovine testes) (1 mg/ml, 4 ml/g of cartilage) at 37°C for 15 minutes in PBS, then trypsin (porcine pancreas) (2.5 mg/ml, 4 ml/g) at 37°C for 30 minutes in PBS, then finally collagenase (*Clostridium histolyticum*) (2.0 mg/ml, 3 ml/g) in serum containing DMEM, for 12-14 hours. Following digestion, cells were passed through a 100 µm cell strainer to remove undigested cartilage. Cells were then centrifuged at 217 x *g* for 10 minutes. The pellet was re-suspended in PBS and centrifuged again at 217 x *g* for 10 minutes. Cells were re-suspended in DMEM containing serum and counted. 3 million cells were plated per T75 cm^2 for passage 1 (P1) chondrocytes or plated at a density of 10,000 per well for 96 well plate or 500,000 per well for 6 well plate for P0 cells. Cells were maintained in culture conditions of 37°C, 5% (v/v) CO_2 for around 10 days till ~70% confluent. Passage (P0) cells grown in flasks were then trypsinised and plated 24 hours prior to use for transfection or transduction at same densities as P0, these became known as P1 cells. P0 cells were serum starved overnight prior to stimulation or inhibitory studies. For serum deprivation, identical conditions were maintained except FBS was excluded.

2.2.2 Cell Line culture

Background

The cell line SW1353 was used for optimisation, subcellular fractionation and over-expression experiments. The cell line HEK293T was used as a packaging cell line for the production of lentiviral particles.

Reagents

- For SW1353 cells:- DMEM-F12 medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin
- For HEK293T cells:- DMEM medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin

Methods

SW1353 cells and HEK293T were maintained in culture conditions of 37°C, 5% (v/v) CO_2. Cells were cultured in T75cm^2 flasks (Corning) until they were 70-80% confluent. Cells were routinely passaged. Cells were washed 3 times in PBS and then 1.5 ml of trypsin added for 5 minutes. Cells centrifuged at 217 x *g* for 10 minutes, re-suspended in serum containing medium and counted using haemocytometer. SW1353 cells were plated at a density of 4,500 per well for 96 well plate or 125,000 per well for 6 well plates for experimental procedures. HEK 293T cells were plated at a density of 25 million in 500 cm^2 plates for lentiviral production.

2.2.3 Lentivirus generation

Background

Recombinant retroviruses are widely used as gene therapy vectors due to their ability to integrate a transgene into the genome of a target cell. Retoviruses can therefore be utilised for basic science, allowing the stable integration of a target gene into the genome of the infected cell. Lentiviral delivery of both over-expression and shRNA plasmids into a target cell has many benefits over transient transfection. Lentiviral transduction of an overexpression or shRNA plasmid into a target cell leads to the incorporation of the gene of interest into the host genome, allowing this to be constitutively expressed. Lentiviruses can also transduce both dividing and non-dividing cells allowing increased chance of delivery. The incorporation and constitutive expression of the gene or shRNA

increases the chance of over-expression or gene silence as the inserted gene is constantly expressed.

Lentivirus can transduce a cell by binding to the host cell membrane. This is instigated by the glycoprotein of the vesicular stomatitis virus (VSV-G) proteins found on the surface of the viral particle. This binding leads to the endocytosis of the viral machinery and RNA into the target cell. Once incorporated, the viral reverse transcriptase converts the over-expression gene or shRNA transcript into DNA, the enzyme integrase then incorporates this into the host genome. This is then constitutively expressed by the host, leading to the production of the gene of interest or shRNA. The constant production increases the chance of gene silence or increases the chance of increased protein synthesis. Multiple modifications to the gene the human immunodeficiency virus (HIV-1) genome have been made to ensure biological safety and prevent the production of replication competent lentiviruses (RCLs).

In this work a second generation lentiviral generation system was used. Here three plasmids were transfected into the packaging cell line HEK293T. These plasmids consisted of an envelope, packaging and vector plasmid. These contained the essential machinery needed to generate transducible lentiviruses which were unable to re-replicate.

Class 2 biosafety approval was received from the Health and safety executive (HSE) for the generation and use of lentiviruses within our laboratory; HSE reference: GM540

Reagents

- HEK293T cells
- Heat inactivated FBS
- DMEM medium containing 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin
- 500 cm^2 square cell culture plate (Corning)
- JetPEI transfection reagent
- 150 mM NaCL
- Lenti-X™ Concentrator kit
- Lenti-X™ qRT-PCR Titration Kit

Method

Lentiviruses were produced on a large scale to ensure continuity between experiments. 25 million HEK293T cells were plated into a 500cm^2 cell culture dishes 24 hours prior to transfection. On day of transfection FBS was heat inactivated by heating to 56°C for 30 minutes. This was then added to supplemented DMEM medium. HEK293T medium was then replaced with this heat inactivated FBS containing medium. In separate tubes equal amounts of 150 mM NaCl were added. To the first tube, the shRNA plasmid, envelope (pMD2.G) and packaging plasmid (pCMV-dR8.91) were added and vortexed. To the next tube, the JetPEI transfection reagent was added at a ratio of 2:1 to plasmid DNA. These were combined by adding the DNA to the JetPEI, this was then vortexed and left for 20 minutes to allow complex formation. The DNA/JetPEI complexes were then added to the HEK293T cells in a drop wise manner. The cells were left for 8-10 hours before the medium was changed. The cells were left for a further 72 hours for viral production. After 72 hours the medium was harvested and cells discarded. The medium was then passed through a 0.45 µm polyethersulfone low protein binding filter to remove cell debris. Viral supernatant was also centrifuged at 1600 × g at 4 °C for 10 minutes to ensure all debris removed. Once viral supernatant was clarified the viral was then concentrated. *Figure 2.1* shows the production of lentiviral particles using a second generation packaging system.

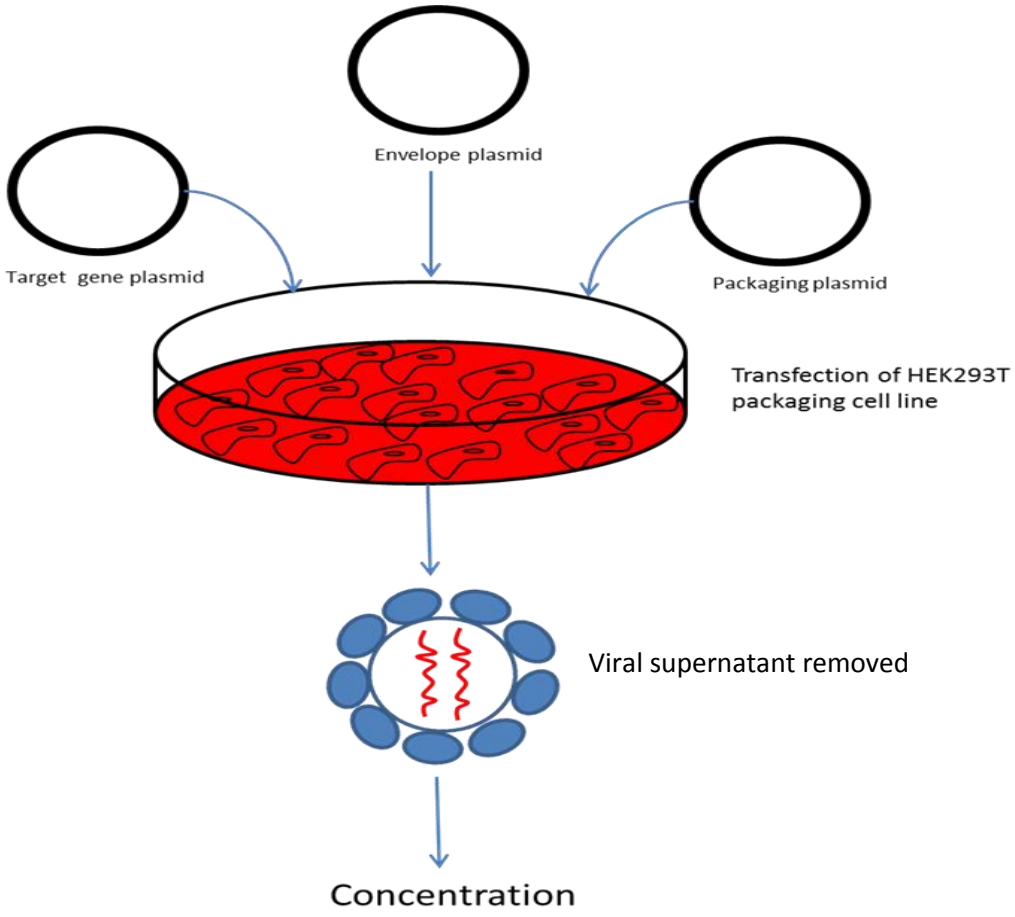


Figure 2.1. Schematic of lentiviral production using the transient transfection of the packaging cell line HEK293T. The envelope plasmid contains the entire components needed to produce the viral envelope. Our envelope plasmid contains the vsv-g gene, which encodes the G-glycoprotein of the vesicular stomatitis virus. This allows the lentivirus generated to transduce a wide range of cell types. The packaging plasmid contains the key genes *gag* and *pol*. *gag* encodes the p24 protein as well as other protomers. These are the major components of the oligomeric viral capsid. The pol gene encodes the two key enzymes involved in incorporation of the viral RNA into the host genome. The third plasmid contains the gene of interest be it an overexpression plasmid or shRNA plasmid. These are all transfected in to the packaging cell HEK293T, which have been modified by the addition of the T antigen of SV-40 to increase replication.

To concentrate the viral supernatant the Lenti-X™ Concentrator kit was used. This kit allows the concentration of lentiviral particles without the need for ultracentrifugation. Supernatant was combined with the Lenti-X™ Concentrator kit solution at a 3:1 ratio of supernatant to concentrator solution. This was left to incubate for up to 1 hour. Once incubation had occurred the solution was centrifuged at 1,500 x *g* for 45 minutes at 4°C. A viral pellet formed and the supernatant was removed. This pellet was then re-suspended in native DMEM medium at a 1:50 concentration, aliquoted into small volumes and stored a -80°C until use. Repeated freeze thawing reduces viral viability.

To calculate the Multiplicity of Infection (MOI) of SW1353 cells and HACs the number of viral particles generated in each viral prep was calculated. To achieve this, the Lenti-X™ qRT-PCR Titration Kit was used. This kit utilises SYBR Green technology to calculate the copy number of viral sample, using a known viral RNA control. An aliquot of viral supernatant was lysed and viral RNA purified, using the NucleoSpin RNA Virus Kit that forms part of the Lenti-X™ qRT-PCR Titration Kit. As the viral particles were produced from a transient transfection rather than stably expressing cell lines, a DNase step was performed to remove any residual plasmid DNA. A one-step reverse transcription-real-time PCR experiment was then performed. Both control and viral RNA undergo 10 fold serial dilutions in dilution buffer. These are then added to a real time PCR plate in duplicate and the enzyme reaction mix added (see *Table 1*). A non-template control was also added to the RT-PCR plate. The plate was then centrifuged at 1200 x g (4°C) for 1 min to remove any bubbles. The samples then underwent the following PCR cycles within the real time PCR machine:

RT Reaction- 42°C 5 min followed by 95°C 10 sec

qPCR x 40 Cycles- 95°C 5 sec followed by 60°C 30 sec

Dissociation Curve- 95°C 15 sec followed by 60°C 30 sec which was followed by (60°C–95°C)

The dissociation curve confirmed that no primers dimers or secondary non-specific PCR artefacts formed in the reaction. *Figure 7.8* (in appendices) shows an example of the dissociation curve of one of the experiments performed.

Reagent	Volume (µl)/ well
RNase-Free Water	8.0
Quant-X Buffer (2X)	12.5
Lenti-X Forward Primer (10 µM)	0.5
Lenti-X Reverse Primer (10 µM)	0.5
Reference Dye LSR	0.5
Quant-X Enzyme	0.5
RT Enzyme Mix	0.5
RNA	2

Table 1. Components, concentrations and volume of viral titre real time PCR master mix. Table indicating the components which made up the reaction mix used for the viral titre calculation.

From the C_t values obtained, the quantity of viral RNA could be calculated. This was achieved by forming a standard curve from the average C_t values of the diluted viral RNA control. This was plotted on a graph with average C_t vs. copy number (log scale). The average C_t values for each duplicate sample dilution were read and the corresponding copy number value from the standard curve was calculated. Then using the equation below, for each dilution, the copy number was calculated. This generated a mean value to determine the RNA genome content of the sample.

$$Copies/ml = \frac{(\text{copies N calculated from Cts})(1000\text{ ml})(50\text{ µl elution})}{(150\text{ µl sample})(2\text{ µl added to each well})}$$

Once the number of copies per ml was calculated the MOI could be calculated. To do this a known number of cells were plated into a 96 well plate and fold dilutions of the viral supernatant was added to each well. From this, using the red fluorescent protein (RFP) or green fluorescent protein (GFP) expressed by the shRNA, the volume needed to transduce all cells plated in one well could be calculated. RFP and GFP were quantified using a Zeiss Axiovert 200M confocal microscope. This volume could then be used to calculate the number of viral particles needed to transduce all cells. An MOI of 30 was calculated for HAC cells. An MOI of 15 was calculated for SW1353 cells. Understanding the MOI allows to correct for any differences between viral preparations and ensure the right volume of viral supernatant is added to the cells to get maximal transduction.

2.2.4 RNA interference

Background

Small interfering (si) and Short hairpin (sh) RNA mediated gene silencing is a robust and well defined method for blocking gene expression. Both methods can be used to silence the expression of a target gene, leading to reduced mRNA expression, which can lead to reduced protein expression. siRNAs can be chemically synthesised as double-stranded RNA and are transfected into the cell in this manner. shRNAs on the other hand can be transfected into the cell in the form of a expression vector or a short hairpin structure. siRNAs and shRNAs can both achieve similar outcomes in gene silencing but are intrinsically different molecules.

siRNAs are double-stranded RNA sequences which are around 20-25 nucleotides in length, these bind through complementarity to the mRNA of a target gene, leading to its degradation. To achieve this, the siRNA must partake in the RNA interference pathway. For this to occur, the dsRNA is transfected into the cell, in this case using a lipid-based vesicle. The dsRNA is unwound and processed into a single stranded 20-25 nucleotide siRNA containing a 2-nucleotide overhang at the 3' end, known as the guide strand. This process is catalysed by the ribonuclease Dicer. Once cleaved, Dicer aids in the translocation of the guide strand and its association with the RNA-induced silencing complex (RISC), which assembles onto the siRNA. The complex is targeted to the complementary gene by sequence complementarity of the siRNA. Once bound the RISC complex cleaves the complementary target gene through its endonuclease activity found within the argonaute protein (Elbashir et al., 2001). This cleavage leads to a reduction in the amount of mRNA of the target gene. A reduction in the levels of mRNA can therefore lead to reduced protein translation and thus reduced protein levels within the cell; this is dependent on protein stability and mRNA transcription rate. The downstream effects of the target gene can then be assessed.

shRNA differs to siRNAs in the manner in which they enter the cell and the form that they are in (Rao et al., 2009). For these reason shRNA undergo different processing within the cell. shRNAs are usually found within an expression vector and enter the cell in this form. Two methods can be utilised in the

expression of the shRNA. These include, transient transfection using a transfection reagent or transduction via viral based incorporation. In this work lentiviral mediated gene delivery was utilised. Here, the shRNA is incorporated into the viral particle and is transduced into the cell as a shRNA rather than an expression vector. Once inserted into the cell, the shRNA must be first converted into a mature siRNA. To this the shRNA must undergo processing within the nucleus of the target cell. The shRNA must undergo cleavage of its short hairpin structure by the RNase III enzyme Drosha (Rao et al., 2009). This converts the shRNA into a dsRNA similar to that of the transfected immature siRNA. This is then exported from the nucleus into the cytoplasm by exportin 5. The dsRNA then enters the RNAi pathway described above.

Reagents

siRNA transfection:

- For SW1353 cells- DMEM-F12 medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin. FBS was excluded for serum free conditions.
- For HAC- DMEM medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 IU/ml nystatin. FBS was excluded for serum free conditions.
- Dharmacon ON-TARGET plus SMARTpool siRNA or Sigma MISSION™ siRNA.
- Dharmacon ON-TARGETplus Non-targeting control siRNA
- DharmaFECT 1 Transfection reagent
- For a list of siRNAs used see *Table 2* and *Table 3*

shRNA transduction:

- For SW1353 cells- Native DMEM-F12 medium or DMEM-F12 medium containing 20% (v/v) FBS, 4 mM L-glutamine, 200 IU/ml penicillin, 100 µg/ml streptomycin.
- For HAC- Native DMEM medium or DMEM medium containing 20% (v/v) FBS, 4 mM L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 80 IU/ml nystatin.
- Polybrene 8 µg/ml

- Doxycyline 3 µg/ml
- For a list of shRNA used see *Table 4*

Methods

Transfection of siRNA

96 well plate format:

Flasks of SW1353 cells or P0 HACs were trypsinised and cells plated out into 96 well plates, 24 hours prior to experiment start point. Several hours prior to transfection, all medium was removed from the HAC or SW1353 cells and replaced with 50 µl of fresh FBS-containing DMEM or DMEM-F12, respectively. For each well, 100 nM siRNA in a total volume of 15 µl FBS-free DMEM or DMEM-F12 was prepared. Separately, 0.26 µl of DharmaFECT 1 lipid reagent in a total volume of 15 µl of FBS-free DMEM or DMEM-F12 was also prepared. These were both combined and incubated at room temperature for 20 minutes before addition to cells, allowing for complex formation. Cells were transfected for 48 hours and serum starved for 12 hours prior to the addition of cytokines for a further 24 hours. Total RNA was isolated and reverse-transcribed as described in *section 2.2.10*. Expression of genes of interest was measured by real-time PCR as described in section 2.2.11. Changes in gene-specific mRNA levels were calculated by comparison of expression levels with cells transfected with 100 nM siCONTROL (siCON).

6-well plate format:

Flasks of SW1353 cells or P0 HACs were trypsinised and cells plated into 6 well plates, 24 hours prior to experiment start point. Several hours prior to transfection, all medium were removed and replaced with 500 µl of serum-containing DMEM or DMEM-F12. For each well, 100 nM siRNA in a total volume of 250 µl serum-free DMEM/DMEM-F12 was prepared. Separately, 3.25 µl of DharmaFECT 1 lipid reagent in a total volume of 250 µl of DMEM/DMEM-F12 was also prepared. These were both combined and incubated at room temperature for 20 minutes before addition to cells, allowing for complex formation. Cells were transfected for 48 hours and serum starved for 12 hours prior to the addition of cytokines for varying time points. Total cell lysates were prepared as described in *section 2.2.14* and proteins were separated by SDS-PAGE as described in *section 2.2.16* and identified using western blotting

(section 2.2.17). Silencing of specific genes was then assessed using antibodies against each protein.

Isoform	Sequence 1 (5'-3')	Sequence 2 (5'-3')	Sequence 3 (5'-3')	Sequence 4 (5'-3')
PKC ζ	CGUCAAAGCCUCCCAUGUU	GCAGGACUUUGACCUAAUC	GACCAAAUUUACGCCAUGA	GAGUAUAUCAACCCAUUAU
PKC ι	AGAAAUCAGUCUAGCAUUA	CAAGUGUUCUGAAGAGUUU	GAGGAGACCUAAUGUUUCA	GCAAUGAACACCAGGGAAA

Table 2. Dharmacon ON-TARGET plus™ SMARTpools® siRNA target sequences for PKC isoforms. 25 nM of four siRNA sequences make up each individual smartpool siRNA.

Isoform	siRNA (1) (5'-3')	siRNA (2) (5'-3')	siRNA (3) (5'-3')
PKD1	CAAGGAAAUUCCUUUAUCU	GUGUAUGUUUGAGACGCCU	GACCAAUUCACCUUGACAA
PKD2	GCUUCUACGGCCUUUACGA	GCUAUACACGGCCCACCGU	CGAUACAUCACGCAUGAGA
PKD3	GGAAGAAGAUCCUUAAUCA	CAUAAACGCUGUGCAUCAA	CCGUAAUGAAGUGGCUAUU
JNK 1	GUUCUUAUGAAAUGUGUUATT		
JNK 2	CUGUAACUGUUGAGAUGUATT		

Table 3. Sigma MISSION™ siRNA Human Kinase Panel sequences. Each sequence were used as individual siRNAs to try and silence each gene of interest at both the protein and mRNA level.

Transduction of shRNA

For both 96 and 6-well plate format:

P0 flasks containing HACs were trypsinised and cells plated out at a density of 10,000 per well in a 96 well plate or 500,000 per well for 6 well plate. SW1353 cells were passaged and plated at a density of 4,500 per well for 96 well plate or 125,000 per well for 6 well plates. For each well of a 96 well plate viral particles were added to native DMEM for HAC or DMEM-F12 for SW1353 cells, at a MOI of 30 for HAC and 15 for SW1353 cells, volumes were made up to 100 µl. Polybrene at a concentration of 8 µ/ml was added to each well. For 6 well plates viral particles were added to native DMEM for HAC or DMEM-F12 for SW1353 cells, at an MOI of 30 and 15 for SW1353 cells, volumes were made up to 1 ml. In some experiments (see *chapter 3, section 3.3.2.4*) spinoculation

(the centrifugal inoculation of cell cultures) was performed. For this cells were centrifuged at 1,200 x *g* for 60 minutes at room temperature immediately after the addition of viral supernatant. Cells were left for 8 hours for transduction. Medium containing twice the normal supplements was then added to each well. If TRIPZ inducible shRNAs were used then this medium was supplemented with doxycycline at varying concentrations. The medium was then changed the following day to normal supplemented medium for GIPZ non-inducible shRNAs or doxycycline containing if TRIPZ based shRNAs. Cells were left for a further 48 hours. Cells were lysed to assess gene silencing by either real-time PCR or western blotting or cells were serum starved for cytokine stimulation. The transduction of cells was assessed by analysing RFP expression for TRIPZ based shRNAs and GFP for GIPZ based shRNAs. Fluorescence was detected using an inverted confocal Zeiss Axiovert 200M confocal microscope, using live cells.

shRNA type	shRNA (1) (5'-3')	shRNA (2) (5'-3')	shRNA (3) (5'-3')
TRIPZ (PKD1)	TTGTTGATAAGATCAATGG	TTCGAAACAATGAGGATTG	TTGAGGTCACAGTGAACGA
GIPZ (PKD1)	AATAGCTACATCTCTTCCT	TTCGAAACAATGAGGATTG	

Table 4. Open Biosystems TRIPZ and GIPZ shRNA sequences. Two different expression vectors were used in this study. Displayed are the sequences for the TRIPZ inducible expression vectors based shRNAs and the non-inducible GIPZ expression vector based shRNAs.

2.2.5 Bacterial transformation

Reagents

- SOCs media
- Mach1TM-T1® chemically competent *E.coli*

Methods

All reagents and glassware were autoclaved prior to use. Antibiotics were added at a final concentration of 100 µg/ml in filter-sterilised water for selection. Standard aseptic technique was used throughout the cloning procedure.

Mach1TM-T1® chemically competent *E.coli* (30 µl) and 0.5 µg of plasmid were added to an eppendorf tube. Cells/DNA mixed by gentle flicking of tube. Agar plate, with appropriate antibiotic, was placed in incubator to warm to 37°C. The

cell/DNA mixture was then placed on ice for 30 minutes. Cells were heat shocked by placing the tube into a 42°C water bath for 30-60 seconds. The tube was incubated on ice for 2 minutes before the addition of 500 µl pre-warmed SOCs (37°C). Tubes were incubated for 1 hour in a 37°C water bath. 50 µl of cell solution was streaked out on pre-warmed antibiotic plates. Plates were allowed to dry for 5 minutes before incubation at 37°C overnight.

2.2.6 Purification of Plasmid DNA by MaxiPrep

Background

To acquire large quantities of plasmid DNA a single transformed bacteria must first be grown in large scale. The bacteria are then lysed and DNA extracted. This was achieved using the Qiagen plasmid MaxiPrep kit.

Reagents

- Plasmid Maxiprep Kit
- Isopropanol
- RNase and DNase free H_2O

Method

Prior to plasmid transfection, large quantities of purified plasmid DNA were prepared. Bacterial stocks were first streaked onto agar plates containing ampicillin (sigma Aldrich) (100 µg/ml) and incubated overnight at 37°C. Individual colonies were first picked and added to 5 ml of LB broth (Sigma Aldrich) containing ampicillin (100 µg/ml) and incubated at 37°C with shaking for 4 hours. The culture was then transferred to a 500 ml conical flask containing 250 ml LB broth supplemented with ampicillin and incubated at 37°C with shaking overnight. The culture was then centrifuged at 6,000 x *g* for 15 minutes at 4°C. All buffers described are part of the Qiagen Maxiprep Kit (Qiagen). Pellets were re-suspended in 10 ml of P1 and RNase, followed by 10ml P2, incubated for 5 minutes, and then 10 ml chilled P3 and incubated for 20 minutes on ice. The lysate was then centrifuged at 20,000 x *g* for 30 minutes at 4°C, and the supernatant transferred to a new tube and centrifuged under the same conditions for a further 15 minutes. A QIAGEN-tip 500 gravity column was equilibrated with 10 ml of Buffer QBT; supernatant was then allowed to drip through the column. The column was subsequently washed twice with 30 ml of Buffer QC, and plasmid DNA eluted using 15 ml of Buffer QF into a fresh

centrifuge tube. DNA was precipitated following the addition of 10.5 ml of isopropanol and the sample was then centrifuged at 15,000 x *g* for 30 minutes at 4°C. The supernatant was then removed and residual isopropanol evaporated. The pellet was re-suspended in 500 µl of RNase- and DNase- free H_2O. 1250 µl (2.5x volume) of 100% ethanol was added and after mixing the solution was incubated on ice for 15-20 minutes. Following a final centrifugation of 17,900 x g for 10 minutes at room temperature, the pellet containing DNA was air dried for 10 minutes prior to re-suspension in 300 µl of RNase- and DNase-free H_2O and quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

2.2.7 Over-expression plasmid transfection

Background

Transient over-expression of a target gene can lead to increased protein expression. The over-expression of the target gene can then be compared to cells expressing the vector backbone only. Changes on downstream signalling pathways and gene expression can be assessed.

Reagents

- JetPEI transfection reagent
- 150 mM NaCl
- For SW1353 cells- DMEM-F12 medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin. FBS was excluded for serum free conditions.
- For HAC- DMEM medium containing 10% (v/v) FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 40 IU/ml nystatin. FBS was excluded for serum free conditions

Method

HAC and SW1353 cells were prepared and cultured as described in section 2.2.2 and 2.2.1. For over-expression plasmid transfections, cells were trypsinized and re-seeded at approximately ~70% confluency, 24 hours prior to transfection. PKD1 over-expression plasmids were used to transfect HAC and SW1353 cells in combination with JetPEI transfection reagent.

96-well plate format:

For all transfections a ratio of 2:1 of transfection reagent to DNA was used. In separate tubes 0.2 µg of plasmid DNA was added to 10 µl of 150 mM NaCl. 0.4 µl of JetPEI was added to 10 µl of 150 mM NaCl in a separate tube. Contents of both tube were added together and vortexed. The mix was then incubated at room temperature for 20 minutes, to allow complex formation. Cells were transfected for 24 hours and serum starved for 12 hours prior to the addition of cytokines for a further 24 hours. Total RNA was isolated and reverse-transcribed as described in section 2.2.10. Changes in gene expression of genes of interest were measured by real-time PCR as described in section 2.2.11. Changes in expression of downstream targets of over-expressed gene were assessed by analysing changes in the mRNA levels of the downstream gene compared with cells transfected with the empty vector, pcDNA3.1.

6-well plate format:

For all transfections a ratio of 2:1 of transfection reagent to DNA was used. In separate tubes 1 µg of plasmid DNA was added to 10 µl of 150 mM NaCl. 2 µl of JetPEI was added to 10 µl of 150 mM NaCl in a separate tube. Contents of both tube were added together and vortexed. The mix was then incubated at room temperature for 20 minutes, to all complex formation. Cells were transfected for 24 hours and serum starved for 12 hours prior to the addition of cytokines for varying time points. Total cell lysates were prepared as described in *section 2.2.14* and proteins were separated by SDS-PAGE as described in *section 2.2.16* and detected by western blotting as described in *section 2.2.17*. Over-expression of specific genes was assessed using antibodies against the over-expressed protein or their specific tag.

2.2.8 Cytotoxicity Assay

Background

Biological inhibitors are useful tool to understand the downstream events regulated by signalling molecules. Many inhibitors have been shown to have toxic effects on certain cell types. Therefore to assess whether the inhibitors used in section 2.2.8 and 2.2.9 were toxic to cells the ToxiLight Bio Assay was performed. This kit utilises bioluminescence to evaluate cell death. The kit works on the principle that on cell damage and cell death the cell will lose plasma membrane integrity. In such case, the cell will release the enzyme,

adenylate kinase (AK), a phospho-transferase. This is released from the cell into the medium. AK converts ADP to ATP; the produced ATP is then used to catalyse a bioluminescent reaction which can be measured and quantified using a luminometer. ATP aids in luciferases conversion of luciferin to oxyluciferin, with light being emitted as a by-product of this. As a consequence, the emitted light intensity is linear in relation to the released AK concentration in the medium and thus the cell death. The intensity of light emitted from the medium and total cell death can then be calculated and a percentage of cell death caused by the inhibitor calculated. All inhibitors were screened for cytotoxicity in HACs and bovine nasal cartilage.

Reagents

- ToxiLight BioAssay Kit: Non-Destructive Cytotoxicity Assay

Method

10 ml of Assay Buffer was first added to the vial containing the lyophilized AK detection reagent. This was mixed gently and was left at room temperature for 15 minutes to equilibrate. SW1353/HACs were grown in 96 well plates; bovine nasal cartilage was plated in 96 well plates. Experiments were performed as though a normal experiment. 100 µl of medium was removed from each well and added to a fresh 96 well plate. The cells in the remaining 100 µl of medium were subjected to multiple freeze-thaw cycles. Freeze-thawing leads to the loss of cell membrane integrity and thus was used as a control for complete cell death. The cell death medium and normal medium was then raised to room temperature. 10 µl of each sample was taken from each sample and transferred to a luminescence-compatible 96 well plate. To each sample, 50 µl of the reconstituted AK detection reagent was added and incubated for 5 minutes. The plate was then placed into the luminometer and light quantified at immediate 1 second integrated readings. From this a percentage cell death was calculated from the released AK in the normal medium compared to total cell death from adding both normal and cell death medium.

2.2.9 Monolayer inhibition

To assess broad-spectrum PKD and STAT3 inhibition on collagenase gene expression, as well as PKC inhibition on PKD phosphorylation broad-spectrum inhibitors were used. Cell grown in monolayer were treated with these inhibitors prior to cytokine stimulation.

Reagents

- DMEM culture medium containing 2 mM L-glutamine 200 IU/ml penicillin, 200 µg/ml streptomycin, 40 IU/ml nystatin.
- NB 142-70
- S3I-201
- DMSO

Method

P0 HACs were used. Cells were used when they reached 70-80% confluency, usually 10 days post-digest. SW1353 cells were plated out 24 hours prior to experiment start point. Cells were serum starved overnight prior to inhibition. The following day, 1 hour prior to stimulation, serum free medium was replaced with serum free medium containing inhibitor or for controls; the vehicle inhibitor was reconstituted in (in all cases DMSO). After 1 hour cells were then stimulate with cytokines as normal. Cells were then lysed and lysates stored at -80°C until used in downstream experiments.

2.2.10 RNA extraction and reverse transcription

Background

Prior to PCR, mRNA was extracted from cultured cell lines and primary chondrocytes grown in monolayer. Quantification of gene expression could then be partaken on the conversion of mRNA to cDNA. RNA was extracted from HACs grown in 96 well plates using the *SideStep™ Kit.* RNA was extracted from SW1353s cells grown in 96 well plates using the Cells-to-cDNA kit. Both kits inactivate RNases in one single step allowing reverse transcription to occur directly from lysates without any further processing.

Reagents

- *SideStep™ Kit*
- Cells-to-cDNA kit
- DNase
- RNase- and DNase-free water
- 5x First Strand Synthesis Buffer
- DTT
- RNaseOut

- Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV)
- Ice-cold PBS

Method

RNA extracted using SideStep™ Kit:

HAC were seeded into 96-well plates at a density of 10,000 cells per well and grown until 70-80% confluent. Cells then underwent experimental conditions. The cell culture medium was removed and cells were washed three times in ice-cold PBS on ice. 20 µl/well of SideStepTM Lysis and Stabilisation Buffer was added to each well and the plate was then vortexed for 2 minutes to ensure complete cell lysis. At this point lysates could be stored at -80°C. Lysates were then diluted 4-fold in RNase- and DNase-free water. 4 µl of the diluted cell lysate was transferred to a new 96-well plate for reverse transcription. To each well of the 96-well plate 1 µl random hexamers ($pd(N)_6$) (0.2 µg/ml) and 3 µl deoxyribonucleotides (dNTPs) (2.5 mM) and 4 µl of Rnase- and DNase-free water were added and the plate heated at 70°C for 5 minutes. The plate was immediately placed on a cold block where 4 µl 5x First Strand Synthesis Buffer, 2 µl Dithiothreitol (DTT) (0.1 M), 0.125 µl RNaseOut (40 U/µl) and 0.5 µl MMLV (200 U/µl) were added to each well. The plate was incubated at 37°C for 50 minutes, followed by 70°C for 15 minutes. cDNA was diluted by adding 30 µl of RNase- and DNase-free water. All cDNA was stored at -20°C until required.

RNA extracted using cell to cDNA™ Kit:

SW1353 cells were seeded into 96-well plates at a density of 4,500 cells per well and grown until 70-70% confluent. Cells then underwent experimental treatments. The cell culture medium was removed and cells were washed once in ice-cold PBS on ice. 30 µl *cells to cDNA™ Kit* was added to each well and the lysates were transferred to a PCR plate. The lysates were then heated to 70°C for 10 minutes to ensure samples were lysed. At this point lystates could be stored at -80°C. For certain experiments a DNase step was performed. If so, 1 µl of RQ1 DNase was added. Lysates were then heated to 37°C for 30 minutes and then heated to 70°C for 10 minutes to inactivate the enzyme. After the DNase step, 8 µl of the cell lysate was transferred to a new 96-well plate for reverse transcription. To each well 3 µl of dNTPs (2.5 mM) and 1 µl $Pd(N)_6$) (10 ng/µl were added and the plate was heated to 70°C for 5 minutes. The plate

was immediately placed on a cold block where 4 µl 5x First Strand Synthesis Buffer, 2 µl DTT (0.1 M), 0.125 µl RNaseOut (40 U/µl), 0.5 µl MMLV (200 U/µl) and 1.375 µl of RNase- and DNase-free water were added to each well. The plate was incubated at 37°C for 50 minutes, followed by 70°C for 15 minutes. cDNA was diluted by add 30 µl of RNase- and DNase-free water. All cDNA was stored at -20°C until required.

2.2.11 Taqman Probe-Based Real-Time PCR

Background

This method for real time quantification of mRNA expression within cells cultured in monolayer relies on the use of a gene specific probe. This lends this method a high degree of specificity. The fluorescent probe is gene specific and binds to a region between both gene-specific primers. The probe contains a 5' prime fluorophore and 3' prime quencher. As real time synthesis of the primer takes place, the 5'-3' endonuclease activity of the Taq polymerase cleaves the 5' fluorophore. The dissociated fluorophore is spatially distanced from the quencher allowing its fluorescence to be emitted. The levels of fluorescence detected are therefore directly proportionate to the amount of product produced by the PCR reaction (Bustin, 2000). Once a threshold level of fluorescence is detected a C_t value is given which indicates the number of PCR cycles taken to reach this threshold. This is inversely proportional to the levels of mRNA expression. The quantification of the gene should have no interference from non-specific binding or primer dimers. To normalise each sample for differences in total RNA present the levels of RNA from a housekeeping gene is used. In these experiments the house keeping gene 18S ribosomal RNA (rRNA) was used. Oligonucleotide probes and primers were designed using Primer Express 1.0 (Applied Biosystems) or Universal ProbeLibrary Assay Design Centre for probe library probes and primers (Roche diagnostics Ltd, West Sussex, UK). All primers were designed to be intron spanning to ensure no genomic DNA was amplified. The exception to this was *c-jun* in which a DNase step was always performed prior to reverse transcription. Probes were either FAM-TAMRA or FAM conjugated. A list of all primers and probes used can be seen in *Table 5*.

Reagents

- TaqMan Universal PCR Master Mix (2x)
- TaqMan Fast Universal PCR Master Mix (2x)

Method

For housekeeping genes cDNA was diluted 1:25 for HAC and 1:100 for SW1353 lysates. 5 µl of cDNA was pipetted into either MicroAMP Fast or normal Optical 96-well reaction plates; this was dependent on the type of assay used. To this 4.5 µl of TaqMan Universal PCR Master Mix (2x) or TaqMan Fast Universal PCR Master Mix (2x) was added dependent on the assay type; these contain Taq polymerase, dNTPs and ROX normalisation dye. To this 0.2 µl of forward and reverse primers were added (final concentration of 300 nM) and 0.1 µl of probe (final concentration of 150 nM). For the TaqMan Universal PCR Master assays, samples were subjected to an initial denaturation stage of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For TaqMan Fast Universal PCR Master assays, samples were subjected to an initial denaturation stage of 95°C for 10 minutes, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. All assays were performed using the ABI prism 7900HT system (Applied Biosystems).

Gene	Forward primer 5'-3'	Reverse Primer 5'-3'	Probe 5'-3'
MMP-1	AAGATGAAAGGTGGACCAAAATT	CCAAGAGAATGGCCGAGTTC	CAGAGAGTACAACTTACATCGTGTTGCGGCTC
MMP-2	TGGCGATGGATACCCCTTT	TTCTCCCAAGGTCCATAGCTCAT	CTCCTGGCTCATGCCTTCGCCC
MMP-8	CACTCCCTCAAGATGACATCGA	ACGGAGTGTGGTGATAGCATCA	CAAGCAACCCTATCCAACCTACTGGACCAA
MMP-9	CCTGGGCAGATTCCAAACCT	GCAAGTCTTCCGAGTAGTTTTGGAT	CTCAAGTGGCACCACCACAACATCACC
MMP-13	AAATTATGGAGGAGATGCCCATT	TCCTTGGAGTGGTCAAGACCTAA	CTACAACTTGTTTCTTGTTGCTGCGCATGA
MMP-14	AGGCCGACATCATGATCTTCTTT	AAGTGGGTGTCTCCTCCAATGTT	CCATGGCGACAGCACGCCCTT
18S rRNA	CGAATGGCTCATTAAATCAGTTATGG	TATTAGCTCTAGAATTACCACAGTT ATCC	TCCTTTGGTCGCTC GCTCCTCTCCC
PRKD1	TGTATTACCCTCTTTCAGAATGACA	CCAGAGACAAAATTTCAGATAAAGG	Human Probe library (Roche) # 38
PRKD2	AGATGCTCTTCGGCCTAGTG	AGCGCTTGTGGTAGTTCAGC	Human Probe library (Roche) #46
PRKD3	TGATTTAAAGCCAGAAAATGTGC	CGTGCAAATCCAAAGTCACA	Human probe library (Roche) #21
jun	CCAAAGGATAGTGCGATGTTT	CTGTCCCTCTCCACTGCAAC	Human probe library (Roche) #19
c-fos	ABi Assay on demand # Hs00170630_m1		
ATF3	TTTGCCATCCAGAACAAGC	CATCTTCTTCAGGGGCTACCT	Human probe library (Roche) #53
NFATc1	CCAAGGTCATTTTCGTGGAG	GGTCAGTTTTCGCTTCCATC	Human probe library (Roche) #45
BMP-2	GACTGCGGTCTCCTAAAGGTC	AGCCCGCTTCAGGATAGAC	Human probe library (Roche) #57
AXUD1	CCTGCCTGACCGTGACTT	GGAAGCAGCAACGCTAGAAG	Human probe library (Roche) #49
EGR2	TGGTTTCTAGGTGACGAGACG	TGGTTTCTAGGTGCAGAGAGACG	Human probe library (Roche) #3
Interferon-α	CCCTCTCTTTATCAACAAACTTGC	TTGTTTTCATGTTGGACCAGA	Human probe library (Roche) #69
Interferon-Y	TGCTCAGCTTTCACTATTGTTG	TTTCTGGGGGCTTACATGAG	Human probe library (Roche) #34

Table 5. List of primers and probes used for Taqman PCR. Oligonucleotide probes and primers were designed using Primer express 1.0 (Applied Biosystems) or Universal ProbeLibrary Assay Design Centre for probe library probes and primers. The c-*fos* assay is an ABi designed assay on demand.

2.2.12 Cytoplasmic and nuclear extraction

Background

The NE-PER Nuclear and Cytoplasmic Extraction kit from Thermo Fisher Scientific was used to separate cytoplasmic and nuclear fractions. The first two (CER I and CER II) reagent within the kit enables the stepwise lysis of cell membrane and the separation of the cytoplasmic protein from the intact nuclei. The second reagent allows the extraction of nuclear proteins from genomic DNA and mRNA.

Reagents

- Ice cold PBS
- Protease inhibitor
- Nuclear and cytoplasmic extraction kit containing
    - Cytoplasmic Extraction Reagent I (CER I)
    - Cytoplasmic Extraction Reagent II (CER II)
    - Nuclear Extraction Reagent (NER)

Method

HACs were cultured as described in section 2.2.1 with 1 million P0 HACs being plated into 6 cm dishes. Following siRNA gene silence and cytokine stimulation, cells were washed twice with ice-cold PBS on ice. Cells were removed from the plastic using a Corning® cell lifter (Sigma Aldrich) in 1 ml of ice-cold PBS and centrifuged at 500 x *g* for 3 minutes to pellet. The supernatant was discarded. Protease inhibitors were added to each reagent. Amounts of reagent used were dependent on pellet volume, as indicated in *Table 6.*

Ice cold CER I containing protease inhibitors was then added to cell pellet and vortexed for 15 seconds (all vortexing performed at the highest setting) and incubated on ice for 10 minutes. Ice-cold CER II was added and sample vortex for 5 seconds. This was then incubated on ice for 1 minute. The pellet was then vortexed for a further 5 seconds before being centrifuged at 16,000 x *g* for 5 minutes. The supernatant (cytoplasmic fraction) was then transferred to a clean pre-cooled eppendorf and kept on ice. The insoluble pellet was then re-suspended in ice-cold NER and vortexed for 15 seconds. The sample was then placed on ice for 10 minutes. The Eppendorf was repeatedly vortexed every 10

minutes for 40 minutes. The tube was then centrifuge for 10 minutes at 16,000 x *g*. The supernatant (nuclear fraction) was then transferred to a clean pre-chilled eppendorf. Fractions were snap-frozen using dry ice and stored at –80^{o}C until required.

Packed cell volume (µl)	CER I (µl)	CER II (µl)	NER (µl)
10	100	5.5	50
20	200	11	100
50	500	27.5	250

Table 6. Volumes of cytoplasmic and nuclear extraction reagents used in relation to pellet size. Volumes of reagents used varied upon size of cell pellet extracted from scraping cell culture dish.

2.2.13 Subcellular protein fractionation

Background

The subcellular protein fractionation kit from ThermoScientific was used to separate the cytoplasmic, membrane, soluble nuclear, chromatin-bound nuclear and cytoskeletal fractions. The first reagent (CEB) added to the cell pellet causes lysis of the cell membrane, releasing soluble cytoplasmic contents. The second reagent (MEB) dissolves the remaining plasma, mitochondria and ER/golgi membranes. This reagent does not dissolve the nuclear membranes. Intact nuclei are recovered by centrifugation and the third reagent (NEB) lyses the nuclear membrane releasing soluble nuclear extracts. NEB is reused in the extraction of chromatin-bound nuclear protein but with the addition of micrococcal nuclease inhibitor and calcium chloride. Finally PEB is used to extract cytoskeletal proteins.

Reagents

- Ice-cold PBS
- Subcellular Protein Fractionation Kit containing:
    - Cytoplasmic Extraction Buffer (CEB)
    - Membrane Extraction Buffer (MEB)
    - Nuclear Extraction Buffer (NEB)

- Pellet Extraction Buffer (PEB)
- Micrococcal Nuclease (≥ 100 units/µl)
- $CaCl_2$ (100 mM)
- Halt™ Protease Inhibitor Cocktail (100X)

Method

HACs and SW1353 cells were cultured as described in *sections 2.2.1* and *section 2.2.2* in 10 cm dishes, with 3 million HAC and 1 million SW1353 cells being plated. Following cytokine stimulation, cells were washed twice with ice-cold PBS. Cells were scraped into 1 ml ice-cold PBS and centrifuged at 500 x g for 3 minutes to pellet. The supernatant was discarded. Protease inhibitors were added to each reagent. Amounts of reagent used were dependent on pellet volume.

Ice-cold CEB buffer containing protease inhibitors was added to the cell pellet and the tube incubated at 4°C for 10 minutes with gentle mixing. The tube was centrifuged at 500 x g for 5 minutes at 4°C and the supernatant (cytoplasmic extract) transferred to a clean pre-chilled tube on ice. Ice-cold MEB buffer containing protease inhibitors was then added to the pellet and vortexed for 5 seconds on highest setting. The tube was then incubated at 4°C for 10 minutes with gentle mixing and centrifuged at 3000 x g for 5 minutes at 4°C. The supernatant (membrane extract) was transferred to a clean pre-chilled tube on ice. Ice-cold NEB containing protease inhibitors was then added to the pellet and vortexed for 15 seconds on the highest setting. This was then incubated at 4°C for 30 minutes with gentle mixing. The tube was centrifuged at 5000 x g for 5 minutes at 4°C and the supernatant (soluble nuclear extract) transferred to a clean pre-chilled tube on ice. The chromatin-bound extraction buffer was prepared by adding 5 µl 100 mM $CaCl_2$ and 3 µl of Micrococcal Nuclease (300 units) per 100 µl of room temperature NEB and added to the pellet. The tube was vortexed for 15 seconds on the highest setting, incubated at room temperature for 15 minutes and centrifuged at 16000 x g for 5 minutes at 4°C. The supernatant (chromatin-bound nuclear extract) was transferred to a clean pre-chilled tube on ice. Room temperature PEB containing protease inhibitors was added to the pellet, vortexed at the highest setting for 15 seconds and incubated at room temperature for 10 minutes. The tube was centrifuged at

16000 x g for 5 minutes at 4^{o}C and the supernatant (cytoskeletal extract) transferred to clean tube. Fractions were snap frozen and stored at –80^{o}C until required.

2.2.14 Whole cell lysis

Background

To extract cellular proteins for western blotting, cells membranes were lysed to extract all cellular proteins. Numerous protease inhibitors were used to prevent protein degradation and protein modifications.

Reagents

- Lysis buffer **:** 50 mM Tris, pH 7.5, 10% (v/v) glycerol, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM 2-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 1 µM microcystin-LR; 0.1% (v/v) 2-mercaptoethanol, 1x protease inhibitor cocktail (Roche).
- Ice cold PBS

Method

Cells were harvested after stimulation at appropriate time points. Cells were washed 2x in ice cold PBS on ice. 120 µl of lysis buffer was added and cells scraped using Corning® cell scrapers (Sigma Aldrich) and added to Eppendorfs. Cells were vortexed at highest setting for 20 seconds and then left on ice for 20 minutes for complete lysis. Cells were then centrifuged for 3 minutes at full speed to remove cytoskeletal debris. Total cell protein lysate were then snap frozen on dry ice and stored at -80°C until use.

2.2.15 Protein Quantification

Background

The Bradford Assay is a colorimetric protein assay which measures protein concentration based on an absorbance shift in the Coomassie protein dye when bound to protein. A standard curve is generated by diluting BSA stock solution in the lysis buffer used for extraction; this is used to estimate the protein concentration of cellular lysates.

Reagents

- 2 mg/ml stock of BSA protein standard
- Bradford assay reagent

Method

A BSA protein standard curve was generated by diluting the BSA stock solution in lysis buffer, with concentrations ranging from 0-4 mg/ml, with 0.4 mg/ml incremental increases. To the diluted BSA or extracted proteins 150 µl of Bradford Ultra (Expedeon, UK) was added to each sample in a 96 well plate. Following a 5 minute incubation, absorbance was read at 595 nm using a Tecan Sunrise microplate absorbance reader (Tecan, Reading, UK). Protein concentration was calculated using the standard curve generated from the BSA standards. All protein samples were then normalised using lysis buffer.

2.2.16 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Background

Protein containing samples, generated from cell lysates that were left whole, subjected to subcellular protein fractionation or nuclear and cytoplasmic protein extraction were separated based on size by SDS-PAGE. This was performed under reducing conditions. Acrylamide was polymerised by the addition of ammonium persulphate (APS) and the base TEMED. TEMED promotes the release of persulphate ions from the APS, allowing the free radicals to initiate the polymerisation of acrylamide. Samples were prepared by boiling in Laemmli loading buffer. This contained β-mercaptoethanol, SDS and the tracking dye Bromophenol blue. β-mercaptoethanol reduces disulphide bonds and prevents the oxidation of cysteines, reducing any tertiary or quaternary structures within the protein samples. SDS is an anionic detergent that denatures protein by disrupting non-covalent bonds, wrapping around the polypeptide backbone at a ratio 1.4 g SDS/g protein. SDS masks any positive charge from the protein and as SDS is an anionic detergents that gives the protein a net negative charge per unit mass. Samples are then separated by utilising two different gel types; the stacking and resolving gels. The stacking gel is the upper gel and this gel concentrates the protein into a sharp band before entering the lower separating gel. This is achieved by utilising the differences between pH and ionic strength

of the different gels as well as the electrophoresis buffer. The pores size of the stacking gel is large allowing free movement of all protein. When charge is applied across the gel, negatively charge glycinate ions, within the electrophoresis buffer, move away from the cathode towards the sample and stacking gel. As they enter the stacking gel the pH reduces so that their velocity decreases. Inside the stacking gel, negatively charged chloride atoms are also moving away from the cathode, these have a higher velocity then the slowed glycinate ions. This creates a tight band in which the sample is concentrated within the glycinate and chloride boundaries. This tight, negatively charged band then moves towards the anode. When this band enters the higher pH of the resolving gel the glycine becomes deprotonated and the mobility of the glycinate ions increase. The protein mobility decreases due to no longer being within this tight negatively charged band. The protein can then be separated upon size, due to changes in mobility due to protein size.

Reagents

- 4 x Lower gel buffer (LGB): 1.5 M TrisHCl pH 8.8, 0.4% (w/v) SDS.
- 4 x Upper gel buffer (UGB): 0.5 M TrisHCl pH 6.8, 0.4% (w/v) SDS.
- Stacking gel: 40% bis/acrylamide diluted to 4.5% with water and 4 x UGB.
- 5 x Laemmli Final sample buffer (FSB): 0.625 mM Tris HCl pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol.
- 10 x Running Buffer: 250 mM Tris, 2 M glycine, 10% (w/v) SDS.
- PageRuler pre-stained protein standards (Thermo Scientific)

Method

Cells were lysed as described in sections 2.2.12, 2.2.13 and 2.2.14. The protein concentration was then quantified as described in section 2.2.15. Samples were then normalised so that maximal amount of protein was ran on each gel. Samples were made to 100µl using DNAse/RNAse free water if required. 20µl of 5x FSB was added to samples and then heated at 105°C for 5 minutes. Electrophoresis was performed in a Bio-Rad Mini-PROTEAN® Tetra Cell using 1.0 mm separator plates. For resolving gel, polyacrylamide-bis-acrylamide 40% (w/v) (37.5:1 acrylamide:bis) solution, was diluted with water and 4 x LGB to the

required percentage. Volumes dependent on number of gels required and percentage volume (gels cast at 10% unless otherwise stated). For one 10% gel 3 ml of dH_2O, 1.5 ml of acryl/bis and 1.5 ml of LGB was used. Gel mixture was polymerised by the addition of TEMED and APS 0.2% (w/v) at a ratio of 1:3 prior to plate pouring (5 µl: 15 µl per gel). A spacer plate and short plate were combined within a casting frame and checked to ensure a seal had formed. The gel was then poured. The lower gel was then overlaid with isopropanol ensure a flat gel surface is achieved and oxygen is expelled, due to oxygen inhibiting polymerisation of the gel. Once set, the isopropanol was washed off using deionised water. The 4.5% bis/acrylamide stacking gel was then polymerised, again using TEMED and APS at a ratio of 1:3. This was laid on top of resolving gel and combs inserted. Once set the gel kit was assembled into the tank and combs removed. The tank and wells were then filled with 1x Running Buffer. Wells were washed in buffer to remove any waste gel. 3.5µl of the molecular weight marker PageRuler pre-stained protein standards was loaded in one well of each gel. 20µl of boiled sample was then added to the wells of the gel as appropriate. Proteins were electrophoresed at 100 mV through the stacking gel, to ensure proteins stacked. The voltage was then increased to 150 mV when proteins entered the resolving gel. Proteins were electrophoresed until the dye front reached the end of the resolving gel.

2.2.17 Western blotting

Background

Following SDS-PAGE, proteins which had been separated by electrophoresis were transferred onto polyvinylidene fluoride (PVDF) membrane. Proteins embedded within the polyacrylamide gel are transferred from the gel onto the PVDF membrane by applying a current across both. The current draws the negatively charged proteins onto the membrane and the proteins bind the membrane through electrostatic and hydrophobic interactions. PVDF is more robust and easier to manipulate compared to nitrocellulose membranes. PVDF membranes can also be stripped, allowing the membranes to be re-probed later. To ensure that specific bind of the primary antibody occurs, the membrane must first be ‘blocked’. This consists of incubating the membrane in skimmed milk protein; this blocks any pores that are not occupied by protein, ensuring

specific binding on the primary antibody. Once blocked, the blot is incubated with a primary antibody that has affinity for the protein of interest. A secondary antibody conjugated to horseradish peroxidase (HRP) is then added; this has high affinity for the primary antibody. The conjugated HRP can then cleave a chemi-luminescent substrate found within a developing solution. This emits light which can be detected, indicating protein levels.

Reagents

- Tris Buffered Saline- Tween (TBS-T): 10 mM TrisHCl pH 7.4, 0.15 M NaCl. 0.2% Tween-20
- Blocking buffer: TBS-T and 5% (w/v) non-fat dry milk
- Stripping buffer: 0.2 M glycine and 2% (w/v) SDS, pH 2.5
- ECL/ ECL prime or Advanced Western Blotting Detection systems according to manufactures instruction (Amersham ™ GE Healthcare UK Ltd)
- ECL plus (MerckMillipore)

Method

The semi-dry iBlot gel transfer system (Life Technologies, Paisley, UK) was used to transfer protein onto PVDF membrane. The kit was used as manufacturer's instruction, at a voltage 25 V of for 7 minutes. Here, the polyacrylamide gel was placed on the lower PVDF containing component. This contained a bottom buffer membrane and copper anode. On top of this was placed filter paper which had been dampened in deionised water. On this was placed the upper buffer membrane which contained a copper cathode. After transfer the membrane was placed in methanol and then deionised water to ensure membrane integrity.

After protein transfer the PVDF membranes were then blocked for 1 hour at room temperature in blocking buffer, with agitation. Blocking solution was then removed and membrane was washed for 5 minutes in TBS-T to remove excess blocking solution. The membrane was then incubate with the primary antibody which had been diluted in TBS-T containing 5% BSA, overnight at 4°C with agitation. The membrane was then washed 3 x 15 minutes and incubated with secondary antibody diluted in TBS-T containing 5 % (w/v) non-fat dried milk for

1 hour at room temperature with agitation. The secondary antibody was discarded and the membrane was washed 3 x 15 minutes in TBS-T. Proteins were then visualised using western blotting detection systems according to manufacturer's instruction. Membranes were visualised using a Syngene G-box detection system. Membranes were stripped and re-probed with antibodies that differed in size to original target, as well as a house keeper gene to determine equal protein loading.

2.2.18 Statistical analysis

Relative levels of gene expression were normalised to the housekeeping gene 18S rRNA gene expression. Data were normalised and plotted as fold induction of target gene expression over basal levels. Data analysed using the $\Delta\Delta C_t$ method. Values are pooled from at least 3 experiments and given as a mean of these data. Standard error of the mean was used (SEM). ANOVA (analysis of variance) with a post hoc Bonferroni was used to analyse significance between independent sample groups. Student one-tailed t-test were used to analyse optical density of western blots, standard deviation (STD) was used. Levels of statistical significance are shown as * $p \leq 0.05$, ** $p \leq 0.01$ and *** ≤ 0.001.

3 Chapter 3: The characterisation of lentiviral-mediated gene silencing and optimisation of the over-expression and siRNA-mediated gene silencing of PKD in human articular chondrocytes

3.1 Introduction

The techniques of RNA interference (RNAi) and over-expression are two powerful molecular biological tools which can be utilised to alter the expression of endogenous mRNA and protein. Both methods can be used to help understand the role of a gene of interest in a particular system. These techniques have been applied for a number of years to understand the role of protein kinases within signalling cascades under cytokine stimulation (Dorsett and Tuschl, 2004). In this chapter I set out to utilise these techniques within primary chondrocytes so that the role of each isoform of PKD can be studied, specifically in the regulation of MMP gene expression under the pro-inflammatory cytokine stimuli of IL-1 in combination with OSM.

In this study two major types of RNAi have been deployed; siRNA- and shRNA-mediated gene silencing. As described in *section 2.2.4* , siRNA and shRNA both exploit the same intrinsic machinery to decrease the expression of a target gene, but differ in the manner in which they are synthesised and introduced into the cell.

siRNAs are double-stranded RNA sequences which are around 20-25 nucleotides in length. siRNAs can be chemically synthesized as double-stranded oligos, which can be transfected into the cell in this form. Design of the siRNA so that it is specific and has no off-target effects is crucial, with many key rules to be followed to enhance specificity (Reynolds et al., 2004). shRNAs on the other hand are hairpin structures, consisting of a stem region of paired antisense and sense strands connected by 7-9 unpaired nucleotides, which form a loop structure. shRNAs can be modified to increase their expression and processing, by making similar to that of a microRNA (miRNA). Modelling them on miRNA adds an additional Drosha processing site to the hairpin construct;

this can increase the gene silencing efficiency of the shRNA (Boden et al., 2004). Adding miRNA flanking sequences either side of the hairpin has also been shown to lead to a 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA design (Silva et al., 2005). All these additions lead to increased chances of gene silencing as increased processing of the shRNA into a siRNA is observed.

To achieve insertion of the siRNA or shRNA into the cell, different techniques can be utilised. siRNA and shRNAs can be directly transfected (non-viral method of insertion) into the cell using multiple methods. These include the use of calcium phosphate, electroporation, cationic polymers, liposomes and other transient transfection methods. This transient transfection directly inserts the siRNA or shRNA into the cell, allowing the RNA to be processed into a single stranded siRNA.

shRNAs on the other hand can be introduced into a cell using two different methodologies. Firstly, they can be transiently transfected into a target cell by a method as described above. Secondly, shRNA can be incorporated into the cell by viral transduction. Recombinant retroviruses are widely used as gene therapy vectors due to their ability to integrate a transgene into the genome of a target cell (Buchschacher and Wong-Staal, 2000). This allows the stable integration of a shRNA into the genome of the infected cell. The expressed shRNA can then be processed by the cell into an active siRNA.

Lentiviruses are one genus of the retroviridae family. One of the many benefits of using lentiviruses over other family members is that lentiviruses have the ability to transduce non-dividing cells (Freed and Martin, 1994), allowing greater transduction capabilities. Lentiviral mediated gene silencing utilises the ability of the virus to encompass a shRNA transcript. The virus can then integrate the gene of interest into the host genome, leading to stable expressing of the shRNA (Sakuma et al., 2012). This results in the long term expression of the transgene. siRNAs can therefore be produced from the shRNAs under the control of Pol III promoters (Brummelkamp et al., 2002); a U6 promoter is most commonly used. The benefit of this method over transient siRNA transfection, is that the cell constantly expresses the shRNA.

Lentiviral-mediated shRNA transduction had not previously been conducted within this laboratory and has only been assessed by a handful of other researchers when trying to transduce HAC (Miot et al., 2010, Li et al., 2004, Gouze et al., 2002). Development of this technique was therefore needed.

As well as the use of RNAi and gene silencing to study the function of a gene of interest, the effect of over-expression of a gene can also be studied. Over-expression of a mutated or native gene can give a similar insight into gene function as that of gene silencing. This method can be utilised by studying the effects of over-expressing a protein on downstream effector molecules and comparing this data to cells expressing normal protein levels. These data can be used by themselves or used to validate data seen with gene silencing, as the opposite effect should be observed. In this study I use the over-expression of native genes to understand their role in the signalling cascade which induces MMP gene expression through the stimulation of IL-1 in combination with OSM.

In this chapter I therefore developed the molecular biological techniques capable of either reducing or increasing the expression of each individual isoform of PKD. This would ensure that any further findings within this thesis could be seen to be specific to each individual PKD isoforms.

3.2 Aims

- Optimisation of gene specific siRNA-mediated PKD isoform silencing
- Optimisation of production of lentiviral particles
- Optimisation of lentiviral transduction and PKD1 gene silencing
- Optimisation of PKD1 over-expression in SW1353s and HAC

3.3 Results

3.3.1 Optimisation of PKD gene silencing using multiple siRNAs

siRNA-mediated gene silencing is a well-established technique for reducing the expression of the mRNA and protein of a target gene. In this work I set out to understand the role of each isoform of PKD in the regulation of collagenase gene expression. Being able to specifically reduce the protein level of each PKD isoform was therefore crucial in this understanding. To achieve this, siRNA was first used. Initial optimisation experiments were performed to establish the

optimum concentration of siRNA and time point needed to achieve the maximal gene silencing. Concentrations of 25, 50, 75 and 100 nM were used at time points of 24, 48, 72 and 95 hours. Here, a final concentration of 100 nM of siRNA added for 48 hours was found to be the optimum concentration and time point for gene silencing.

3.3.1.1 siRNA mediated gene silencing of PKD1

To establish the specificity and capacity of each siRNA, in the silencing of each isoform of PKD, the effect of each siRNA on the mRNA and protein expression of each PKD isoform was assessed. To find specific and effective siRNAs a total of 9 siRNAs, with 3 targeting each isoform of PKD were used. To assess changes in mRNA and protein levels, RT-PCR and western blotting were used.

Figure 3.1.A shows that the PKD1 siRNAs #1, #2 and #3 all reduce the mRNA expression of PKD1 by 55%, 68% and 53% respectively (*Figure 3.1. A*). These data suggest that each siRNA is significantly reducing the mRNA levels of PKD1 within HAC. To establish the specificity of each PKD1 siRNA, the effect of each siRNA on the gene expression of PKD2 and PKD3 was examined. No effects on the gene expression of PKD2 or PKD3 were seen when cells were treated with these siRNAs (*Figure 3.1. B and C*).

When examining the effects of each siRNA targeting PKD1 on the protein expression of PKD1, no changes to the protein expression of PKD1 was observed (*Figure 3.1. D*). These data suggest all 3 of the siRNAs were capable of silencing PKD1 at the mRNA level, but not at the protein level. To establish protein silencing of PKD1 further siRNAs were used (ON-TARGETplus siRNA were purchased and targeted PKD1: siRNA codes L-005028-00-0005, l-004197-00-0005, L-005029-00-005). These, similar to the data shown here were either unspecific or had no effect on the protein expression of PKD1 (data not shown).

3.3.1.2 siRNA mediated gene silencing of PKD2

Figure 3.1. B shows that the PKD2 siRNAs #1, #2 and #3 all reduce the mRNA expression of PKD2 by 59%, 72% and 55% respectively (*Figure 3.1. B*). These data suggest that each siRNA significantly reduced the mRNA levels of PKD2 within HAC. These data also confirm that each siRNA was specific, with no

effects on the gene expression of PKD1 or PKD3 being observed with these siRNAs (*Figure 3.1. A and C*). This shows specificity of the siRNA against each isoform, indicating these siRNAs to be effective tools for specifically silencing PKD2 at the mRNA level.

When examining the effect of each PKD2 siRNA on the protein level of PKD2, marked reduced expression of PKD2 is observed (*Figure 3.1. D*). These data indicated that the PKD2 siRNA #3 had the greatest and most consistent silencing of PKD2 at the protein level. For this reason PKD2 siRNA #3 was used in all following experiments in this thesis.

3.3.1.3 siRNA mediated gene silencing of PKD3

Figure 3.1. C shows that the PKD3 siRNAs #1, #2 and #3 all reduce the mRNA expression of PKD3 by 50%, 48% and 48% respectively (*Figure 3.1. C*). When examining the specificity of each PKD3 siRNA (*Figure 3.1. A and B*), it was observed that PKD3 siRNA #3, also reduced the gene expression of PKD1. These data therefore indicate this siRNA to be non-specific and could therefore not be used in this study. The PKD3 siRNAs, #1 and #2, were seen to be specific and therefore effective tools for specifically silencing PKD3 at the mRNA level.

When examining the effects of each PKD3 siRNA on the protein level of PKD3 a clear reduction in the protein expression of PKD3 is seen (*Figure 3.1. D*). HACs treated with the PKD3 siRNAs #1, #2 and #3 all have reduced protein expression of PKD3. All 3 siRNAs reduce the level of PKD3 to a similar degree but due to the lack of specificity of #3, therefore it was decided that PKD3 siRNA #2 would be used for all subsequent experiments.

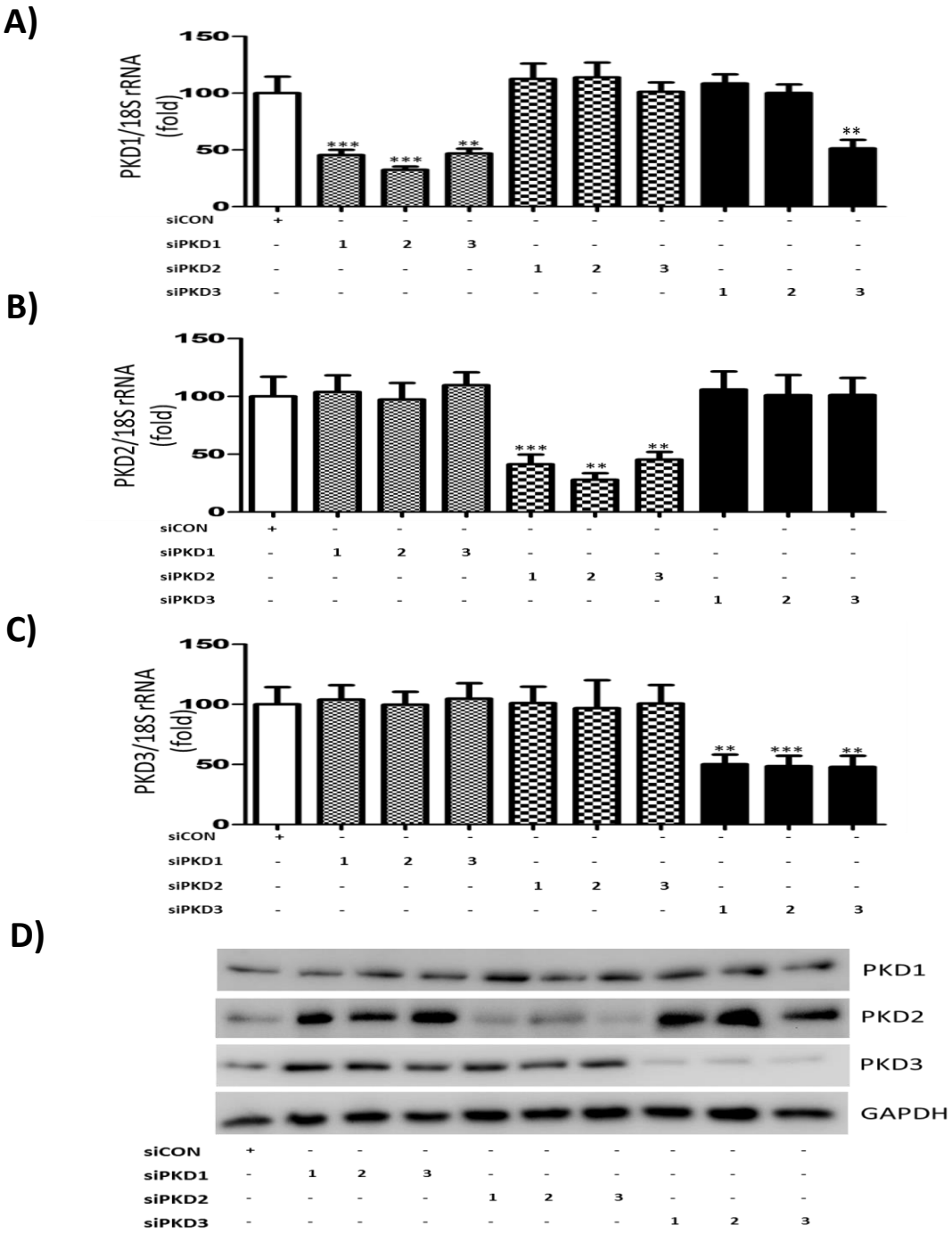


Figure 3.1. siRNA mediated gene silencing of each isoform of PKD. P1 HAC in 96 well plates were treated with 9 individual siRNA specific to PKD1, PKD2 or PKD3 (3 siRNAs per isoform) or non-targeting siCON (100 nM) for 48 hours. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *PKD1*, (B*) PKD2* and (C) *PKD3*, as described in the Materials and Methods. Data plotted are the mean ± S.E.M. of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control, transfection, where PKD expression in control was normalised to 100% expression. ***, p≤0.001, **, p≤0.01 vs siCON. (D) P1 HAC in 6 well plates were treated as above. Cell lysates were then immunoblotted using antibodies against PKD1, PKD2 and PKD3, as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

3.3.2 Lentiviral characterisation and optimisation

As described in *section 3.3.1.1*, the silencing of PKD1 at the protein level was unsuccessful. To try and silence PKD1 at the protein level multiple, siRNAs and varied experimental conditions were used. These changes and the use of new reagent still failed to silence PKD1 at the protein level. To overcome this, it was decided a new approach to reduce the protein expression of PKD1 would be utilised. It was decided that lentiviral mediated shRNA gene delivery would be used. The production of lentiviral particles containing shRNA had not previously been performed within this laboratory. This technique therefore needed to be characterised, with the production of viral particles needing to be optimised as well as the transduction of HAC. The purpose of this was to establish lentiviral mediated delivery of shRNA, using virus produced in-house and capable of silencing PKD1 at the protein level.

3.3.2.1 Initial production of GFP containing lentivirus

The production of lentiviral particles was first established. Here, using a second generation packaging and envelope plasmid system, the production of lentiviral particles was optimised. Initial lentiviral particles were produced using a plasmid which expressed a GFP protein. Transduced SW1353 cells or HAC could therefore by identified by GFP expression using live cell imaging. This ensured that living, transduced, cells could be identified. As *Figure 3.2* shows, when lentiviral particles were generated using the method described in *section 2.2.4*, viral particles were produced capable of transducing both SW1353 cells and HAC, although HAC did not transduce as readily as SW1353 cells..

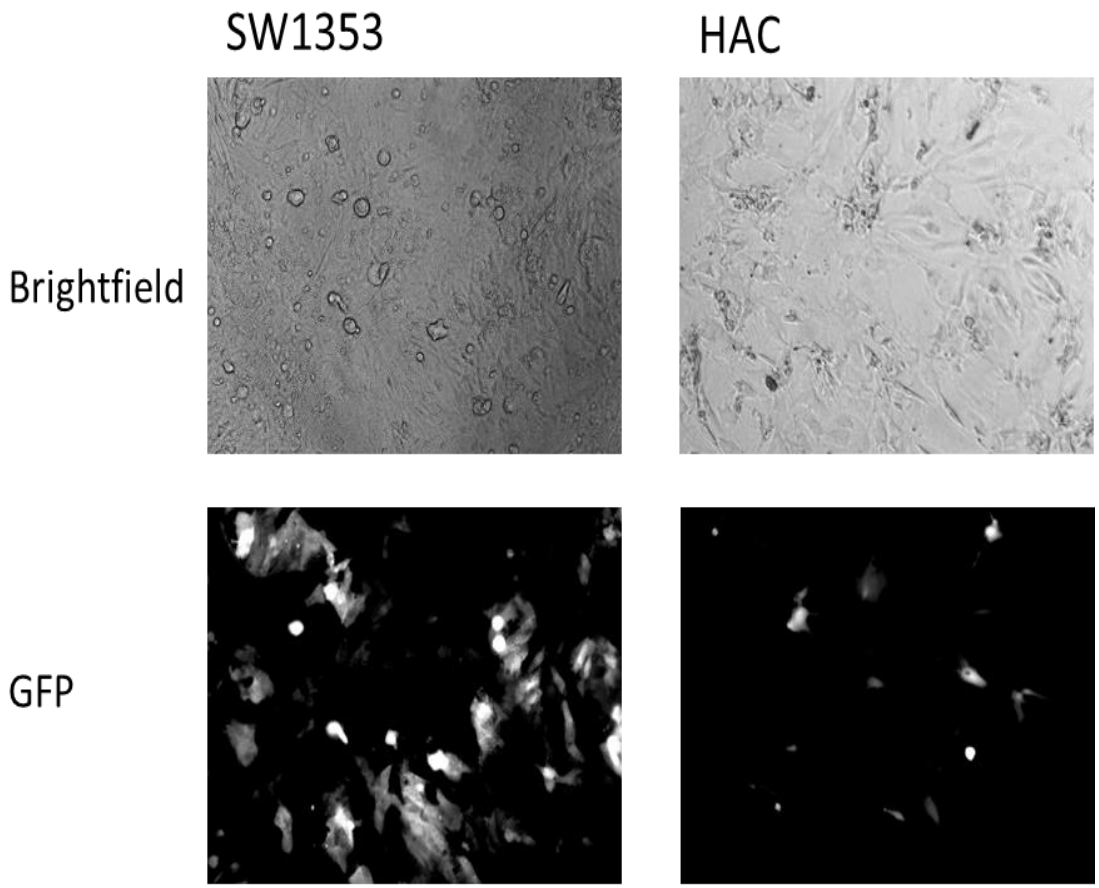


Figure 3.2. Characterisation of lentiviral transduction of SW1353 cells and HAC using confocal microscopy. SW1353 cells or P0 HAC were plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 100 µl of viral supernatant total. Supernatant was replaced after 24 hours and cells were visualised after a further 48 hours. Levels of transduction were equated to expression of GFP protein. The bright field image indicates the cells present, the GFP image indicates the levels of GFP .

3.3.2.2 Polybrene (hexadimethrine bromide) increases viral transduction

Polybrene is a cationic polymer which increases the efficiency of viral transduction by neutralising the negative charge of the virion and cell membrane. Polybrene has therefore been shown to increase viral transduction at varying concentrations (Davis et al., 2004). Initial experiments were performed to find the optimum concentration of Polybrene to yield the greatest transduction of HAC cells. To achieve this, Polybrene was added to cells at the same time point as transduction at a range of 2-10 µg/ml. After observing the number of GFP-expressing cells between each condition and further insight from the literature, a concentration of 8 µg/ml was decided on as the optimum concentration of Polybrene (*Figure 3.3*).

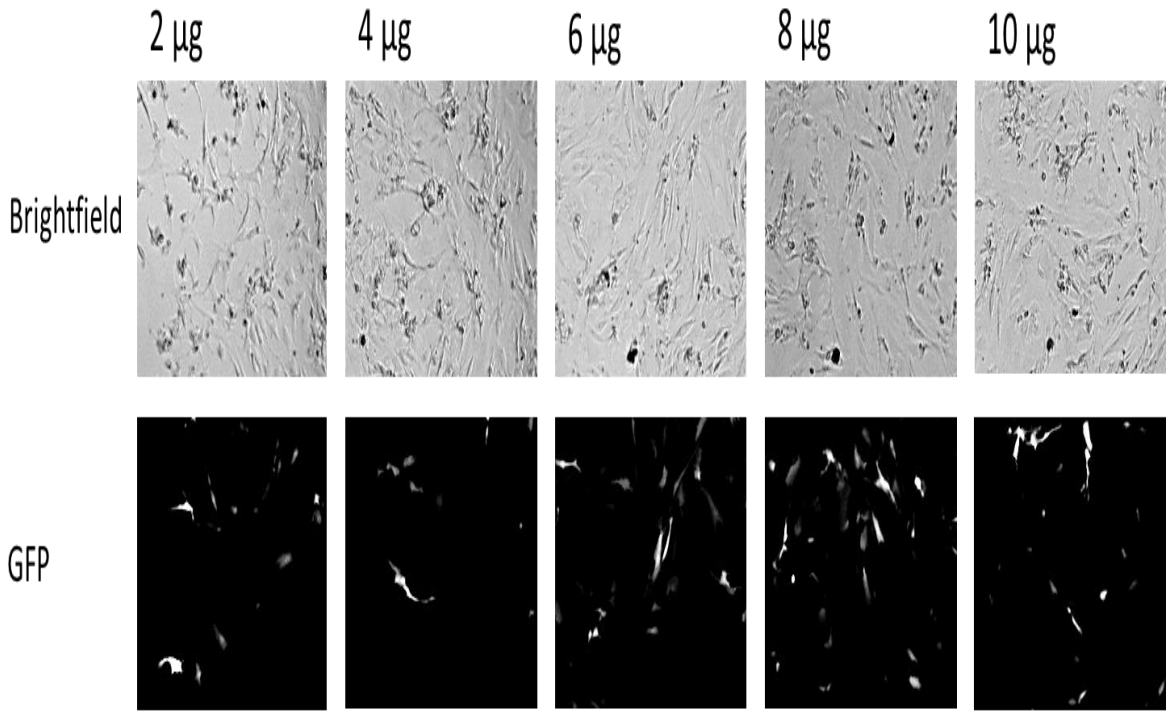


Figure 3.3. Characterisation of the optimum Polybrene concentration in HAC. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 100 µl of viral supernatant total. To this supernatant varying concentrations of Polybrene were added (ranging from 2-10 µg/ml). Supernatant was replaced after 24 hours and cells were visualised after a further 48 hours. Levels of transduction were equated to expression of GFP protein. The brightfield image indicates the cells present and the GFP image indicates the levels of GFP.

3.3.2.3 Lentiviral concentration

Initial viral preparations showed poor transduction of both HAC and SW1353 cells, indicated by the low GFP expression. To increase viral transduction, increased volumes of the viral preparations were used, and moderate increases in the amount of GFP expression were observed (data not shown). Therefore, to increase lentiviral transduction further, lentiviral viral preparations were concentrated following production. This was performed using the Clontech Lenti-x concentrator kit as described in *section 2.2.3*. Viral preparations were concentrated 50-fold, allowing for an increase in the number of viral particles added per well. Once concentrated, viral supernatant was added at varying volumes to both HAC and SW1353 cells (data not shown). *Figure 3.4* represents the increase in viral transduction observed with the concentrated viral particles.

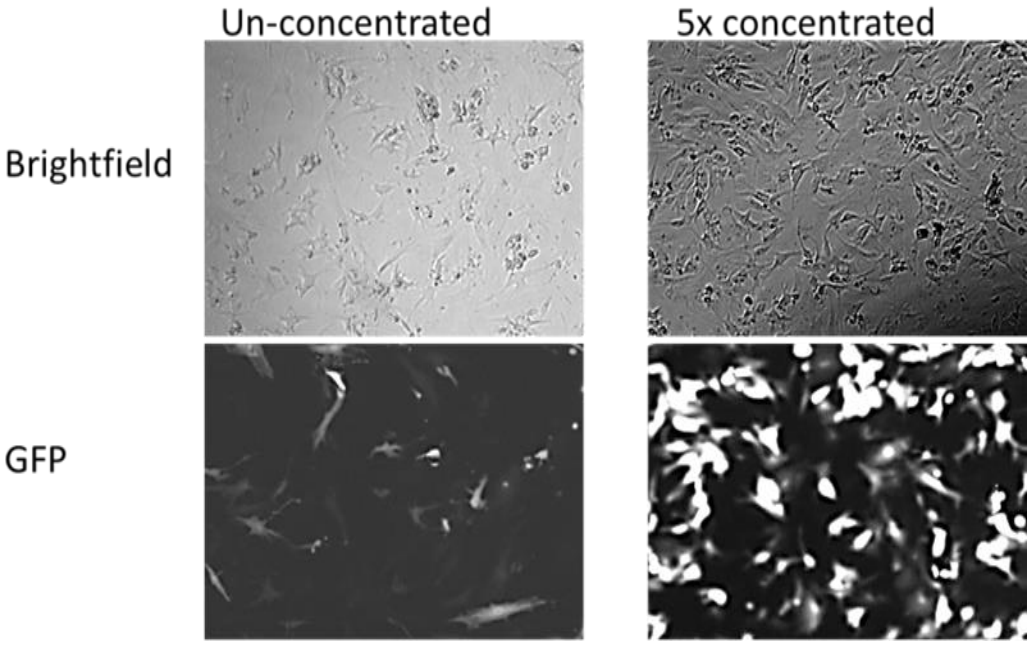


Figure 3.4. The effect of lentiviral concentration on lentiviral transduction in HAC. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with either 100 µl of viral supernatant total or 10 µl of concentrated lentiviral supernatant in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replaced after 24 hours and cells were visualised after a further 48 hours. Levels of transduction were equated to expression of GFP protein. The bright field image indicates the cells present and the GFP image indicates the levels of GFP .

3.3.2.4 Centrifugation of cells transduced with lentiviral particles

To further optimise the transduction of HAC the technique of spinoculation was used. This technique utilises centrifugation to increase transduction efficiency, this technique has been shown to dramatically increase the transduction of difficult to transfect cells (O'Doherty et al., 2000). However, this effect is not always replicated (Strang et al., 2005). As *Figure 3.5* shows, centrifugation of HAC post addition of viral supernatant had no positive effect on the number of cells transduced.

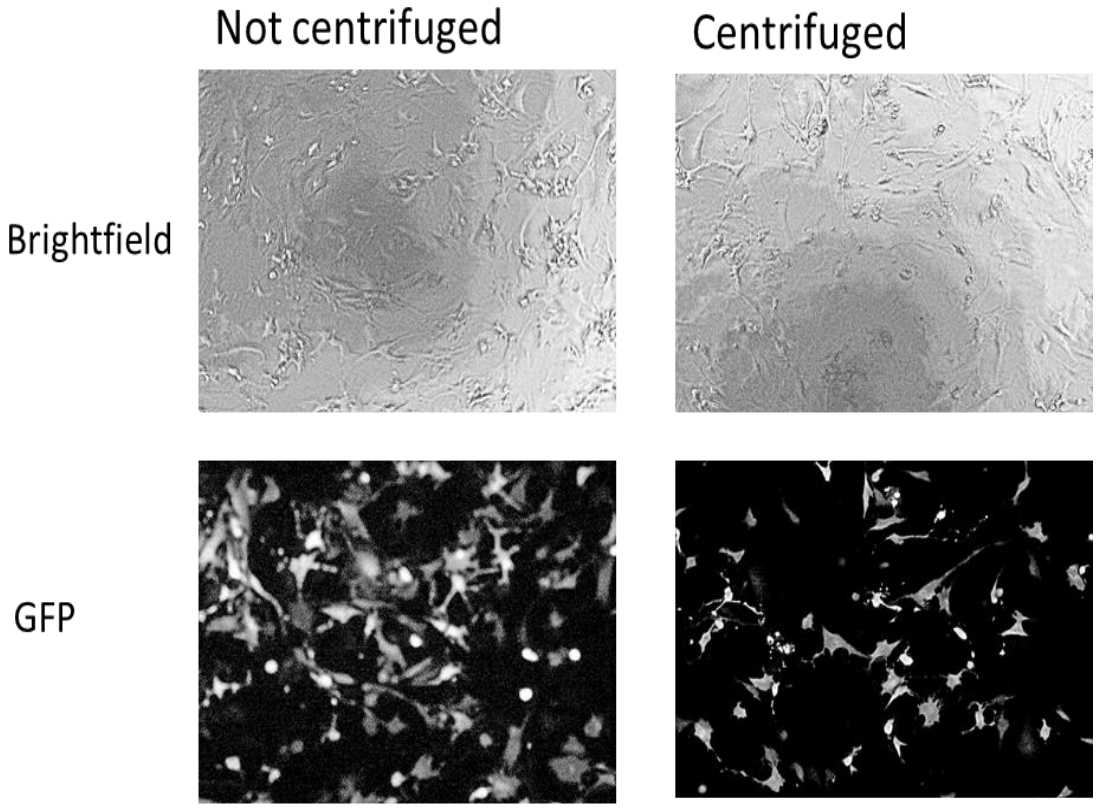


Figure 3.5. The effect of centrifugation on lentiviral mediated transduction of HAC. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 10 µl of concentrated lentiviral supernatant in a total of volume of 100 µl of SFM. Polybrene at a concentration of 8 µg/ml was then added. Cells were then left or centrifuged at 1200 *g* for 60 minutes at room temperature. Supernatant was replaced after 24 hours and cells were visualised after a further 48 hours. Levels of transduction were equated to expression of GFP protein. The bright field image indicates the cells present and the GFP image indicates the levels of GFP.

3.3.2.5 Time course of transduction

Once it was established that lentiviral preparations were capable of transducing both HACs and SW1353 cells, achieving this using lentiviral preparations containing a shRNA which targeted PKD1 was optimised. shRNA expression plasmids were therefore purchased from Open Biosystems; these were TRIPZ-based PKD1 shRNA plasmids. These plasmids use a tet-on operator, in which doxycycline must be present for the promoter of the shRNA to become active and expression of the shRNA to occur. This operator contains a tetracycline response element (TRE) and a transactivator (rtTA3). The transactivator binds to the TRE promoters in the presence of doxycycline leading to expression of the shRNA. These plasmids facilitated the expression of RFP instead of GFP, but only when the shRNA was being expressed. Viral preparations containing these shRNAs were produced. To optimise the experimental conditions needed to achieve maximal expression of the shRNA the cell line SW1353 were first used. These cells were readily available in large quantities, unlike HAC. As the presence of doxycycline was needed to induce the expression of the shRNA, the optimum time point needed for maximal transduction and shRNA expression was established. A time course study was therefore performed. Here, using the expression of RFP as an indicator of shRNA expression, the number of RFP positive cells was observed. The same well of a 96 plate was examined for up to 72 hours, and from this it was established that cells left for 72 hours achieved a maximal transduction (*Figure 3.6*), with no increase in viral transduction observed in cells left beyond this time (data not shown).

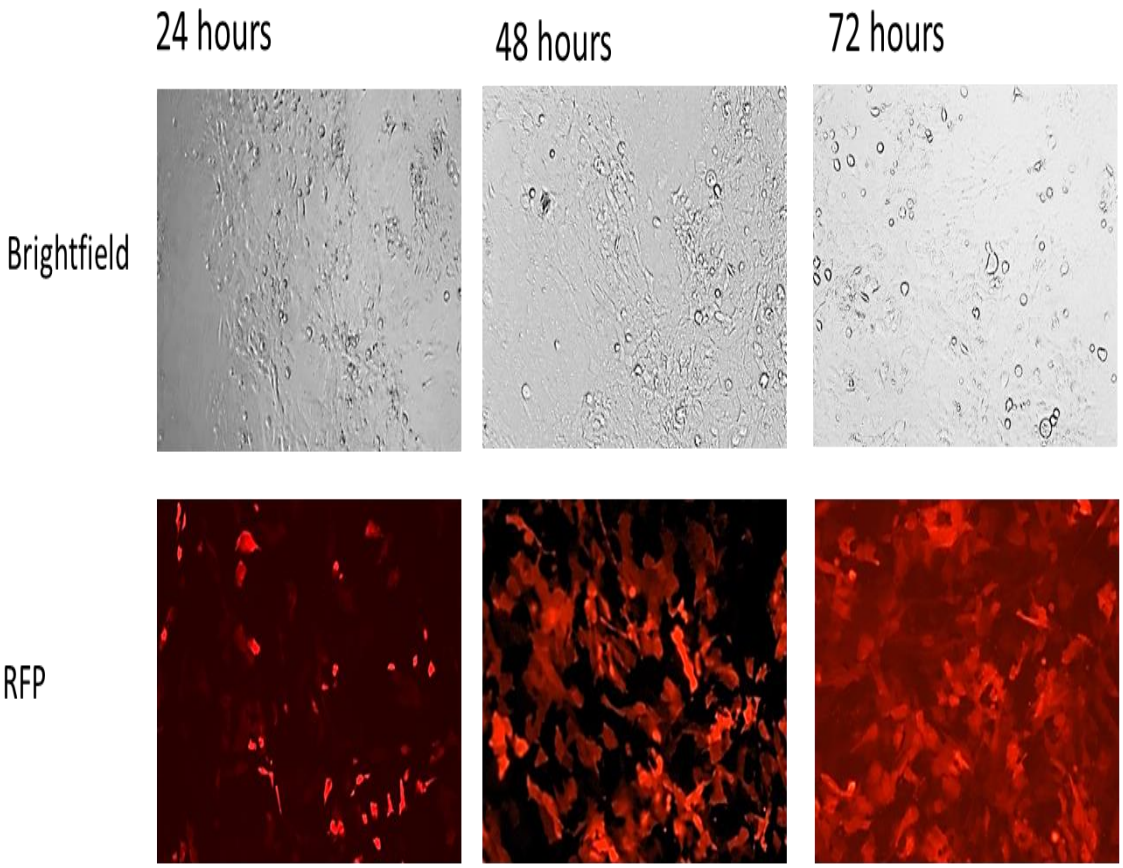


Figure 3.6. Optimum time point for maximal viral transduction in SW1353 cells. SW1353 cells were plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 5 µl of concentrated lentiviral supernatant in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replaced after 8 hours with serum containing medium which also contained 2 µg/ml of doxycycline. Cells were then repeatedly visualised every 24 hours for a further 72 hours. Levels of shRNA expression were equated to expression of RFP protein. The bright field image indicates the cells present and the RFP image indicates the levels of RFP .

3.3.2.6 Doxycycline concentrations

To further optimise the expression of the shRNA, the optimum concentration of doxycycline was assessed. To achieve this, a concentration response experiment for doxycycline was set up. Here cells were treated for 72 hours post viral treatment with 0-3 µg/ml of doxycycline. No doxycycline was used as a control, to confirm that the promoter was not leaky and doxycycline was needed to express the shRNA. As *Figure 3.7* shows, a concentration of 3 µg/ml of doxycycline induced the greatest expression of the RFP, indicating the greatest expression of the shRNA. Higher concentrations of doxycycline were used but no further increase in RFP expression was observed (data not shown)

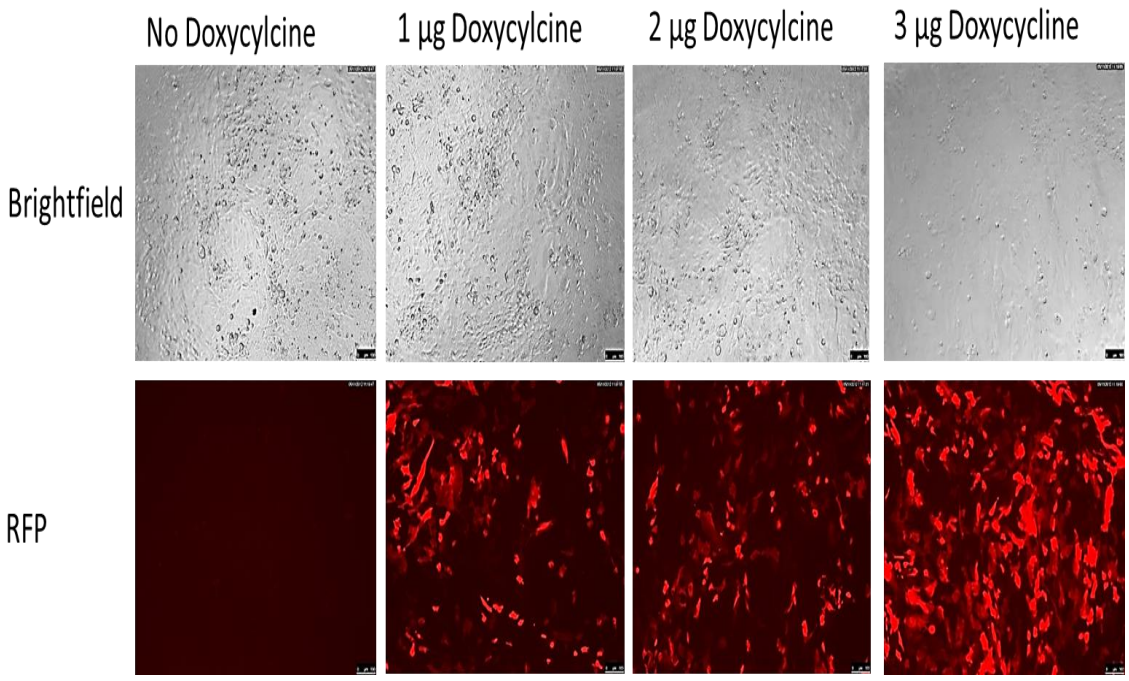


Figure 3.7 The effect of different concentrations of Doxycycline on RFP expression in SW1353 cells. SW1353 cells were plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 5 µl of concentrated lentiviral supernatant in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replaced after 8 hours with serum containing medium which also contained either no, 1, 2 or 3 µg/ml of doxycycline. Cells were then visualised after 72 hours. Levels of shRNA expression were equated to expression of RFP protein. The bright field image indicates the cells present and the RFP image indicates the levels of RFP.

3.3.2.7 Lentiviral mediated TRIPZ shRNA gene silencing in SW1353 cells

Once it was established that SW1353 cells could be transduced to around 80-90%, with tet-on shRNAs, the effect of these shRNA on the gene expression of PKD1 were assessed. Using three different viral preparations, each containing 1 of the 3 shRNAs, gene silencing of each isoform of PKD was examined. As *Figure 3.8. A* shows, when doxycycline is not present there is no effect on the gene expression of PKD1. The effect of each shRNA in the presence of doxycycline was then assessed. As *Figure 3.8. B* shows, PKD1 shRNA #1, #2 and #3 reduced PKD1 mRNA expression by 65%, 54% and 52%, respectively, when compared to cells transduced with a scrambled shRNA. To confirm the specificity of each shRNA, the effect on PKD2 and PKD3 gene expression was examined; the shRNAs had no effect on the other two isoforms *(Figure 3.8. C* and *D*). These data indicated that all 3 of the shRNAs were specific and effective at silencing PKD1 only.

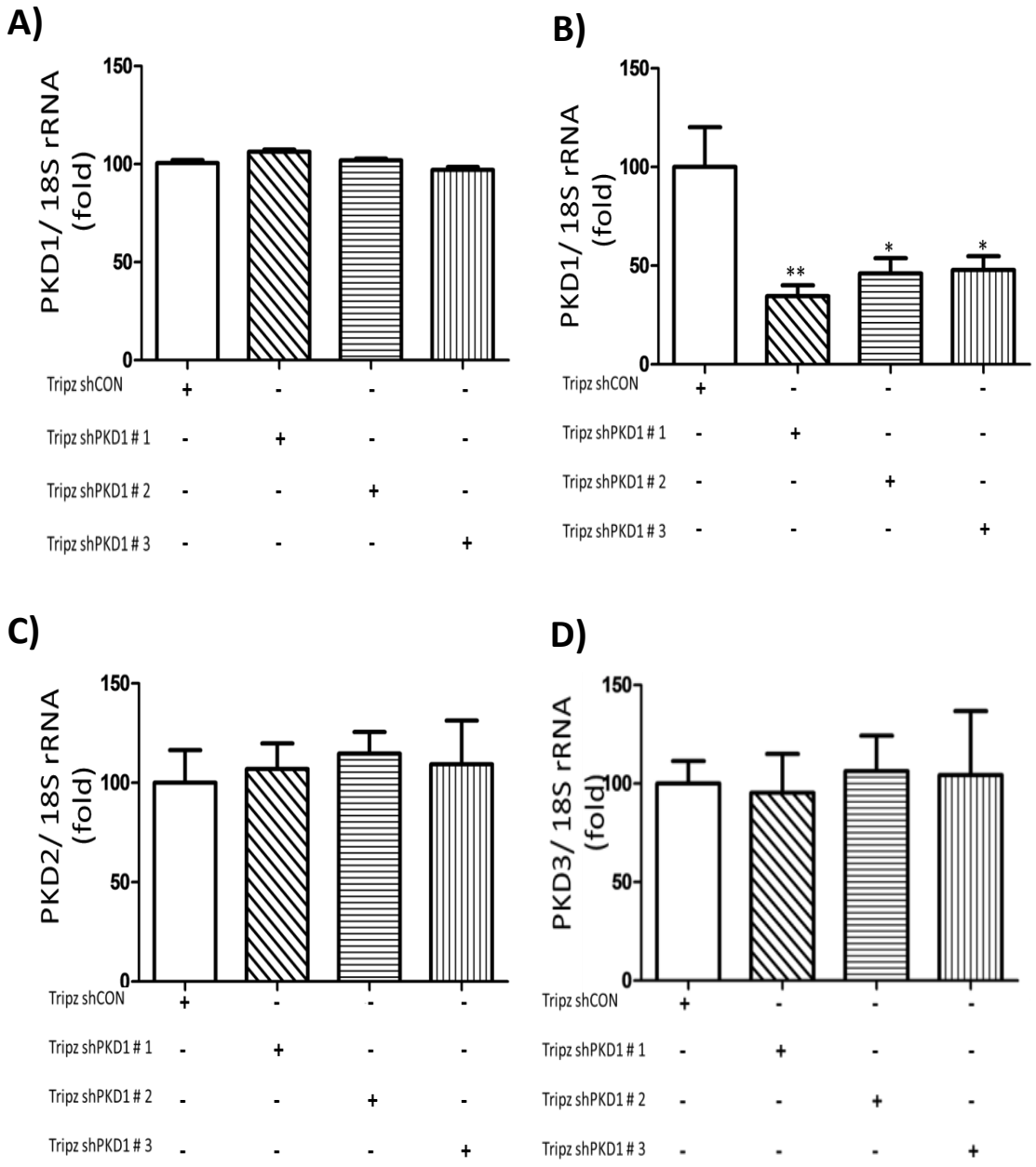


Figure 3.8. The effect of lentiviral mediated delivery of TRIPZ shRNA on PKD gene expression in SW1353 cells. SW1353 cells were plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 5 µl of concentrated lentiviral supernatant of either control, shRNA PKD1 #1, #2 or #3 in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replaced after 8 hours with serum containing medium which also contained either (A) no doxycycline or (B), (C) and (D) 3 µg/ml of doxycycline. Cells were then visualised after 72 hours to confirm shRNA expression. Cells were then lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *PKD1*, (B) *PKD1* (C) *PKD2* and (D) *PKD3* as described in the Materials and Methods. Data plotted are the mean ± S.E.M. of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control, transfection, where PKD expression in control was normalised to 100% expression.**, p≤0.01, *, p≤0.05 vs shCON.

3.3.2.8 Lentiviral mediated TRIPZ shRNA delivery in HAC

Once the optimum conditions needed to maximally transduce SW1353 cells were established, these conditions were used as a starting point for HAC transduction. Varying time points and concentrations of doxycycline were also used. When the levels of RFP expression in HACs transduced with the tet-on shRNAs were examined, low levels of RFP expression were observed (*Figure 3.9. A*). This indicated one of two possibilities; poor shRNA expression due to the doxycycline not inducing expression, and/or poor transduction of HAC. As these viral particles had been shown to transduce SW1353 cells and HACs had previously been shown to be transduced well with GFP expressing lentiviruses, transduction was not thought to be the problem. These data therefore implied that the doxycycline was not capable of inducing expression in HAC. To try and overcome this, a new batch of doxycycline was purchased. Transduced HAC were therefore treated with either batch of doxycycline. As *Figure 3.9. B* shows, no increases in the levels of RFP were observed in the cells treated with the new doxycycline. Changes in viral concentration, doxycycline concentrations and duration of incubation with doxycycline also had no effect on viral transduction (data not shown).

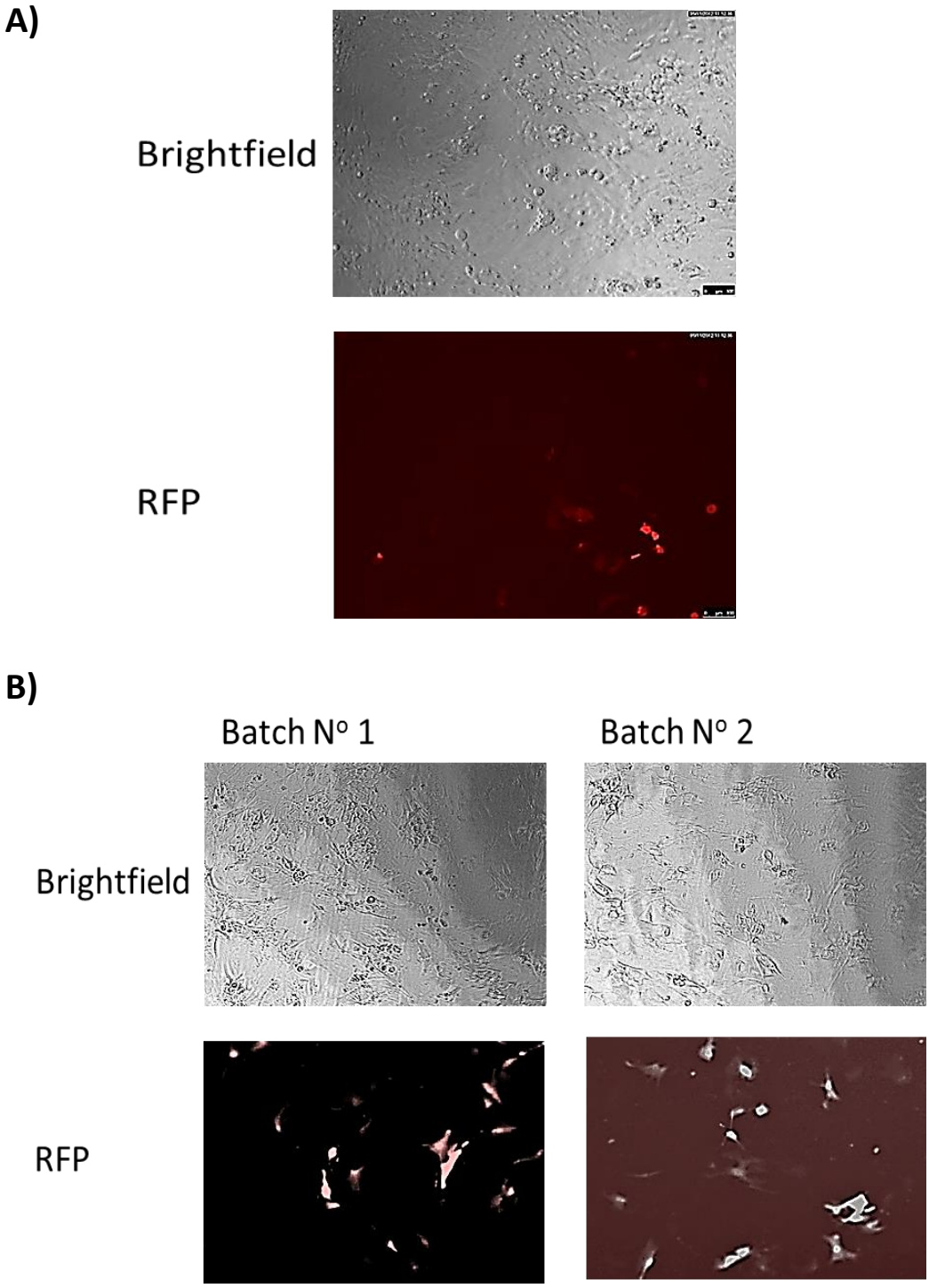


Figure 3.9. Lentiviral mediated shRNA expression in HAC. P0 HAC were plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with 10 µl of concentrated shCON lentiviral supernatant in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. (A) Supernatant was replaced after 8 hours with serum containing medium which also contained 3 µg/ml of original doxycycline. (B) Supernatant was replaced after 8 hours with serum containing medium which also contained 3 µg/ml of original doxycycline or a new batch of doxycycline. Cells were then visualised after 72 hours. Levels of shRNA expression were equated to expression of RFP protein. The brightfield image indicates the cells present and the RFP image indicates the levels of RFP.

3.3.2.9 Lentiviral mediated delivery of GIPZ shRNA into HAC

Due to time restraints and lack of improvement of the shRNA expression in HAC using the TRIPZ based tet-on shRNAs, new shRNA expression plasmids were purchased. These constructs did not require doxycycline to induce shRNA expression. Initial experiments set out to confirm whether HAC could be transduced with these new viral preparations and whether they expressed the shRNA. Experiments showed that viral particles containing these shRNAs were capable of transducing HAC to levels similar to that of the TRIPZ shRNAs in SW1353 cells, albeit when a larger volume of viral supernatant was used. Therefore, to reduce the variability between viral preparations and increase the reproducibility of this work, the multiplicity of infection (MOI) of HAC and SW1353 cells was calculated. To achieve this, the Clontech Lenti-X qRT-PCR Titration kit was used (see *section 2.2.3* for details). As *Figure 3.10* shows, an MOI of 30 led to the maximal transduction of HAC, with no increase in the number of transduced cells observed when cells were treated with virus equating to a MOI of 45. An MOI of 15 was found to be optimal for SW1353 cells (data not shown). With every new batch of lentivirus made, the number of viral particles per ml was calculated and from this the volume of viral supernatant needed to transduce both HAC and SW1353 cells was calculated. This increased the reproducibility of viral transduction between different viral preparations.

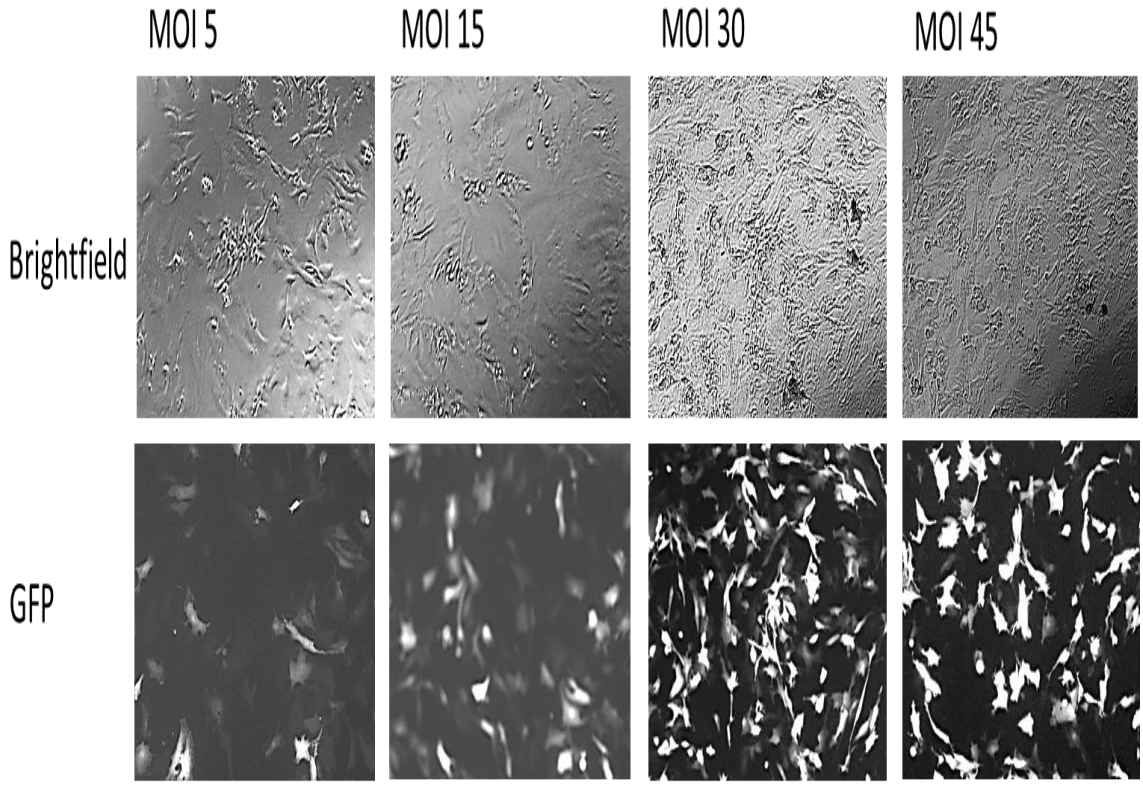


Figure 3.10. Calculation of the MOI of HAC. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with shCON viral supernatant equating to a MOI of 5, 15, 30 or 45 in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised after 48 hours. Levels of shRNA expression were equated to expression of GFP protein. The bright field image indicates the cells present and the RFP image indicates the levels of GFP.

3.3.2.10 The effect of lentiviral mediated shRNA delivery of a PKD1 shRNA on PKD1 gene and protein expression in HAC

Once it was established that transduction of HACs using the GIPZ-based shRNA plasmids could be achieved, the effects of these two PKD1 shRNAs on PKD gene and protein expression were assessed.

Here cells were treated with two viral populations; these expressed either the GIPZ based PKD1 #1 shRNA or the GIPZ based PKD1 #2 shRNA. Transduction of HAC with GIPZ PKD1 #1 shRNA and GIPZ PKD1 #2 shRNA led to a 54% and 64% reduction in PKD1 gene expression, respectively *(Figure 3.11. A*). Although there were no effects on PKD2 gene expression with either shRNA for PKD1 (*Figure 3.11. B*), GIPZ PKD1 #1 shRNA did reduce the expression of PKD3 (*Figure 3.11. C*). A clear reduction in the protein level of PKD1 was observed when cells were transduced with PKD1 #2 shRNA. This shRNA was therefore capable of specifically reducing PKD1 at both the mRNA and protein level in HAC and was therefore used in subsequent experiments.

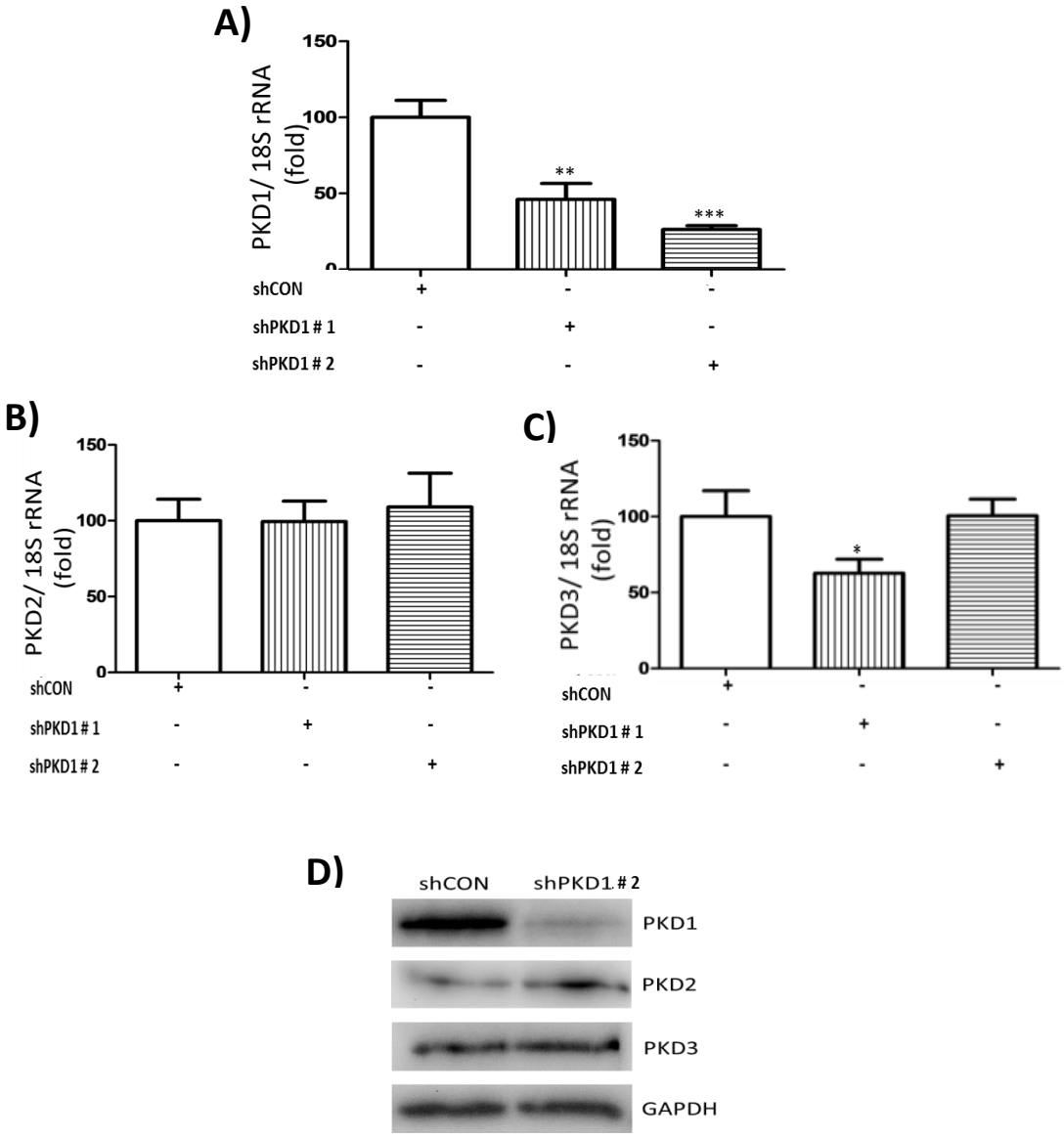


Figure 3.11 The effect of lentiviral mediated GIPZ based PKD1 shRNA on PKD1 mRNA and protein expression in HAC. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then medium was removed and replaced with viral supernatant containing either shCON, PKD1 #1 or PKD1 #2 shRNA at a MOI of 30, in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *PKD1*, (B) *PKD2* (C) *PKD3* as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control, transfection, where PKD expression in control was normalised to 100% expression. ***, p≤0.001, **, p≤0.01, *, p≤0.05. (D) HACs were treated as above (using only PKD1 #2 shRNA), except plated into 6 well plates in a total of 1 ml SFM. Cell lysates were then immunoblotted using antibodies against PKD1, PKD2 and PKD3, as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

3.3.3 PKD1 over-expression optimisation

Whilst optimising the conditions needed to silence PKD1 at the mRNA and protein level, the technique of protein over-expression of PKD1 was optimised to complement my research and understand the role of PKD1 in the regulation of collagenase gene expression. To achieve this, the optimum experimental conditions and transfection reagent for the over-expression of PKD1 were established. A PKD1 expression plasmid was purchased (Addgene). The PKD1 plasmid was used in all experiments and included the backbone of pcDNA3.1, whilst transfection of the empty vector was used as a control. The plasmid contained a HA tag which aided in the identification of over-expression. The HA tag, unfortunately, could not be detected in HACs.

Optimisation began by identifying the best transfection reagent to over-express the PKD1 plasmid; several commercially available transfection reagents were used. The magnetic bead based transfection reagent Magnet Assisted Transfection (MATra) transfection reagent (IBA, Goettingen, Germany), a cationic polymer based transfection reagent Exgene 500 (Thermo Scientific), a non-liposomal based transfection reagent Fugene HD (Promega) and the cationic polymer based transfection reagent JetPEI were all used.

Experiments were first optimised in SW1353 cells. As *Figure 3.12. A* shows, the greatest expression of PKD1 when compared to control plasmid was achieved using Exgene 500 (both the magnetic transfection reagent and Fugene HD were cytotoxic at 4 µg of DNA). The optimum quantity of plasmid DNA and volume of Exgene 500 to use in transfection experiments was then sought. As *Figure 3.12. B* shows, little differences in the amount of over-expressed PKD1 was observed when different amounts of DNA were used, a concentration of 3 µg of DNA was chosen, to ensure over-expression of PKD1 was achieved. From the data observed in *Figure 3.12. C* it was decided that 9.87 µl of Exgene 500 should be used to transfect in the PKD1 plasmid.

Unfortunately, after this optimisation process, Exgene 500 became unavailable. A new cationic polymer based transfection reagent was therefore sought, as this reagent type had been shown to achieve the greatest transfection in both cell types. A new cationic polymer based transfection reagent, JetPEI, was

purchased. When comparing the expression of PKD1 in cells transfected with JetPEI to that of Exgene 500 no differences in over-expression were observed *(Figure 3.12. D*). JetPEI transfection reagent was therefore used in all further experiments performed in this thesis. Over-expression of PKD1 was achieved using 1 µg of DNA to 2 µl of JetPEI in SW1353 cells, without cytotoxicity. JetPEI was also used to over-express PKD1 in HAC, where 3 µg of plasmid DNA at 1:2 ratio of transfection reagent successfully over-expressed PKD1 in HAC (*Figure 3.12. E)*

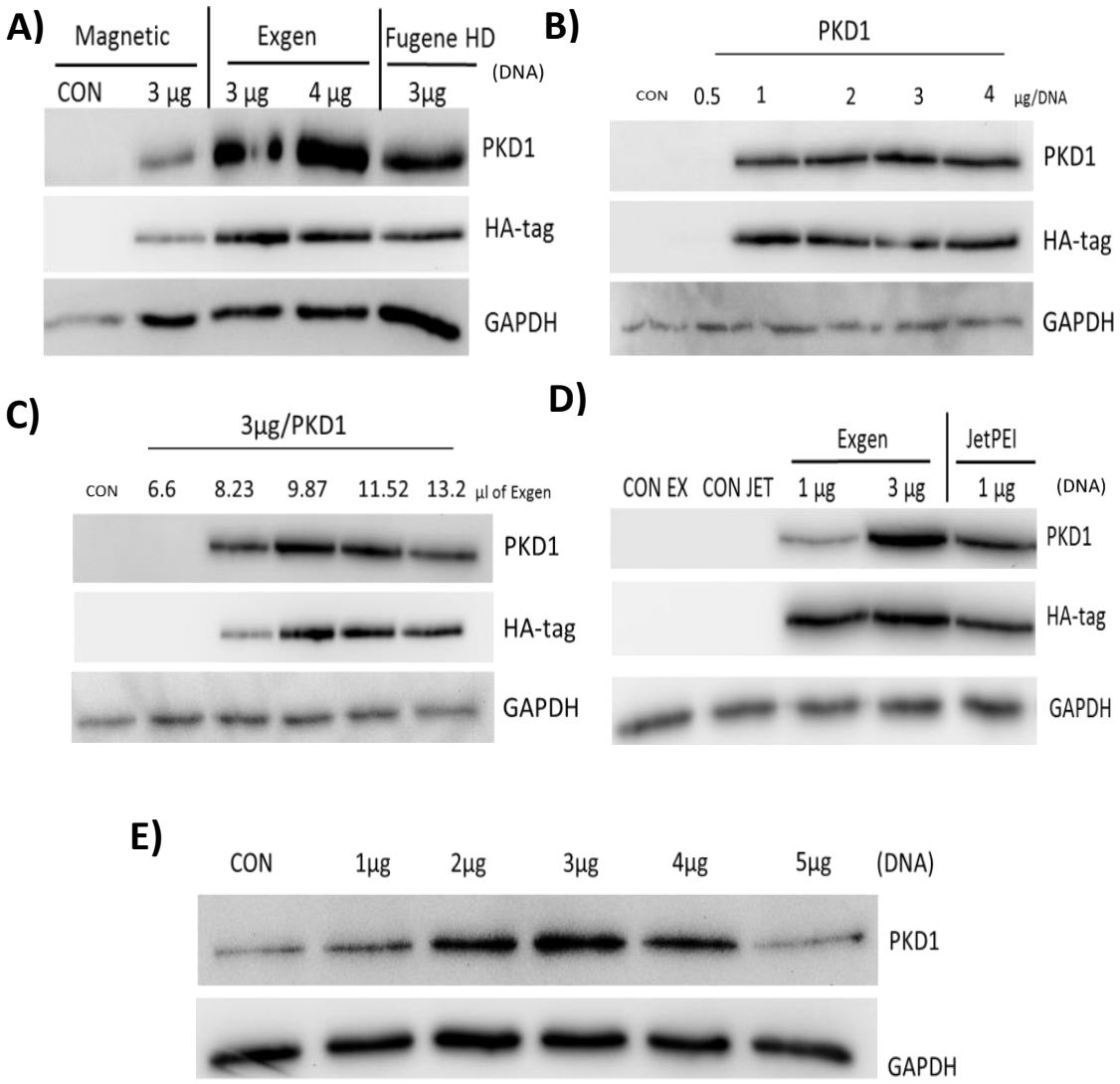


Figure 3.12 Optimisation of the over-expression of PKD1 in SW1353 cells and HAC. SW1353 cells were plated into 6 well plates, and left over-night. (A) The following day cells were transfected using MATra based magnetic transfection, Exgen 500, Fugene HD as described in the manufactures instructions. Cells were transfected with either 4 µg of control backbone plasmid or 3 or 4 µg of PKD1 plasmid DNA. (B) Cells were transfected using Exgen 500 at 1:3 ratio using either 4µg of control plasmid or PKD1 plasmid DNA as indicated. (C) Cells were transfected using Exgen 500 using volumes of as indicated and 3 µg of control and PKD1 plasmid DNA. (D) Cells were transfected using either 9.87 µl of Exgen 500 and 3 µg of control plasmid or 1 or 3 µg of PKD1 plasmid DNA or JetPEI at 1:2 ratio of DNA to transfection reagent. 1µg of control plasmid or PKD1 plasmid DNA were used. (E) P0 HAC were trypsinised and plated into 6 well plates and left over-night. Cells were transfected using JetPEI at 1:2 ratio of DNA to transfection reagent. 5 µg of control plasmid DNA was transfected along with PKD1 plasmid DNA as indicated. Cells were lysed and separated by electrophoresis and then immunoblotted using antibodies indicated, as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

3.4 Discussion

Understanding the isoform specific roles of PKD was one of the major aims of this thesis. Therefore, ensuring that the biological techniques used to modulate the expression levels of each PKD isoforms were both robust and specific was the major initial aim. Much time was therefore spent on the optimisation and characterisation of RNAi and over-expression. These methods have long been established as important molecular biological techniques, enabling the understanding of the downstream effects of protein signalling molecules, within a particular system.

It was first established that PKD2 and PKD3 could be silenced at the mRNA and protein level using siRNA specific for each isoform. PKD1 could be silenced at the mRNA level but not at the protein using siRNA. Off target effects were also seen when using some of these siRNAs.

The siRNAs or shRNAs purchased were validated against each isoform. Even though these were validated reagents, off target effects were observed. Birmingham *et al.,* using bioinformatics, observed off-target effects were often caused between match of the seed region of a siRNA (position 2-7) and the 3'UTR of the non-specific gene. As this sequence is often not checked for when designing a siRNA or shRNA this could explain the off target effects observed, especially as all isoforms of PKD are closely related and may have similar 3'UTRs (Birmingham et al., 2006).

As well as bioinformatical studies, the final concentration of siRNA used can have a profound effect on siRNA efficiency. Increased concentration of siRNA is seen to increase the likelihood of off-target effects (Semizarov et al., 2003). To try and overcome this optimisation experiments performed. When the siRNA was used at lower concentrations than the 100 nM used, these reagents were seen to be less efficient and reductions in their off target effects were not observed. SMARTpool siRNAs from ThermoScientific were also used. These use a pool of 4 siRNAs and therefore when using 100 nM of siRNA each of the 4 siRNAs are at a concentration of 25 nM, potentially reducing any off target effects. Off target effects were still observed when using these siRNAs (data not

shown). Therefore it was concluded that all siRNAs must be screened to confirm specificity at both the mRNA and protein level.

Also of note is the increased expression of the other isoforms of PKD when an alternative isoform was silenced. This can be seen when cells are treated with both PKD2 and PKD3 siRNAs. An increase in the expression of PKD1 and PKD3 is observed when PKD2 was silenced; PKD1 and PKD2 protein expression also increased when PKD3 was silenced (*Figure 3.1*). This suggests a compensatory mechanism in which the reduction in the expression of one isoform is compensated for by increased expression of another. The effects of the PKD1 siRNA also appear to have this affect, as these reagents weren't used in further experiments and the shRNA used appears to have no effect (*Figure 3.11. D*), these data were not of concern.

One of the major problems to overcome in this study was to find a molecular biological tool capable of silencing PKD1 at the protein level. Research shows the importance of studying both the mRNA and protein expression of a target gene when using siRNA (Aleman et al., 2007). The levels of mRNA and protein expression are often thought of as very linear in relation to one another, with increased mRNA expression leading to increased protein expression. This, however, is not always the case, with the translational machinery dictating protein levels (Schwanhausser et al., 2011). As well as this, it is also observed that decreases in mRNA expression, as observed in these data, do not always correlate with decreased protein expression (Morgan et al., 2006) and are shown to have temporal differences in expression (Fournier et al., 2010). Therefore one explanation for the decrease in mRNA levels but not protein could be that the protein half-life of PKD1 exceeded the half-life of the mRNA. This means that any mRNA silencing by the siRNA was not sufficient in duration to cause a decrease in total protein. This effect has been noted in previous studies, with mRNA loss but no effect on protein expression (Dani et al., 1984, Franch et al., 2001).

As PKD1 protein stability may exceed its own mRNA half-life, to try and increase the possibility of protein silencing the incubation time of SW1353 cells and HAC with the siRNA was increased. When siRNA incubation time was

increased no effect on the protein levels were observed (data not shown). This is unsurprising as the half-life of an siRNA is thought to be around 48 hours with the complete removal of the siRNA being observed after 96 hours (Pushparaj and Melendez, 2006). Therefore to try and overcome this problem, lentiviral mediated delivery of shRNA was used. This incorporated shRNA into the host genome allowing constitutive expression of the shRNA, increasing the duration of siRNA expression.

The use of lentiviral mediated gene expression has many advantages over transient transfection of siRNA and shRNA, as well as the delivery of shRNA by other retroviral and adenoviral methods of transduction. Adenoviruses have been shown to transduce HAC and express the *lacZ* gene (Doherty et al., 1998). However, adenoviruses only transiently transduce their target cell, with no incorporation of the shRNA in to the host genome. Retroviruses, unlike lentiviruses, require cells to be dividing in order to transduce. HACs are known to divide and grow slowly, suggesting lentiviral mediated transduction to be a better method to incorporate a shRNA into HAC than other retroviral methods. This has been shown, with Li *et al.,* showing that lentivirus vectors gave up to three fold higher transduction efficiency in chondrocytes than retrovirus vectors (Li et al., 2004). For these reasons it was decided that lentiviral mediated gene silencing was the most appropriate technique to use.

To optimise the lentiviral mediated transduction of SW1353 cells and HAC numerous steps were used. To ensure transduction and expression of the shRNA of interest could be quantified, all used virions were capable of inducing RFP or GFP expression. This enabled the identification of the cells which had been transduced.

To increase the likelihood of transduction, viral preparations produced were concentrated. The use of the VSV-G viral coat protein gives the virus the capability to be concentrated (Burns et al., 1993). This stable and strong viral coat allows the integrity of the virus to be maintained when concentrated. The concentration of the virus allows more viral particles to come into contact with a cell increasing the likelihood of transduction, as shown in *Figure 3.4*. One problem with using VSV-G as the coat for the viral particles is the effect that

serum complement can have on these particles. VSV-G pseudotypes have been shown to be inactivated by serum complement (DePolo et al., 2000), and for this reason heat-inactivated FBS was used in the production of the lentiviral particles. All transduction of HAC or SW1353 cells were performed in native medium to also prevent inactivation in the initial steps of transduction.

Much work has demonstrated poor transduction efficiency of retroviruses to be due to the slow diffusion and rapid inactivation. Viral particles are thought to be able to diffuse only a few microns before losing their bioactivity, for these reasons a large proportion of the viral particles are inactive before they come into contact with the cells (Davis et al., 2004). To therefore reduce these effects and increase viral transduction Polybrene was used. Polybrene masks the net negative charge, reducing charge repulsion (Coelen et al., 1983). The addition of Polybrene was seen to increase viral transduction (*Figure 3.3*). The technique of spinoculation was also used. Spinoculation works by concentrating virions onto the surface of target cells reducing diffusion; this technique should therefore increase the binding of viruses to target cell, enhancing the efficacy of virus-based incorporation of shRNA (O'Doherty et al., 2000). However, a decrease in the levels of transduction was seen when centrifuged; this may have been due to increases in cell death due to centrifugation of the cells

Once it was established that a protocol to transduce both SW1353 cells and HAC had been achieved, I next sought to optimise the delivery of shRNA into the host genome. To achieve this, shRNA which were under the control of tet-on operator, in which doxycycline was needed, were first used. As *Figure 3.7* and *Figure 3.8* show, the conditions needed to successfully transduce SW1353 cells with these shRNAs and also silence PKD1 specifically at the mRNA level were achieved. However when this technique was extrapolated into HAC, even when two different batches of doxycycline were used, little to no expression of the shRNA is observed. As previously high levels of viral transduction had been seen, it was believed that the doxycycline was not initiating promoter activity. One explanation for this could be due to the doxycycline not being able to enter primary cells, but doxycycline at a concentration 10 µg/ml has been shown to enter and have an effect on HAC (Shlopov et al., 2001). As high levels of

transduction were seen in SW1353 cells with the same viral populations and doxycycline, the reason for this limitation remains unclear.

It is also of note that doxycycline can inhibit MMP activity and expression (Liu et al., 2003, Curci et al., 2000, Shlopov et al., 2001). As both control and PKD1 shRNA expressing cells were treated with doxycycline, the effects of the doxycycline would likely have been normalised. But because of the problems with the optimisation of this system and the potential alterations in the results from the doxycycline I decided not to continue with the Tet-on based shRNAs.

To continue this work, a set of GFP expressing, non-inducible shRNAs were used. The GFP expression was therefore used to calculate the MOI of SW1353 cells and HAC (*Figure 3.10)*. The calculation of the MOI was important to ensure confidence in the reproducibility of results between different viral preparations. Calculating the number of viral particles per ml for every viral preparation made enabled the same number of viral particles to be added in each experiment, increasing reproducibility and protein silencing. Once this was calculated the effect of the GIPZ based shRNA was assessed. As *Figure 3.11* shows, the shRNA GIPZ PKD1 #2 was capable of both silencing PKD1 at the mRNA and protein level.

Whilst optimising lentiviral mediated gene silencing of PKD1, the technique of over-expression was utilised. Data show that the use of cationic polymers based transfection reagents were the best transfection reagents to use for over-expressing PKD1 in SW1353 cells and HAC. The change of transfection reagent from Exgen 500 to JetPEI had no effect on experiments performed; JetPEI was then used in all further experiments.

The tools needed to silence each isoform of PKD specifically were eventually found and optimised, as well as the over-expression of PKD1. The work achieved in this chapter laid the foundation for the rest of the work in this thesis, increasing confidence that any results obtained were specific to the isoform stated.

3.4.1 Summary

Studies in this chapter have shown:

- Isoforms PKD2 and PKD3 can be silenced at the mRNA and protein level using siRNA.
- Using GIPZ expression plasmids containing a shRNA against PKD1 was capable of silencing PKD1 at both the mRNA and protein level.
- Optimum PKD1 over-expression was observed using cationic polymer based transfection reagents.

4 Chapter 4. The role of PKD isoforms in the regulation of MMP gene expression

4.1 Introduction

As mentioned previously, the irreversible degradation of the cartilage matrix which lines articular joints leads to the loss of normal joint function and homeostasis. This process is characterised by the proteolytic degradation of collagen and proteoglycan found within the ECM of this cartilage. The degradation of proteoglycan is rapid yet reversible (Dingle et al., 1987) and for this reason the irreversible degradation of cartilage collagen, albeit slower, is thought of as a key step in cartilage breakdown (Jubb and Fell, 1980). The major family of enzymes involved in this process are a sub group of the MMPs known as the collagenases. The collagenases consist of MMP-1, -8, -13 and -14. Of these, MMP-1 and MMP-13 are thought to be of greatest importance in the cleavage of type II collagen during arthritis progression.

To understand the regulation of these proteolytic enzymes within chondrocytes in inflammatory context, the inflammatory cytokines IL-1 and OSM were used as a model stimulus. These two pro-inflammatory cytokines have been shown to synergise, leading to the induction of collagenase gene expression. Various studies from within our laboratory have shown the induction of collagenase gene expression by these pro-inflammatory cytokines to correlate with cartilage degradation in human, murine and bovine models (Barksby et al., 2006, Cawston et al., 1995b, Rowan et al., 2001, Rowan et al., 2003, Koshy et al., 2002b).

The precise mechanism by which these pro-inflammatory cytokines regulate collagenase gene expression within chondrocytes remains unclear. Only a few studies have explored the signalling cascades that are activated by this combined stimulus (Catterall et al., 2001, Litherland et al., 2010, Litherland et al., 2008). Work within this chapter therefore sets out to assess the role of the PKD family in the induction of collagenase gene expression under the stimulation of IL-1 in combination with OSM. From these data and our own

previous work indicating the PKC family as collagenase regulators (Litherland et al., 2010), I wanted to establish the role of this PKC substrate in the regulation of collagenase gene expression.

I therefore set out in this chapter to discover the role of each individual isoform of PKD in the regulation of collagenase gene expression in HACs. To perform this work the PKD inhibitor kb NB 142-70 was first used to elucidate the role of PKD as an entity within chondrocytes. Then using RNAi and over-expression techniques the role of each isoform of PKD on collagenase expression (as well as other matrix degrading enzymes) was assessed.

4.2 Aims

The aim of this chapter was to elucidate the role of each isoform of PKD in the regulation of collagenase gene expression under the synergistic stimulus of IL-1 in combination with OSM.

The specific aims were:-

- Identify whether PKD regulates collagenase gene expression within HAC
- Identify the isoform-specific roles of PKD on collagenase gene expression
- Identify other MMPs potentially regulated by each isoform

4.3 Results

4.3.1 Identifying PKD as a regulator of collagenase gene expression

Litherland *et al.,* have tentatively implicated PKD in the regulation of collagenase gene expression within HAC (Litherland et al., 2010). PKD inhibition using the PKC/PKD inhibitor Gö6976 showed a reduction in collagenase gene expression within HACs stimulated with IL-1 in combination with OSM. Collagen release and collagenase activity was inhibited in bovine nasal cartilage by (Litherland et al., 2010). Gö6976 inhibits PKD (Gschwendt et al., 1996) as well as PKCα and PKCβ (Martiny-Baron et al., 1993). Since Litherland *et al.,* concluded that aPKC isoforms were most important in collagenase gene regulation; I wanted to confirm or otherwise a specific role for PKD in the regulation of collagenase expression. To achieve this, the PKD inhibitor kb NB 142-70 was used. This inhibitor has been shown to be PKD

specific, with similar IC_{50s} for each isoform of PKD (Bravo-Altamirano et al., 2011)

4.3.1.1 PKD inhibition modulates collagenase expression

The cytotoxic effect of kb NB 142-70 in HAC was first assessed. Using the ToxiLight assay the levels of cell death were equated in HAC treated with the inhibitor vs inhibitor vehicle. No increased cytotoxicity was observed when using the inhibitor at concentrations up to 5 μM (*Figure 7.1* supplementary data). To reduce the possibility of cytotoxicity and off target effects a concentration of 1 μM was used. As *Figure 4.1. A* and *B* shows, a significant decrease in MMP-1 and MMP-13 gene expression was observed in HAC in basal conditions and when stimulated with IL-1 alone or in combination with OSM when treated with the inhibitor.

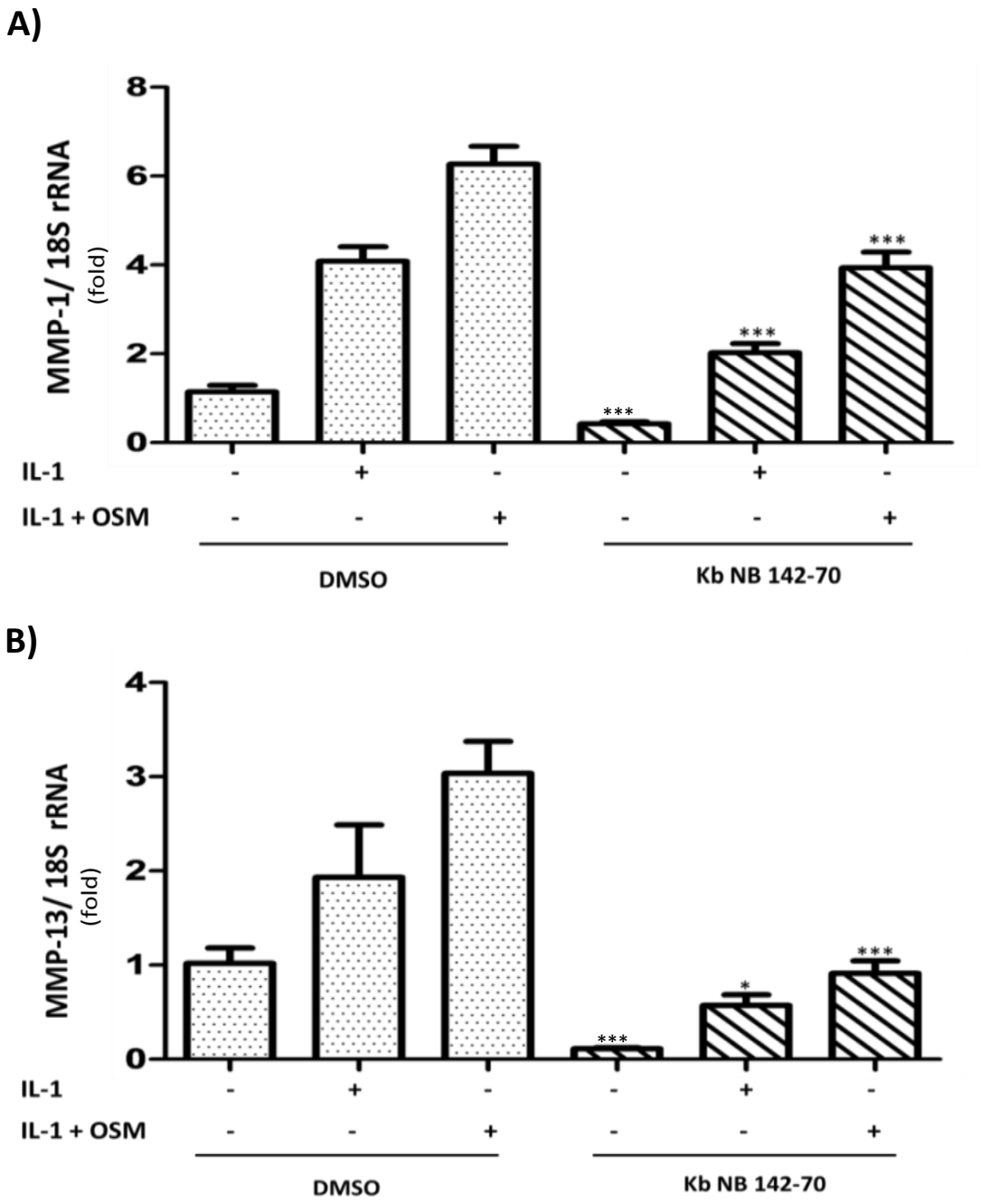


Figure 4.1. The effect of PKD inhibition on collagenase expression in human articular chondrocytes. P0 HAC plated into 96 well plates were grown to ~70% confluency and then serum starved overnight. HAC were pre-incubated with 1 μM kb-NB 142-70 or the same volume of DMSO vehicle for 1 hour prior to stimulation with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, as described in the Materials and Methods. Data (mean ± S.E.M.) are representative of at least three combined chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific for PKD inhibitor versus control DMSO treated, where ***, p≤0.001, *, p≤0.05 vs DMSO.

4.3.2 The isoform specific roles of PKD on collagenase gene expression

Once a role for PKD in the regulation of collagenase gene expression was established, I next sought to elucidate the effect of each isoform of PKD on collagenase gene expression. Using over-expression and RNAi of PKD, I set out to understand how each isoform of PKD regulated collagenase gene expression within chondrocytes stimulated with IL-1 alone or in combination with OSM. The effect of PKD1 over-expression and PKD1, PKD2 and PKD3 gene silencing were assessed using western blotting to confirm over-expression or gene silencing (as seen in *chapter 3*)

4.3.2.1 The effect of PKD1 on collagenase gene expression

PKD1 over-expression in HAC stimulated with IL-1 alone and in combination with OSM led to a significant reduction in the gene expression of MMP-1 and MMP-13 (*Figure 4.2 A* and *B*). To validate the overexpression data, PKD1 was specifically silenced using lentiviral-mediated delivery of shRNA. PKD1 silencing led to a significant increase in expression of MMP-1 and MMP-13 when stimulated with the synergistic stimuli IL-1 in combination OSM; no increase was seen with IL-1 stimulation alone (*Figure 4.3 A* and *B*). These two complementary techniques therefore confirmed PKD1 to down-regulate collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. The over-expression and shRNA-mediated gene silencing of PKD1 had the same effects in the chondrocytic cell line SW1353 (*Figure 7.2 and Figure 7.3* supplementary data).

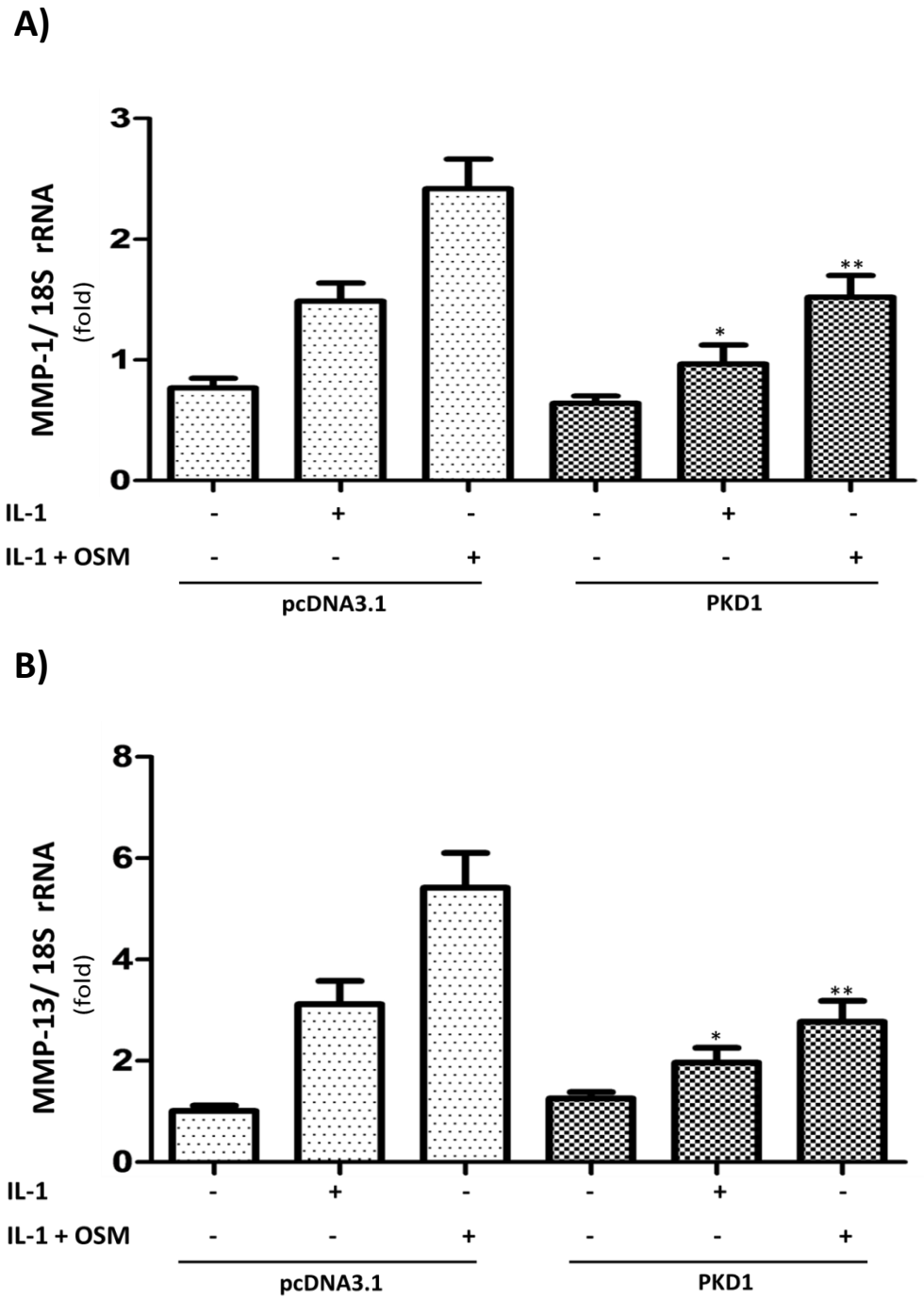


Figure 4.2. The effect of PKD1 over-expression on collagenase expression in human articular chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, and left overnight. The following day cells were transfected with pcDNA3-PKD1 or pcDNA3.1, and then serum starved overnight. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 48 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control over-expression, where **, p≤0.01, *, p≤0.05 vs transfected vector

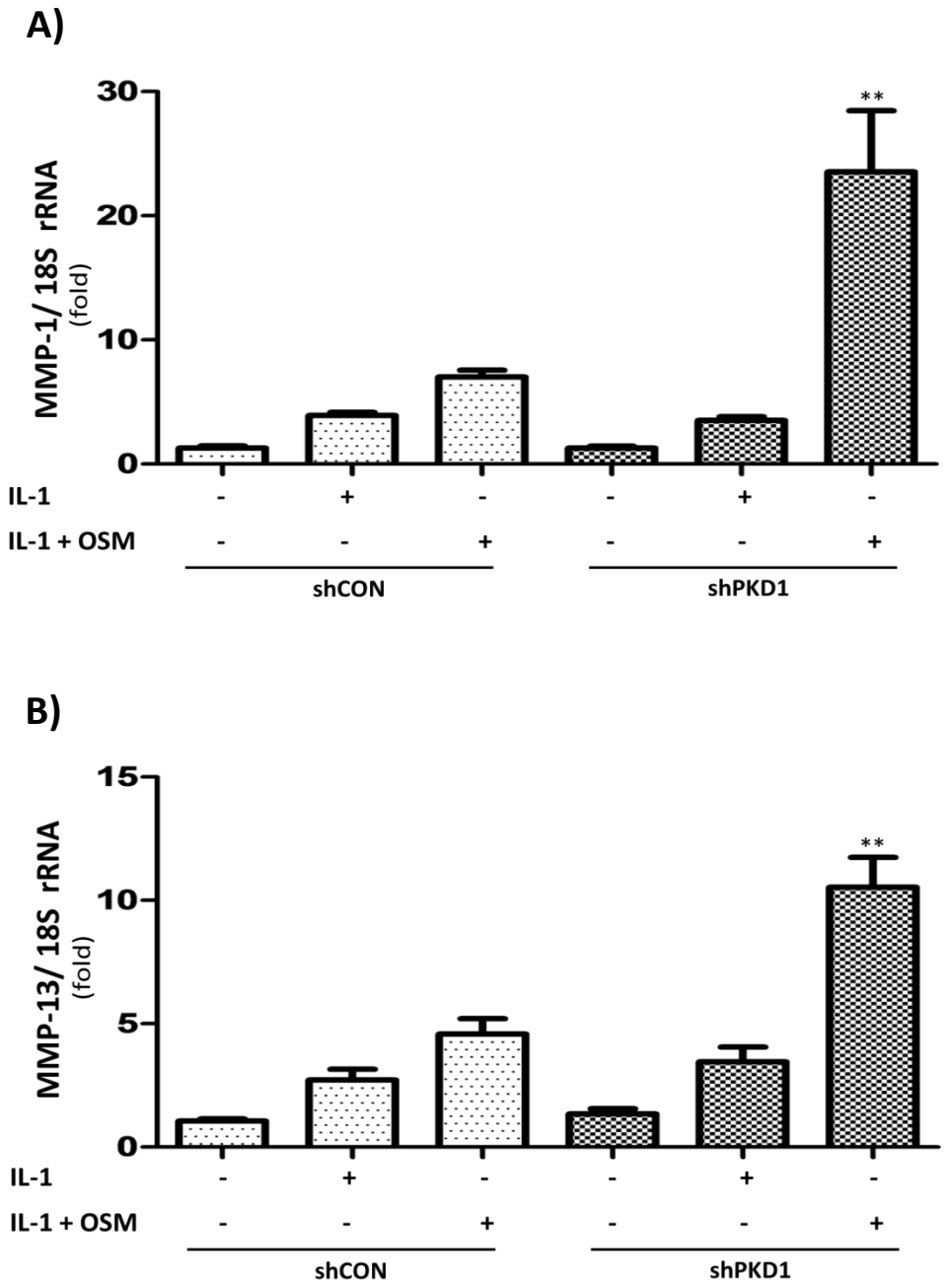


Figure 4.3. The effect of PKD1 gene silencing on collagenase expression in human articular chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then mediumwas removed and replaced with viral supernatant containing either PKD1 shRNA #2 or shCON at a MOI of 30, in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 hours. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 96 h after start of transduction as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transduced PKD isoform versus control transduced, where **, p≤0.01 vs shCON.

4.3.2.2 The effect of PKD2 silencing on collagenase gene expression

Specific PKD2 gene and protein silencing was achieved using siRNA against PKD2. When PKD2 was silenced no effects on MMP-1 or MMP-13 gene expression were observed in HACs under either stimulus (*Figure 4.4 A* and *B*).

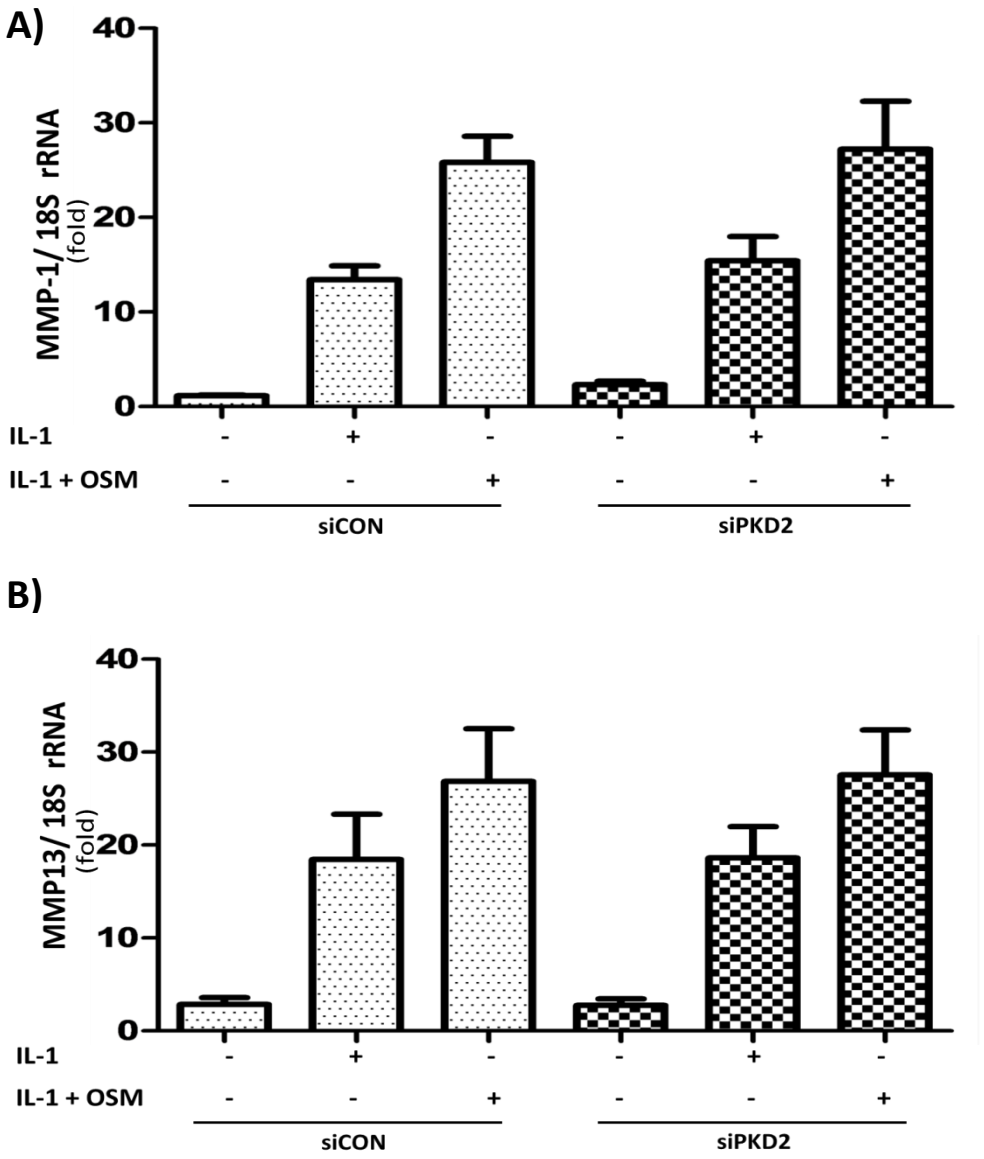


Figure 4.4. The effect of PKD2 gene silencing on collagenase expression in human articular chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, and left over-night. The following day cells were transfected with siRNA specific to PKD2 #3 or siCON (100 nM). 48 hours later cells were serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP*-1 and (B) *MMP-13*, 72 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene.

4.3.2.3 The effect of PKD3 silencing on collagenase gene expression

Specific PKD3 gene and protein silencing was achieved using siRNA specific against PKD3. Gene silencing of PKD3 led to a significant decrease in the gene expression of MMP-1 and MMP-13 when stimulated with IL-1 alone and in combination with OSM (*Figure 4.5 A* and *B*). A more profound effect of PKD3 silencing was seen on MMP-13 than MMP-1 gene expression. PKD3 silencing led to a 43% reduction in MMP-1 gene expression compared to a 72% reduction in MMP-13, when stimulated with IL-1 in combination with OSM. The same is observed for the simple stimulus of IL-1 alone, a 37% reduction in MMP-1 gene expression is seen compared to a 62% reduction for in MMP-13. PKD3 activity therefore leads to the induction of both MMP-1 and MMP-13 in chondrocytes stimulated with IL-1 in combination with OSM. The MMP-13 data suggests that synergy is nearly completely abrogated when PKD3 is silenced.

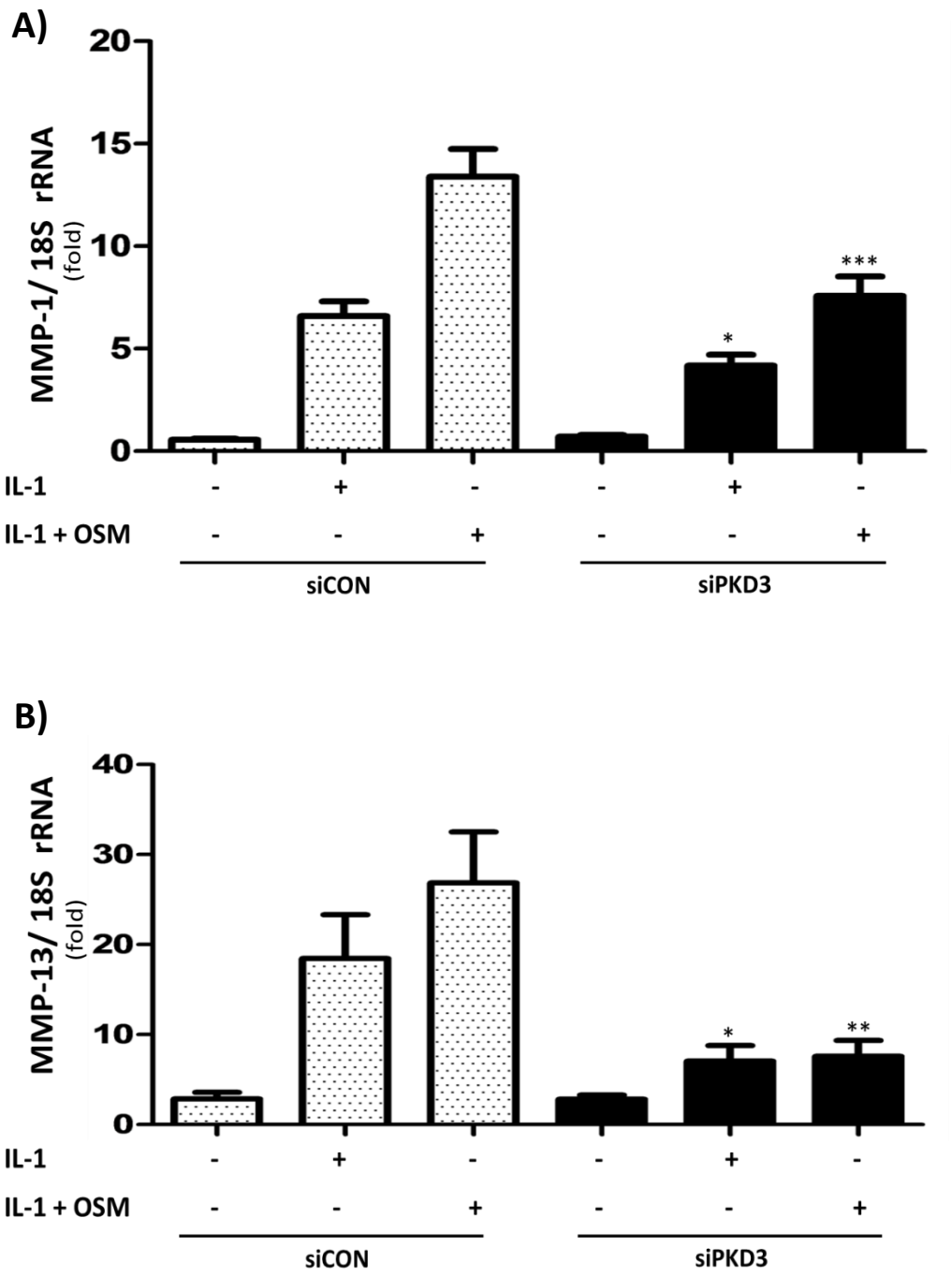


Figure 4.5. The effect of PKD3 gene silencing on collagenase expression in human chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, and left over-night. The following day cells were transfected with siRNA specific to PKD3 #2 or siCON (100 nM). 48 hours later cells were serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 72 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control transfection, where ***, p≤0.001, **, p≤0.01, *, p≤0.05 vs siCON.

4.3.3 Isoform specific roles of PKD on wider MMP gene expression

As both PKD1 and PKD3 silencing had profound effects on the expression of the classical collagenases MMP-1 and MMP-13, I wanted to further assess their role in regulating other collagen degrading MMPs. This was to elucidate whether these findings were specific to the classical collagenases or the regulation of collagen degrading MMPs in general. Understanding the global effects of PKD1 and PKD3 in the regulation of MMP gene expression would provide further information about the consequences of PKD1 and PKD3 inhibition, if either were taken further as possible therapeutic targets.

To understand the regulation of further MMPs in HAC by PKD1 and PKD3, a range of MMPs were examined, many of which had previously been seen to be induced by IL-1 in combination with OSM in chondrocytes (Koshy et al., 2002b). These specific MMPs were chosen as they belonged to the three major subclasses of MMPs believed to be involved in native fibrillar collagen degradation (the collagenases, gelatinases and membrane bound MMPs) (Gioia et al., 2007). The role of PKD1 and PKD3 in the regulation of MMP-8 and MMP-14 was assessed as they are known to have similar substrate specificity as the classic collagenases. MMP-14 is also a member of the membrane bound MMP family which has high affinity for cleavage of type I and III collagen, as well as being able to cleave type II collagen (Gioia et al., 2007, Ohuchi et al., 1997). MMP-2 was also studied as it belongs to the gelatinase subgroup of the MMPs. This enzyme is capable of cleaving both native and denatured collagen (Creemers et al., 1998, Aimes and Quigley, 1995). This member was again chosen to give further insight into the regulation of collagen degrading MMPs as a whole. Understanding whether PKD1 and PKD3 can regulate these collagen degrading MMPs will give greater insight into the specificity of the MMPs regulated by each isoform of PKD within HACs.

4.3.3.1 The effect of PKD1 silencing on the gene expression of other collagen degrading MMPs

To determine whether the observed effects of PKD1 silencing were specific to MMP-1 and MMP-13 a range of collagen degrading MMPs were assessed (*Figure 4.6*). PKD1 silencing led to decreased gene expression of MMP-2 and MMP-14 in cells stimulated with both IL-1 alone and in combination with OSM. No effect on MMP-8 gene expression was observed. These findings indicate a level of selectivity with respect to PKD1 following cytokine stimulation of human chondrocytes, showing differences in the regulation of MMPs gene expression by this isoform of PKD.

4.3.3.2 The effect of PKD3 on the gene expression of other collagen degrading MMPs

PKD3 silencing led to decreased gene expression of MMP-2 and MMP-8 in HAC stimulated with both IL-1 alone and in combination with OSM, whilst no effect on MMP-14 gene expression was observed (*Figure 4.7*). These findings indicate some specificity with respect to PKD3 following cytokine stimulation of human chondrocytes. These data also imply differences in MMP gene regulation via each isoform of PKD under the stimulus of IL-1 in combination with OSM.

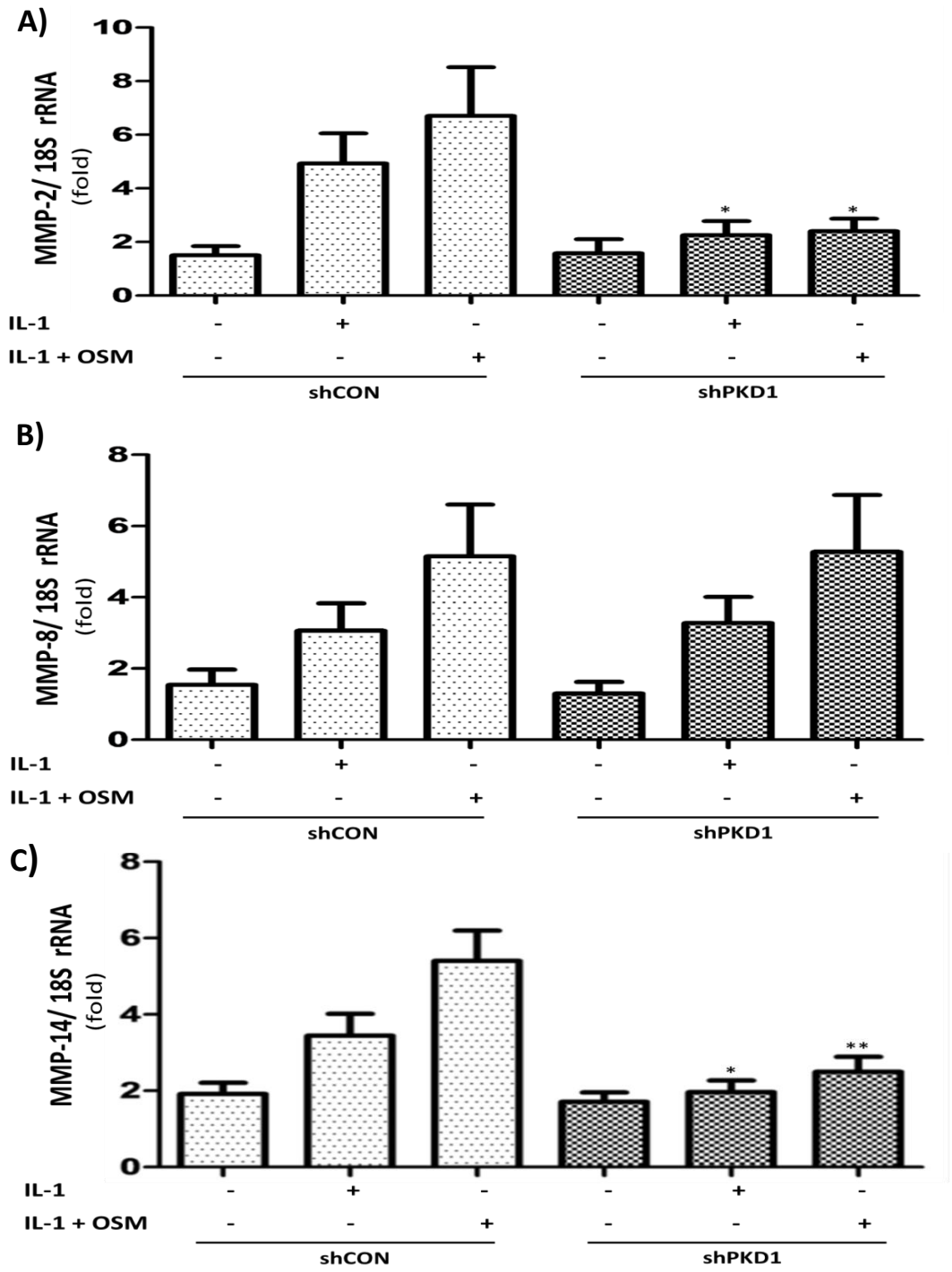


Figure 4.6. The effect of PKD1 gene silencing on MMP expression in human articular chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, allowed to adhere overnight and then mediumwas removed and replaced with viral supernatant containing either a PKD1 shRNA #2 or shCON at a MOI of 30, in a total volume of 100 µl SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 hours. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-2*, (B) *MMP-8*, (C) *MMP-14*, 96 h after start of transduction as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transduced PKD isoform versus control transduced, where **, p≤0.01, *, p≤0.05 vs shCON.

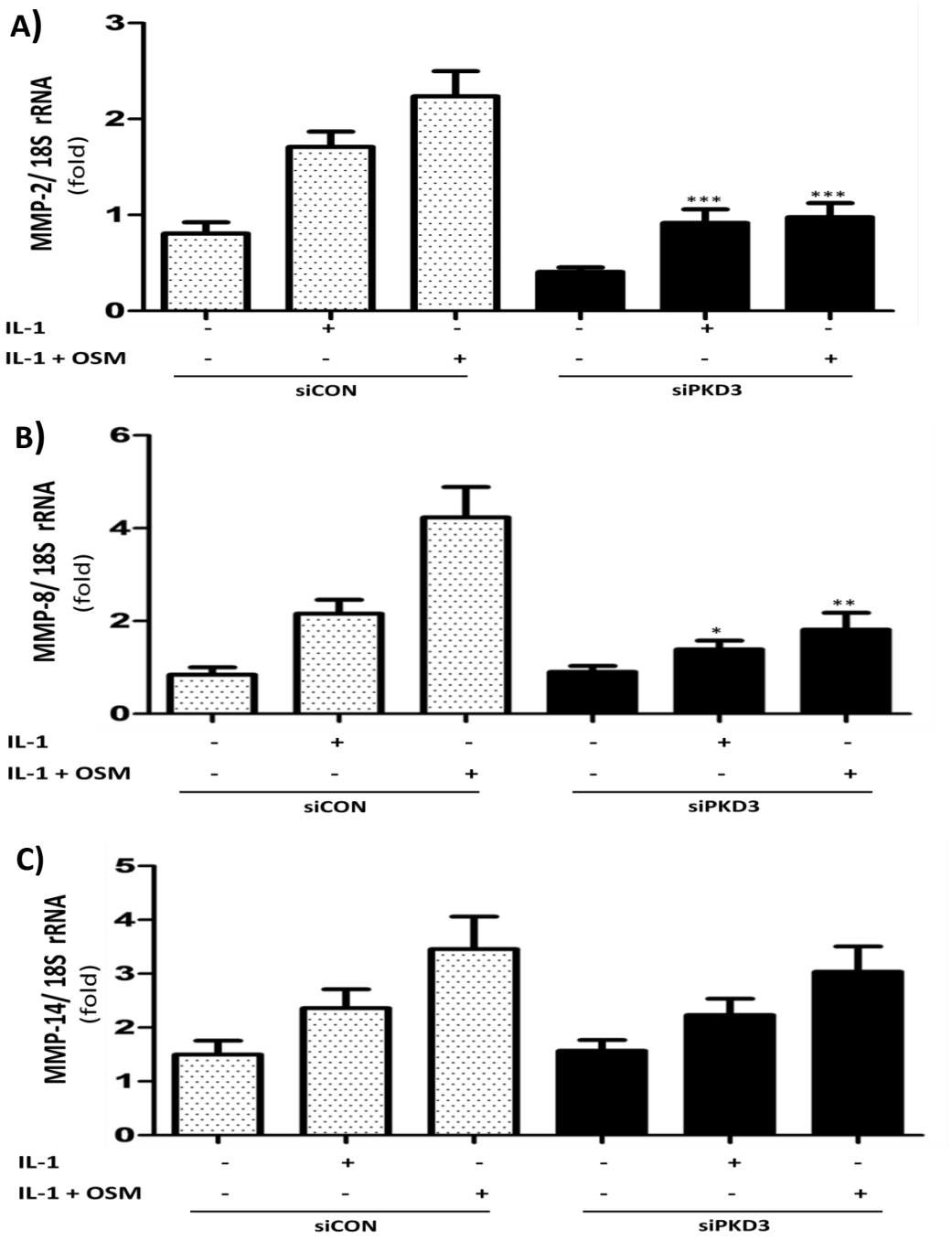


Figure 4.7. The effect of PKD3 gene silencing on MMP expression in human articular chondrocytes. P0 HAC were trypsinised and plated into 96 well plates, and left over-night. The following day cells were transfected with siRNA specific to PKD3 #3 or siCON (100 nM). 48 hours later cells were serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-2*, (B) *MMP-8*, (C) *MMP-14*, 72h after start of transduction as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control transfection, where ***, p≤0.001, **, p≤0.01, *, p≤0.05 vs siCON.

4.4 Discussion

IL-1 in combination with OSM has been shown to be a potent cytokine stimulus of cartilage ECM degradation (Cawston et al., 1998b). This degradation has been shown to be catalysed by the MMPs (Cawston and Wilson, 2006). These enzymes have high affinity for type II collagen, with this cleavage initiating the irreversible degradation of the ECM of cartilage. The signalling events involved in the induction of MMP gene expression induced by this pro-inflammatory stimulus have not been well studied, with only a few studies examining the signalling events involved (Litherland et al., 2008, Litherland et al., 2010, Catterall et al., 2001).

In this chapter I examined the role of a protein kinase family; PKD, in the induction of these matrix degrading enzymes. The role of PKD in the expression of MMPs has previously been shown to be important in metastasis in cancer (Biswas et al., 2010, Eiseler et al., 2009), this process has not previously been studied in relation to cartilage destruction. In this chapter I therefore assessed the role of each isoform of PKD on collagenase gene expression, establishing whether the effects observed are specific against the major collagenases involved in initiating cartilage degradation.

4.4.1 The effect of PKD inhibition on collagenase gene expression

To establish whether PKD regulated the expression of MMP-1 and MMP-13 within chondrocytes stimulated with IL-1 alone or in combination with OSM, the PKD inhibitor, kb NB142-70 was used. Kb NB 142-70 is a more potent analogue of the non-competitive ATP inhibitor CID 755673, the first specific pan-PKD inhibitor to be classified. This inhibitor was identified by high throughput screening of the National Institutes of Health small molecule repository library (Sharlow et al., 2008). Further study of the structure-activity relationships (SAR) of this compound structure led to the development of the more potent inhibitor kb NB 142-70 (Bravo-Altamirano et al., 2011). PKD inhibition using kb NB 142-70 showed for the first time that pan PKD inhibition within chondrocytes leads to decreased collagenase gene expression. These data implicate PKD activity in the regulation of collagenase gene expression within chondrocytes in basal conditions and when stimulated with the pro-inflammatory cytokines IL-1 alone

or in combination with OSM. However, the use of this inhibitor does not yield useful information in regards to which isoforms of PKD are regulating these effects. Understanding the role of each isoform of PKD within chondrocytes was next sought.

4.4.2 The effect of PKD1 over-expression and gene silencing of PKD1 on MMP gene expression

Using the over-expression of PKD1 and shRNA mediated gene silencing of PKD1, this isoform was found to inhibit the expression of the collagenases MMP-1 and MMP-13 (no effect on MMP-8) in HACs stimulated with the pro-inflammatory cytokines IL-1 in combination with OSM. The same results were observed for MMP-1 and MMP-13 (no other MMPs assessed) in the human chondrosarcoma cell line SW1353 (*Figure 7.2* and *Figure 7.3* supplementary data). These data imply that the pro-inflammatory cytokine stimulus of IL-1 in combination with OSM can lead to a down-regulation of these catabolic enzymes. The role of PKD1 in the regulation of collagenase gene expression under the simple stimulus of IL-1 is not as clear. The over-expression of PKD1 appears to lead to a decrease in gene expression MMP-1 and MMP-13 when stimulated with IL-1 alone, whilst PKD1 silencing had no effect in cells stimulated with IL-1 alone. These results do not show a clear role for PKD1 in IL-1 dependent collagenase gene expression.

The reason for this observation is not clear, but could be due to limitations within both techniques. One reason for these opposing results could be due to artificial results being observed in the over-expression data. Increasing the levels of PKD1 may lead to regulatory interactions occurring between signalling molecules which would not normally occur within HAC. This has been previously reported as a problem with transient over-expression (Gibson et al., 2013). A simpler explanation could be that PKD1 silencing may not be sufficient to cause signalling inhibition through PKD1 in IL-1 stimulated chondrocytes. This could however be simply explained by differences in the regulation of collagenase gene expression within the different cell types. However, as the gain of function and loss of function results do not give reciprocal effects in the context of IL-1, the role of PKD1 in classical collagenase gene expression under this stimulus is still unclear.

As well as the inhibition of MMP-1 and MMP-13, PKD1 is seen to induce the expression of both MMP-2 and -14. No effects on MMP-8 gene expression were observed. MMP-8 is not well expressed in primary chondrocytes [14] and for this reason is not thought to play a major role in the cartilage destruction via the stimulation of chondrocytes with IL-1 in combination with OSM. These experiments were performed to gain a further understanding of the MMPs that PKD1 signalling may regulate.

MMP-2 belongs to the gelatinase family of the MMPs, with the role of this MMP in the progression of arthritis being an area of much debate. This enzyme can cleave type IV collagen, the major structural component of basement membranes, as well as other denatured collagens and gelatines (Aimes and Quigley, 1995). In MMP-2 knockout mice increased joint erosion, bone defects, altered osteoblast and osteoclast regulation is observed (Mosig et al., 2007), implying that MMP-2 may inhibit the progression of arthritis, within murine models of arthritis. As well as this, mutations within MMP-2 are shown to lead to arthritis syndrome (Martignetti et al., 2001). However, there are contradictory results, with MMP-2 being implicated with disease progression through bone erosion (Hill et al., 1995) and increased severity of septic arthritis (Puliti et al., 2012). The pathological role of MMP-2 induced by PKD1 in chondrocytes is therefore open to debate, with previous data implying MMP-2 could both decrease and increase cartilage destruction. The effects of the induction of MMP-2 by PKD1 in a disease context are therefore unclear.

MMP-14 (also known as MT-MMP1) belongs to the membrane bound MMP subfamily. This enzyme has the capability to cleave type II collagen at the same efficiency as MMP-8 (Konttinen et al., 1998) as well as the cleavage of other collagen molecules (Ohuchi et al., 1997, d'Ortho et al., 1997). MMP-14 also has the capability to increase cartilage degradation by activating both proMMP-13 and proMMP-2, increasing collagen degradation (Knauper et al., 1996a, Murphy et al., 1999). The role in the up-regulation of MMP-14 in chondrocytes implies PKD1 signalling could induce cartilage damage though increased expression of this cartilage-degrading enzyme. These results therefore show contrasting findings to the inhibitory effects seen in PKD1 regulation of MMP-1 and MMP-13, which are thought of as the major initiators of cartilage degradation.

Also of note is the finding that PKD1 has the opposite effect on collagenase gene expression compared to pan PKD inhibition using kb NB 142-70. The reason for this remains unclear as the potency of the inhibitor against each isoform of PKD is similar (with the IC_{50} values are 28.3, 58.7 and 53.2 nM for PKD1, 2 and 3 respectively (Bravo-Altamirano et al., 2011)). The expression of each isoform of PKD was also assessed to understand whether there was a dominant isoform of PKD expressed in HAC. RNA sequencing data showed the expression of PKD1 within both healthy and OA chondrocytes to be higher compared to that of PKD2 and relative in comparison to PKD3 (DA Young, personal communication). These data therefore imply that differences between the expression of each isoform could not alone account for these results. Alternatively, the kinase activity of PKD3 towards MMP-regulating effects in chondrocytes may be greater than that of PKD1, despite slightly lower transcription. The reason for these results remains unclear but does suggest that pan PKD inhibition within chondrocytes has the potential to reduce the expression of MMPs stimulated with pro-inflammatory cytokines. The design of isoform specific inhibitors may lead to a greater inhibition of collagenase expression within chondrocytes.

4.4.3 The effect of PKD2 gene silencing on collagenase gene expression

Next, the role of PKD2 in the regulation of MMP-1 and MMP-13 gene expression was examined. PKD2 has been implicated in the induction of MMP gene expression through the regulation of ERK and JNK signalling pathways (Bernhart et al., 2013). Regulation of MMPs -1, -9 and -14 has been previously observed via PKD2 (Bernhart et al., 2013, Zou et al., 2012). PKD2 gene silencing in HACs had no effect on MMP-1 and MMP-13 gene expression under any stimuli. These data imply PKD2 does not regulate collagenase gene expression within primary chondrocytes when stimulated with IL-1 in combination with OSM. This data is further implied by the fact that PKD2 gene silencing also appears to increase the expression of PKD1 and PKD3; these isoforms have opposing roles in the regulation of collagenase gene expression. Therefore, as no increase or decrease in MMP gene expression is seen, these data further suggest PKD2 to have no effect on collagenase gene expression.

During the optimisation of these experiments the well-established chondrocyte like cell line SW1353 was used. This cell line was used to optimise many of the siRNAs used against each PKD isoform. This was due to HAC being technically difficult, due to their difficulty to obtain and variable responses. Previous work from our group has demonstrated variation in the responses of chondrocytes to the stimulation of IL-1 in combination with OSM (Barksby et al., 2006). These difficulties were overcome by the use of multiple HAC populations and the removal of any populations that did not respond well to cytokine stimulation. The SW1353 cell line has been shown to be a useful model to study collagenase response to IL-1 stimulation, with MMP-1 and MMP-13 responding in a similar manner under this stimulus to HAC (Gebauer et al., 2005). These cells were therefore used as a model for collagenase expression within primary chondrocytes when HACs were unavailable. When examining the effects of PKD2 gene silencing in SW1353 cells, a reduction in collagenase expression of both MMP-1 and MMP-13, under both stimuli is seen (*Figure 7.4* supplementary figures). This again shows cell specificity in the actions of each isoform of PKD, indicating that PKD2 regulation of collagenase gene expression is dependent on cell type. This may explain some of the conflicting roles of PKD within the literature. These data also show the value of using primary cells in studying a disease, as cell lines may not exhibit the same behaviour.

An explanation for these differences could be due to the expression profile of each isoform of PKD varying between different cell types. As previously mentioned, PKD1 is epigenetically suppressed in many tumour cells. This implies that cellular regulation of PKD expression can lead to the modulation of downstream effectors such as the expression of the MMPs. When comparing the expression profile of PKD1, PKD2 and PKD3 within normal and osteoarthritic cartilage, the expression pattern of each does not change (D A Young, personal communication). However, there is around 70% less PKD2 gene expression in both OA and normal cartilage compared to PKD1 and PKD3. RNA sequencing from the Young group, compared 6 samples of normal cartilage from neck of femur (NOF) fractures to 10 samples of osteoarthritic cartilage from patients undergoing total hip replacement (for methods see (Bui et al., 2012)). These data could explain why PKD2 silencing had no effect on

collagenase gene expression compared to the other two isoforms of PKD within primary chondrocytes. This may also explain why PKD2 has an effect in SW1353 cells, as the expression pattern of PKD2 may be different in these cells compared to primary chondrocytes, although such data are not available.

4.4.4 The effect of PKD3 gene silencing on MMP gene expression

The role of the PKD3 in the regulation of collagenase gene expression was then examined. siRNA mediated gene silencing of PKD3 led to decreased expression of MMP-1, MMP-2, MMP-8 and MMP-13, but had no effect on MMP-14. The same results were observed for MMP-1 and MMP-13 (no other MMPs assessed) in SW1353 (data not shown). The up-regulation of MMP-8, but lack of regulation of the gene expression of MMP-14, implies isoform specific regulation of MMP gene expression between PKD1 and PKD3, with each kinase regulating the expression of individual MMPs rather than the MMP family as a whole. As well as the expression of the MMPs studied, MMP-9 gene expression was examined. Unfortunately, when examining the effects of PKD1 silencing on the expression of MMP-9 the data were inconclusive, with the expression of MMP-9 being undetectable in many of the populations of HAC studied. PKD3 silencing was, however, shown not to regulate the expression of MMP-9 in HAC (*Figure 7.6* supplementary figures).

These data imply that PKD1 and PKD3 have opposing roles in regulating collagenase gene expression in HACs stimulated with IL-1 in combination with OSM. However, silencing of both PKD1 and PKD3 led to a decrease in the expression of MMP-2, implying that the mechanisms by which the expression of each MMP family member is regulated by is different; each isoform of PKD may therefore be regulating different MMPs via the same or distinct signalling pathways. Clues into the differential regulation may lie in the study of the transcription factors binding elements in different MMP promoters. These data shows the necessity to study not only each isoform of PKD and their regulation of MMP-1 and -13 gene expressions but to study the effects on other MMPs to fully elucidate their role in cartilage degradation.

The role of each individual isoform of PKD, in one study, within the same cell type has not previously been reported. However, two individual studies have

looked at the individual isoforms of PKD in the same prostate cancer cell lines. Biswas *et al.*, used PC3 cells and DU145 cells to elucidate the role of PKD1 in MMP gene expression, whereas Zou *et al.,* used both cell lines to study the roles of PKD2 and PKD3 in MMP regulation (Biswas et al., 2010, Zou et al., 2012). These two papers show that within these prostate cancers cell lines, all isoforms of PKD up-regulate the expression of MMP-9. These two papers suggest opposing roles for the expression of MMP-9 in these cancer cells, implying MMP-9 to either induce or suppress cancer progression. Other data within the literature in which different cell types have been used show conflicting effects of PKD1 (Wille et al., 2013). PKD1 is seen to both induce and suppress MMP-2 (Biswas et al., 2010, Onishi et al., 2012, Eiseler et al., 2009), with data presented in this chapter showing isoform specific regulation of this MMP. PKD1 has also been shown to down-regulate the expression of MMP-13 (Eiseler et al., 2009), as observed in the data presented here. However, the data presented within that paper showed PKD1 to also down regulate MMP-2 (Eiseler et al., 2009), opposing the data presented in this chapter. These data therefore imply that PKD1 dependent MMP regulation is dependent on cell type as well as the MMP studied. This is consistent with the data in this chapter, in which different MMPs are regulated in opposing manners through the same isoform under the same stimulus. The literature and the work performed in this chapter imply the need to study each isoform of PKD as well as the role of these on individual MMPs. Also of importance is the use of primary cells when studying a disease. If the cell line SW1353 had been primarily used, PKD2 would have thought to play a major role in collagenase gene induction in chondrocytes, and in HAC this is not the case.

The aims of this chapter were to elucidate the role of each PKD isoform in the regulation of collagenase gene expression. Here, I show for the first time opposing roles of PKD1 and PKD3 in regulating MMP-1 and MMP-13 gene expression within HAC. PKD2 appears to be poorly expressed within chondrocytes compared to the other isoforms; this may be an explanation for PKD2 not regulating collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. Data imply that the use of a pan-PKD inhibitor leads to reduced collagenase gene expression, indicating this inhibitor as a potential

therapeutic lead against the induction of these matrix degrading enzymes. However, this inhibitor is known to inhibit PKD1, which from the data shown in this study appear to suggest PKD1 to have the potential to be an anabolic kinase. Design of a PKD3 specific inhibitor may therefore be a better therapeutic. In the following chapter, I set out to further understand the signalling pathways which are regulated by each isoform of PKD under the stimulation of IL-1 in combination with OSM, in order to elucidate how PKD regulates collagenase expression under this pro-inflammatory stimulus.

4.4.5 Summary

Studies in this chapter have shown:

- PKD inhibition via the pan-PKD inhibitor kb NB 142-70 leads to the loss of MMP-1 and MMP-13 gene expression in chondrocytes stimulated with IL-1 alone or IL-1 in combination with OSM.
- PKD1 overexpression leads to a decrease in the expression of MMP-1 and MMP-13 in chondrocytes stimulated with IL-1 alone or IL-1 in combination with OSM.
- PKD1 down regulates the expression of the classical collagenases MMP-1 and MMP-13. PKD1 can also increase the expression of MMP-2 and MMP-14, no effect on MMP-8 is observed. This data suggests PKD1 can up and down regulate cartilage degrading enzymes.
- PKD2 does not regulate MMP-1 and MMP-13 gene expression within chondrocytes stimulated with IL-1 or IL-1 in combination with OSM.
- PKD3 signalling leads to the induction of MMP-1, -2, -8 and -13. No effect on MMP-14 gene expression is observed. PKD3 signalling induces the expression of cartilage degrading enzymes under a pro-inflammatory stimulus.

5 Chapter 5: The role of PKD in regulating signalling pathways and transcription factors involved in MMP gene induction

5.1 Introduction

Data presented in Chapter 4 demonstrated that collagenase gene expression is regulated in a PKD isoform specific manner. To understand the specific roles of each PKD isoform in the regulation of MMP gene expression, the modulation of signalling pathways known to promote collagenase gene expression were studied. Using gene silencing and over-expression of the individual isoforms of PKD, the regulation of these signalling pathways was studied in HAC and SW1353 cells. These cells were stimulated with IL-1, OSM or both in combination. These combined stimuli were used to represent the complex inflammatory milieu of cytokines elevated within an arthritic joint during disease.

MAPKs, STATs, PKC and the PI3K pathways have all been implicated in the regulation of collagenase gene expression under the complex stimulus of IL-1 in combination with OSM (Catterall et al., 2001, Litherland et al., 2008, Litherland et al., 2010, Richards et al., 2001). These signalling pathways have been shown to regulate the activity and expression of the AP-1 transcription factor, leading to MMP gene induction, under the stimulation of IL-1 in combination with OSM (Catterall et al., 2001, Litherland et al., 2010). Work performed in this chapter therefore was performed to understand how each isoform of PKD lies within these signalling cascades. The consequences of PKD signalling on the expression of the AP-1 transcription factor components and further latent transcription factors recently implicated in MMP gene induction were also examined.

IL-1 in combination with OSM has been shown to induce the gene expression of multiple MMPs in HAC (Koshy et al., 2002b). The first paper to try to elucidate the signalling consequences which regulate their induction within a chondrocyte setting was by Catteral *et al.,* 2001. Here, the effects of IL-1 in combination with OSM were studied on MMP-1 gene expression; data implicated that the STAT signalling pathway and AP-1 family transcription factors were important in this

regulation. To understand the induction of MMP-1 gene expression induced by IL-1 in combination with OSM, the binding of transcription factors to the proximal promoter of MMP-1 was studied. The proximal MMP-1 promoter has two AP-1 binding elements located at -181 and -72bp upstream from the transcriptional start site. A STAT binding element (SBE) is also found adjacent to the -72 AP-1 site (Yan and Boyd, 2007). When AP-1 and STAT binding to the proximal region (-517/+63) of the MMP-1 promoter was assessed using EMSA, it was found that AP-1 proteins were constitutively associated with this promoter, under basal conditions. No STAT proteins could be detected bound to the SBE element (Catterall et al., 2001). However, data did imply a role for the STATs in the induction of MMP-1. The over-expression of protein inhibitor of activated STAT-3 (PIAS-3) completely abolished OSM dependent MMP-1 promoter activity. These data implied that STAT-3 regulated MMP-1 gene expression, albeit not through direct binding to the MMP-1 promoter. Unpublished EMSA data from our laboratory suggest that STAT-1, -3 and -5 are all capable of binding to the c-*fos* promoter under synergistic conditions (Rowan, personal communication). This work implies that the STAT pathway regulates MMP-1 gene expression via the induction of c-*fos*.

The regulation and induction of AP-1 transcription factors is not only dependent on OSM-dependent STAT induction; IL-1 and OSM can also induce AP-1 component expression via the regulation of the MAPK pathways.

The ERK pathways can be activated by both IL-1 and OSM (Vincenti and Brinckerhoff, 2002, Heinrich et al., 2003). This pathway can induce the expression of Fos as well as inducing post-translational modifications, increasing the transcriptional capabilities of this transcription factor. ERK, upon activation, translocates to the nucleus, inducing the phosphorylation of Elk-I (Gille et al., 1995), leading to increased Fos transcription. ERK is also seen to directly phosphorylate Fos at Ser374, leading to increased stability and transactivation activity (Chen et al., 1993). ERK inhibition was shown to significantly reduce Fos protein expression in cells stimulated with IL-1 in combination with OSM; this was suggested to abrogate collagenase gene

expression (Litherland et al., 2010). All of these data implicate ERK signalling in the regulation of collagenase gene expression.

The JNK signalling pathway is the major regulator of the Jun family of transcription factors. Phosphorylation and activation of JNK leads to its translocation to the nucleus. Within the nucleus JNK phosphorylates Jun at Ser63 and Ser73, enhancing the transactivation potential of Jun (Hess et al., 2004). This implicates the regulation of Jun via JNK as an important signalling cascade in the induction of collagenase gene expression. This is important due to Jun/Fos dimers being seen as the most potent AP-1 complex to drive MMP-1 gene expression (Bakiri et al., 2002). JNK can also regulate the expression of c-*fos* through Elk-1 phosphorylation (Cavigelli et al., 1995), further illustrating the importance of this signalling molecule in AP-1 component expression and activity.

p38, the final member of the MAPK family can also induce c-*fos* gene expression. p38, like ERK, is known to phosphorylate Elk-1 enhancing its transcriptional activity, leading to the induction of c-*fos* (Whitmarsh et al., 1997). This induction has been shown to modulate MMP gene expression (Reunanen et al., 2002, Lim and Kim, 2011). Unpublished data from this laboratory has shown p38 gene silencing to reduce the expression of both MMP-1 and MMP-13 when stimulated with IL-1 in combination with OSM (Litherland and Rowan, personal communication), indicating the importance of this signalling pathway.

The PI3K signalling pathway has also been implicated in the regulation of collagenase gene expression in chondrocytes stimulate with IL-1 in combination with OSM. These data implicated PI3K class IA of PI3Ks as the regulators of collagenase expression; this activation was shown to be via Akt activation (Litherland et al., 2008). PKC, a further downstream target of both PI3K signalling and IL-1 has been implicated in the regulation of collagenase gene expression. PKC, the upstream activator of PKD has been shown to regulate collagenase expression through the induction of the STAT and ERK pathways, leading to changes in AP-1 component gene expression (Litherland et al., 2010).

All of these signalling pathways have been implicated in the regulation of collagenase gene expression, with stimulation leading to changes in the expression and stability of the components of the AP-1 transcription factor. Understanding the role of each PKD isoform in the regulation of these signalling cascades will provide mechanistic data into the induction of collagenase gene expression modulated by PKD. Using our model of the inflammatory milieu of cytokines involved in arthritis, I will examine the role of each PKD isoform in the regulation of signalling pathways implicated in the modulation of collagenase gene expression.

5.2 Aims

- Understand whether PKD is phosphorylated in HAC stimulated with either IL-1 or OSM.
- Establish whether PKC regulates PKD phosphorylation in HAC and the isoforms which are involved.
- Establish the consequences of PKD1 over-expression and silencing, as well as PKD2 and PKD3 silencing on the signalling pathways known to regulate collagenase gene expression under the pro-inflammatory stimulation of IL-1 and OSM alone or when in combination.
- Establish the role of each isoform of PKD on the expression of the AP-1 family members; c-*jun* and c-*fos* and other newly identified post-AP-1 factors.

5.3 Results

5.3.1 Phosphorylation and activation of PKD by IL-1 and OSM

To assess the phosphorylation of PKD by IL-1 or OSM, two PKD phosphorylation sites were studied; Ser744/748 and Ser916. The Ser744/748 site is phosphorylated directly by PKC whilst the Ser916 is an autophosphorylation site which indicates PKD activation. Although these are the sites of PKD1 phosphorylation, both antibodies cross-react with each PKD isoform (except Ser916 in PKD3, as this site is not present within this isoform).

As *Figure 5.1. A* shows, IL-1 stimulation of HACs led to a transient activation of PKD. Phosphorylation is identified with both antibodies at the 20 minute time point. This is nearly completely abrogated by 45 minutes, with levels of phosphorylation at both sites being diminished to levels only slightly higher than the basal conditions. For OSM, phosphorylation at Ser744/748 occurred from 20 minutes, and persisted until 120 minutes. The peak phosphorylation was at 20-45 minutes, with 45 minutes also being the time point for maximal phosphorylation at Ser916 which was much more transient (*Figure 5.1. B*).

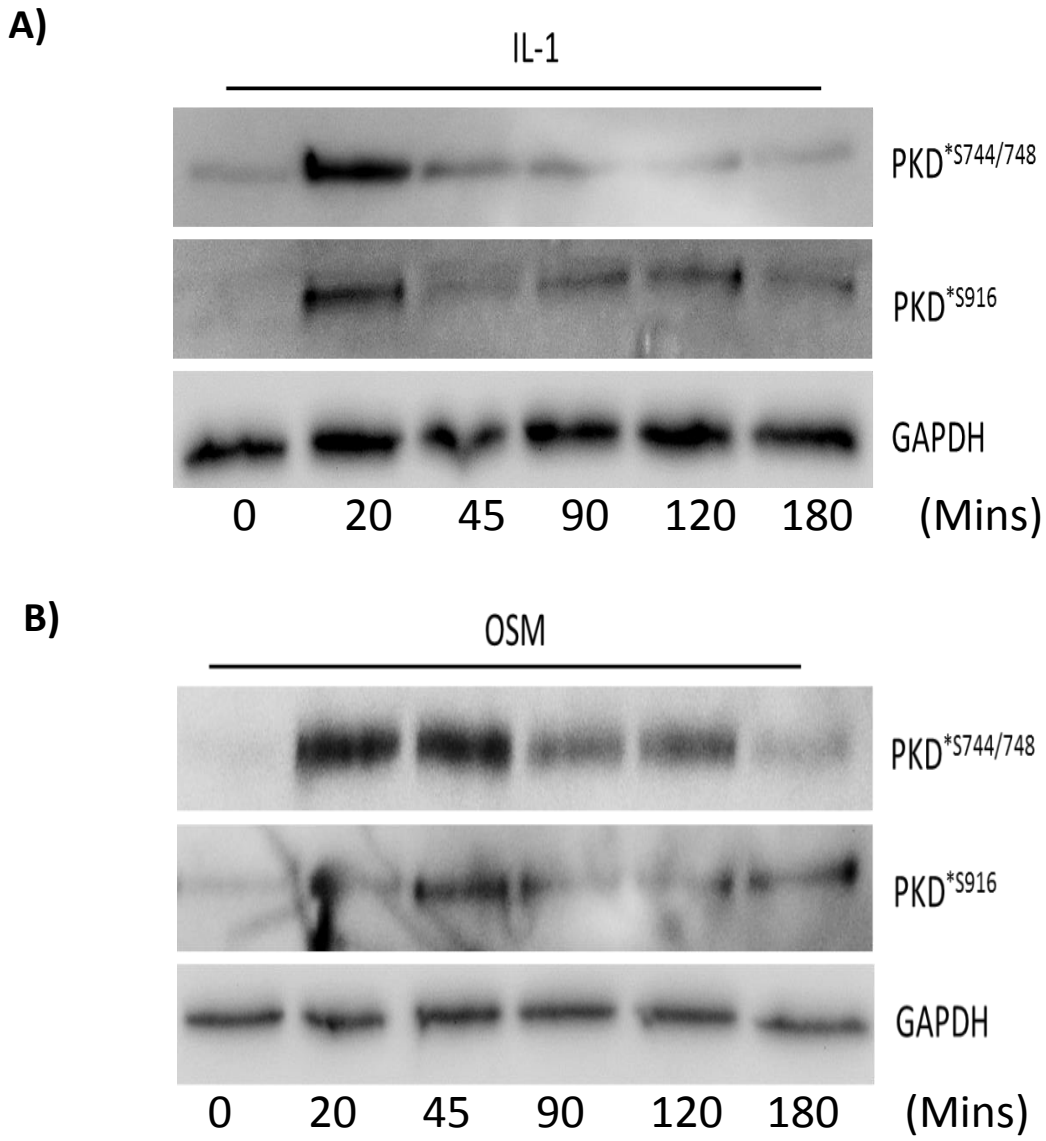


Figure 5.1. The phosphorylation of PKD in chondrocytes stimulated with IL-1 or OSM. P0 HAC plated in 6 well plates were grown to ~70% confluency, and then serum starved over-night. The following day cells were stimulated for various time points with either (A) IL-1 (0.2 ng/ml) or (B) OSM (10 ng/ml) for the times shown. Cell lysates were then immunoblotted using the antibodies shown, as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

5.3.2 The subcellular distribution of each isoform of PKD

The subcellular distribution of each isoform of PKD in SW1353 cells, when stimulated with IL-1 in combination with OSM, was examined. As *Figure 5.2* shows, all isoforms of PKD were detected in the cytoplasmic, membrane and nuclear fractions (PKD isoforms could not be detected in the chromatin bound or cytoskeletal fractions (data not shown)). Stimulation with IL-1 in combination with OSM led to a small increase in the protein levels of each isoform in the cytoplasmic fraction, but appeared to have little effect on the subcellular distribution of each PKD isoform.

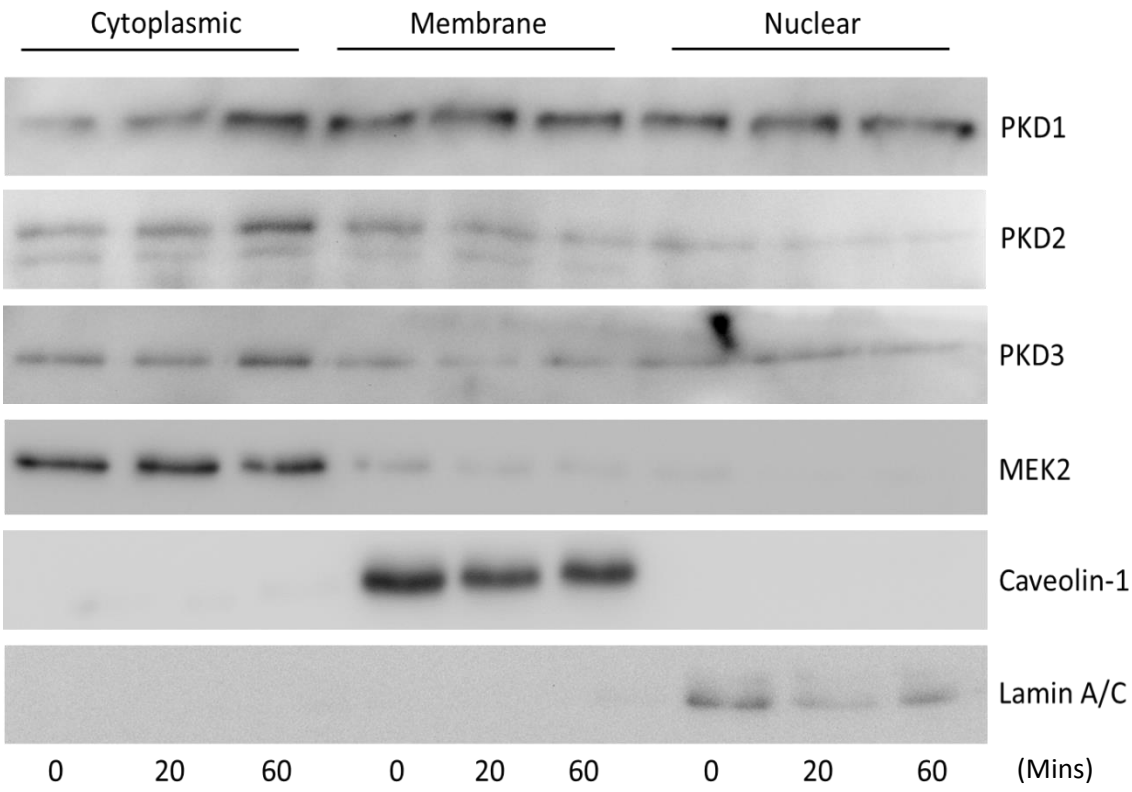


Figure 5.2. The subcellular distribution of each PKD isoform when stimulated with IL-1 in combination with OSM in SW1353 cells. SW1353 cells were plated into 10 cm dishes and grown to ~70% confluency, and then serum starved over-night. The following day cells were stimulated for various time points with IL-1 (0.5 ng/ml) in combination with OSM (10 ng/ml) for the time points shown. Cells were scraped into PBS and separated into subcellular fraction using the Pierce subcellular fractionation kit as described by the manufacturer's instructions. Fractions were then separated by electrophoresis. Protein was then transferred to PVDF membrane and immunoblotted using the antibodies shown, as described in the Materials and Methods. Data are representative of at least two separate chondrocyte populations. MEK2 was used as a cytoplasmic loading control, Caveolin-1 was used as a membrane loading control and Lamin A/C was used as a nuclear loading control.

5.3.3 The regulation of PKD via PKC

Previous data within our group have demonstrated a role for the PKCι and PKCζ in the regulation of collagenase gene expression in HAC stimulated with IL-1 in combination with OSM (Litherland et al., 2010). As PKD is well established as a downstream signalling target of PKC (Waldron et al., 2004, Iglesias et al., 1998, Zugaza et al., 1996), it was hypothesised that PKD may be regulated by one/both of these proteins.

5.3.3.1 Broad spectrum PKC inhibition

To first establish whether PKC phosphorylates PKD in chondrocytes stimulated with IL-1 or OSM, HACs were treated with the PKC inhibitor Gö6983. This inhibitor is a potent and reversible ATP-competitive inhibitor that does not effectively inhibit PKD at low concentrations. The addition of Gö6983 completely abolished PKD phosphorylation at both phosphorylation sites. This implicated PKC in the direct phosphorylation of PKD at the Ser744/748 site as well as PKD activation, as indicated by loss of phosphorylation at the Ser916 site (*Figure 5.3. A*).

5.3.3.2 Isoform specific roles of PKC in the regulation of PKD

Following effective PKCι and PKCζ silencing (*Figure 5.3. B*), loss of PKD phosphorylation at the PKC dependent phosphorylation site, Ser744/748, was only observed with PKCι depletion (*Figure 5.3. C*)

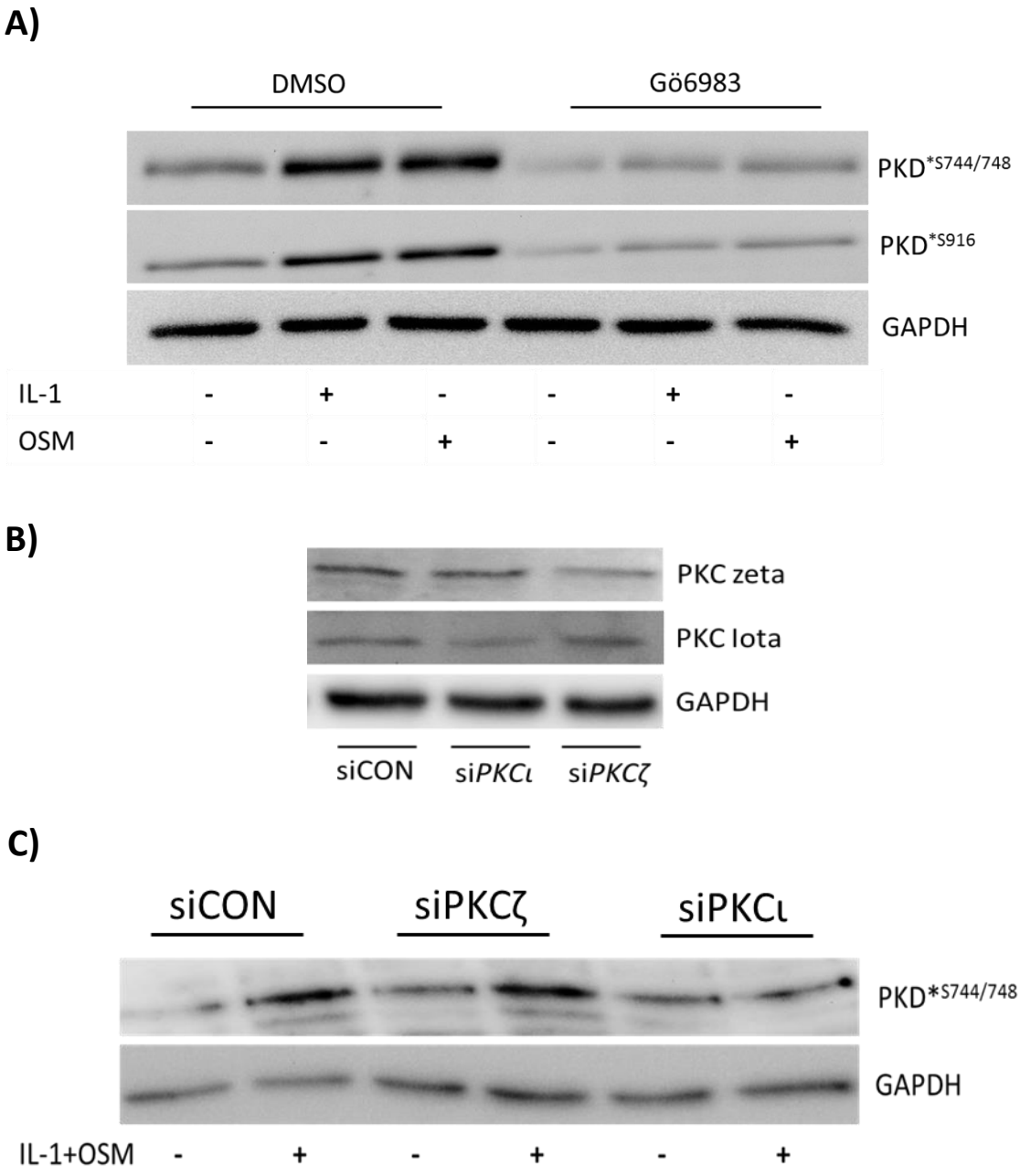


Figure 5.3. The role of PKC in the regulation of PKD activity. (A) P0 HAC were plated into 6 well plates and grown till ~70% confluency, and then serum starved over-night. The following day cells were pre-treated with Gö6983 (20 μM) for 1 hour prior to being stimulated with either IL-1 (0.2 ng/ml) or OSM (10 ng/ml) for 20 minutes and then lysed. (B) P0 HAC were trypsinised and plated into 6 well plates and then left over-night. The following day cells were transfected with siRNA against PKCζ, PKCι or siCON (100 nM) for 48 hours. Cells were then serum starved over-night. Cells were then either lysed or (C) stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 minutes and then lysed. Lysates were then separated by electrophoresis. Protein was then transferred to PVDF membrane and immunoblotted using the antibodies shown, as described in the Materials and Methods Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

5.3.4 PKD regulates multiple signalling pathways involved in MMP gene expression

Once the individual role of each isoform of PKD in the regulation of collagenase gene expression was established (see *chapter 4*), I next sought to understand how each isoform of PKD could be regulating these changes. To achieve this each isoform of PKD was either silenced or over-expressed and the effects of this on known signalling pathways involved in MMP gene expression were studied.

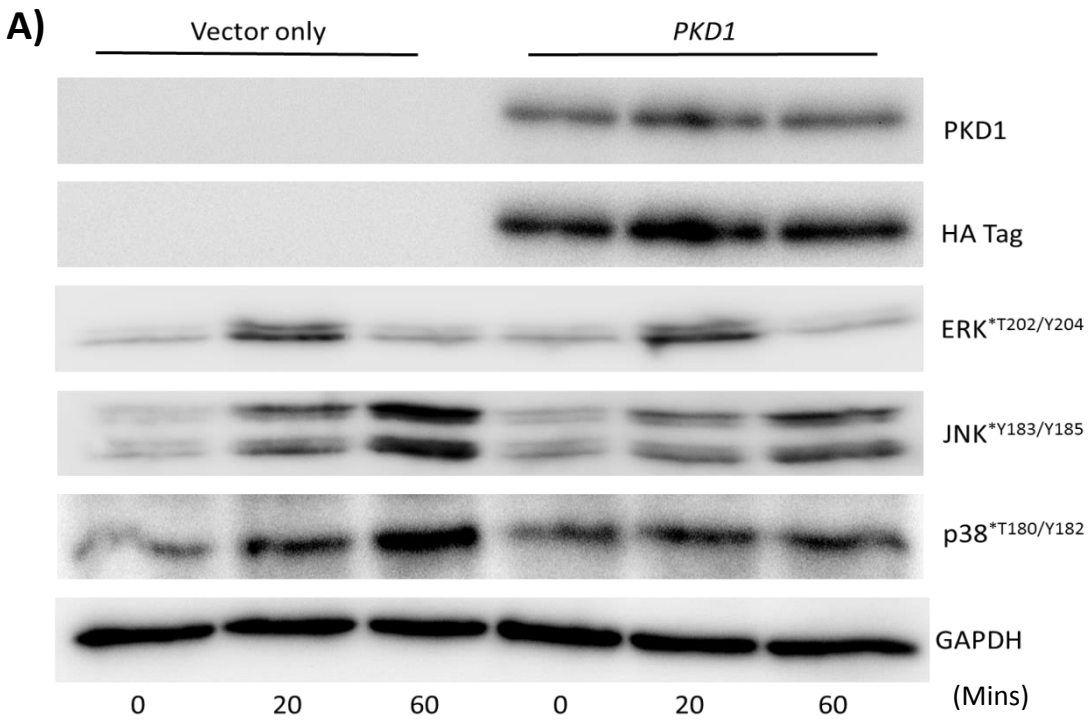
5.3.4.1 The effect of PKD1 overexpression on IL-1- ± OSM stimulated signalling pathways in SW1353

Over-expression of PKD1 was confirmed by the identification of the HA tag, which was covalently linked to the over-expressed PKD1. PKD1 expression was also used to confirm expression. PKD1 was not identified in control cells due to the high expression of PKD1 in the over-expressed cells, preventing the identification of the native PKD1 be western blotting.

As can be seen in *Figure 5.4*, when PKD1 was over-expressed in SW1353 cells stimulated with IL-1 alone, the phosphorylation of p38 and JNK was abrogated. No changes in the phosphorylation of ERK were observed.

As *Figure 5.5* shows, when PKD1 was over-expressed in SW1353 cells stimulated with OSM alone, a reduction in Akt phosphorylation occurred, suggesting reduced Akt signalling. PKD1 over-expression had no effect on the phosphorylation of ERK, STAT-1 or STAT-3 at their tyrosine phosphorylation site.

When PKD1 was over-expressed in SW1353 cells stimulated with IL-1 in combination with OSM, a reduction in the levels of phosphorylation of both p38 and JNK was seen (*Figure 5.6*). Interesting to note is the change in kinetics for the time points at which JNK and p38 are phosphorylated when comparing IL-1 alone to IL-1 in combination with OSM. No effects on the phosphorylation of Akt, ERK, STAT-1 or STAT-3 at their tyrosine phosphorylation site were observed.



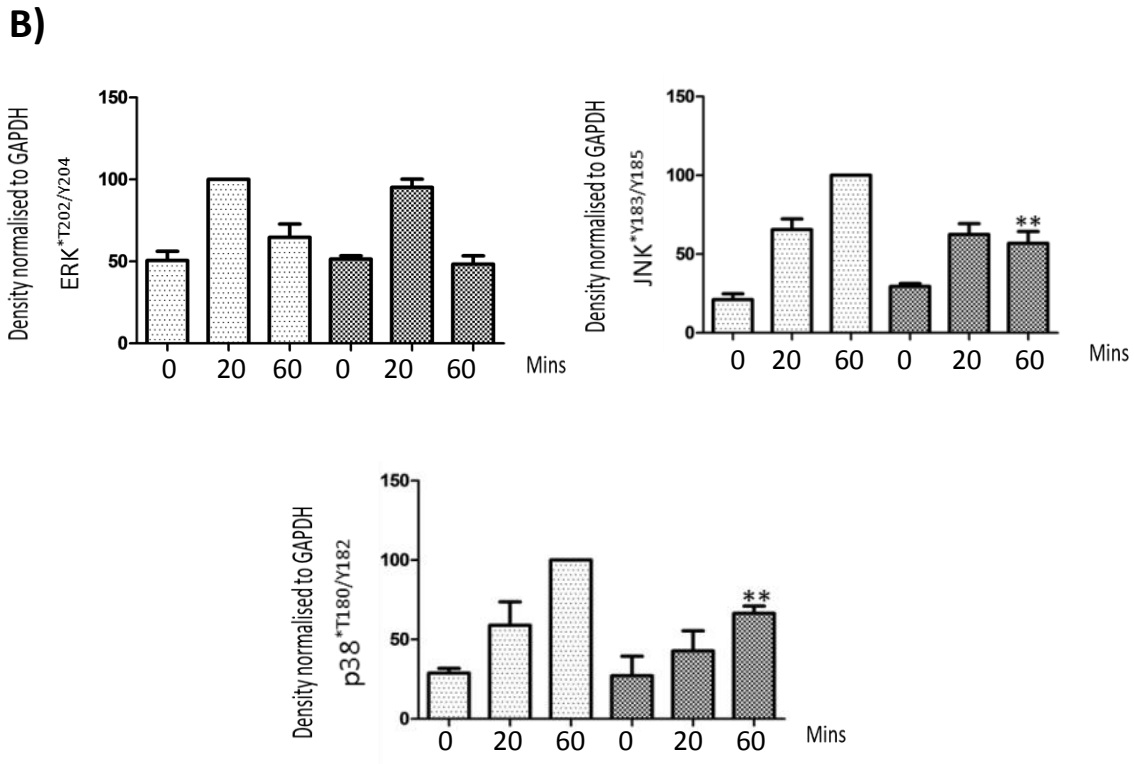


Figure 5.4. The effect of PKD1 over-expression on the signalling pathways activated by IL-1. (A) SW1353 cells were plated into 6 well plates and left over-night. The following day cells were transfected using 1 µg of PKD1 plasmid DNA with JetPEI, at 1:2. 1µg of vector was used as a control. Cells were left for 24 hours and then serum starved over-night. The following day cells were stimulated with IL-1 (0.5 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH levels presented as a percentage of the cytokine-induced expression. Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) **, p≤0.01, vs transfected vector.

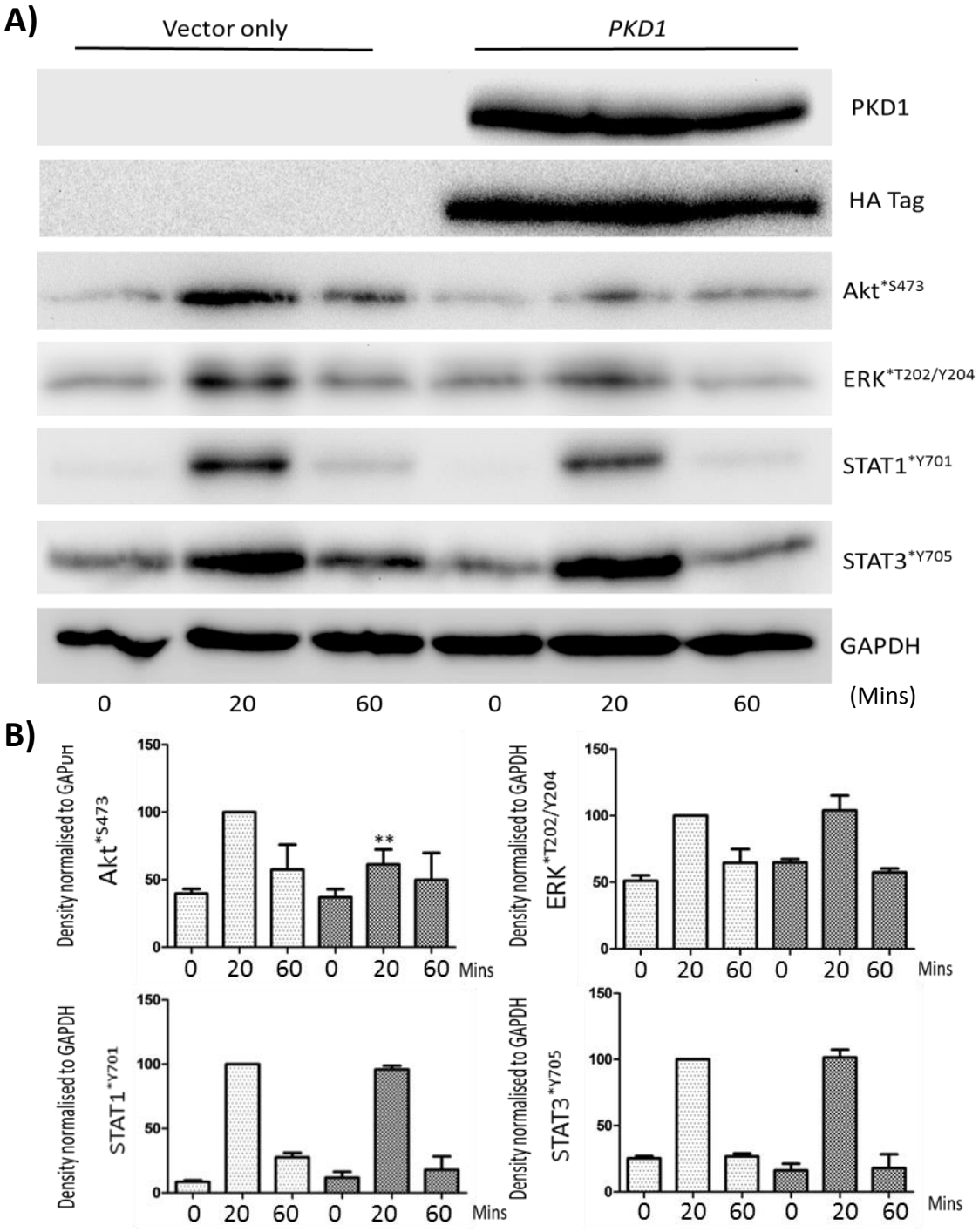


Figure 5.5. The effect of PKD1 over-expression on the signalling pathways activated by OSM. (A) SW1353 cells were plated into 6 well plates and left over-night. The following day cells were transfected using 1 µg of PKD1 plasmid DNA with JetPEI, at 1:2. 1µg of vector was used as a control. Cells were left for 24 hours and then serum starved over-night. The following day cells were stimulated with OSM (10 ng/ml) for 20 or 60 minutes. Cells were lysed and separated by electrophoresis. Proteins were then transferred to PVDF membrane and immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH levels presented as a percentage of the cytokine-induced expression. Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) **, p≤0.01, vs transfected vector.

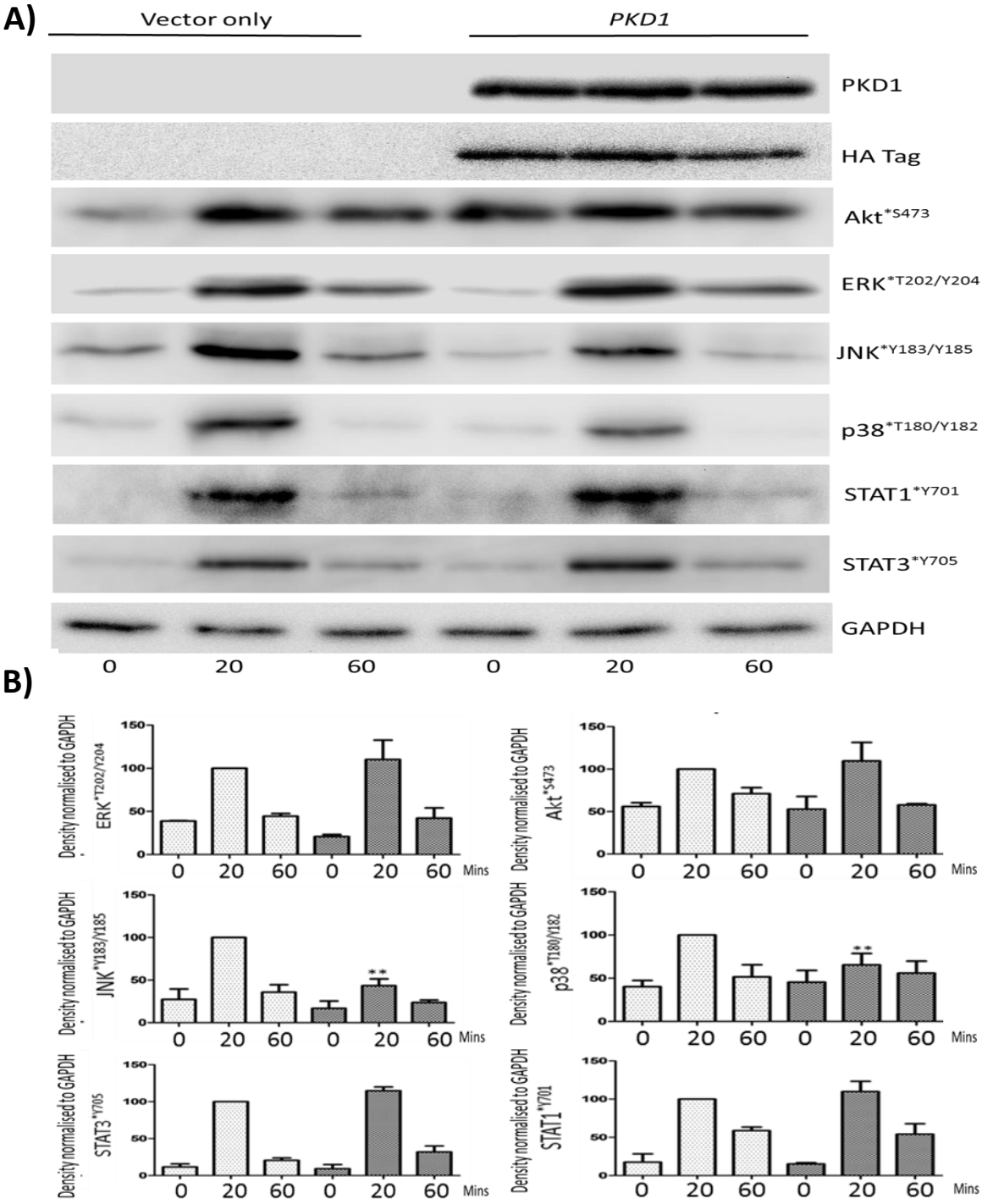


Figure 5.6. The effect of PKD1 over-expression on the signalling pathways activated by Il-1 in combination with OSM. (A) SW1353 cells were plated into 6 well plates and left over-night. The following day cells were transfected using 1 µg of PKD1 plasmid DNA with JetPEI, at 1:2. 1µg of vector was used as a control. Cells were left for 24 hours and then serum starved over-night. The following day cells were stimulated with IL-1 (0.5 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH, levels presented as a percentage of the cytokine-induced expression. Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) **, p≤0.01 vs transfected vector.

5.3.4.2 The effect of PKD1 gene silencing on IL-1- ± OSM stimulated signalling pathways in HAC

After the optimisation of the gene silencing of PKD1 using lentiviral-mediated delivery of shRNA, I used this technique to establish the role of PKD1 in HAC. This work only studied the effects of PKD silencing on signalling pathways induced by IL-1 in combination with OSM. Unlike the work using PKD1 over-expression, these experiments were performed in HAC. As *Figure 5.7* shows, PKD1 gene silencing led to a decrease in the phosphorylation of ERK, JNK and p38. However, PKD1 gene silencing also increased the levels of Akt, STAT-1 phosphorylation at the Tyr701 and Ser727 sites. No effect on the phosphorylation of STAT-3 at Ser727 or Tyr705 was observed.

NFκB signalling was then assessed. Unfortunately, due to poor detection of NFκB protein by the antibody, these results were only replicated in two HAC populations. As *Figure 5.8* shows, when PKD1 was silenced in two HAC populations an increase in the levels of phosphorylated p65 were detected.

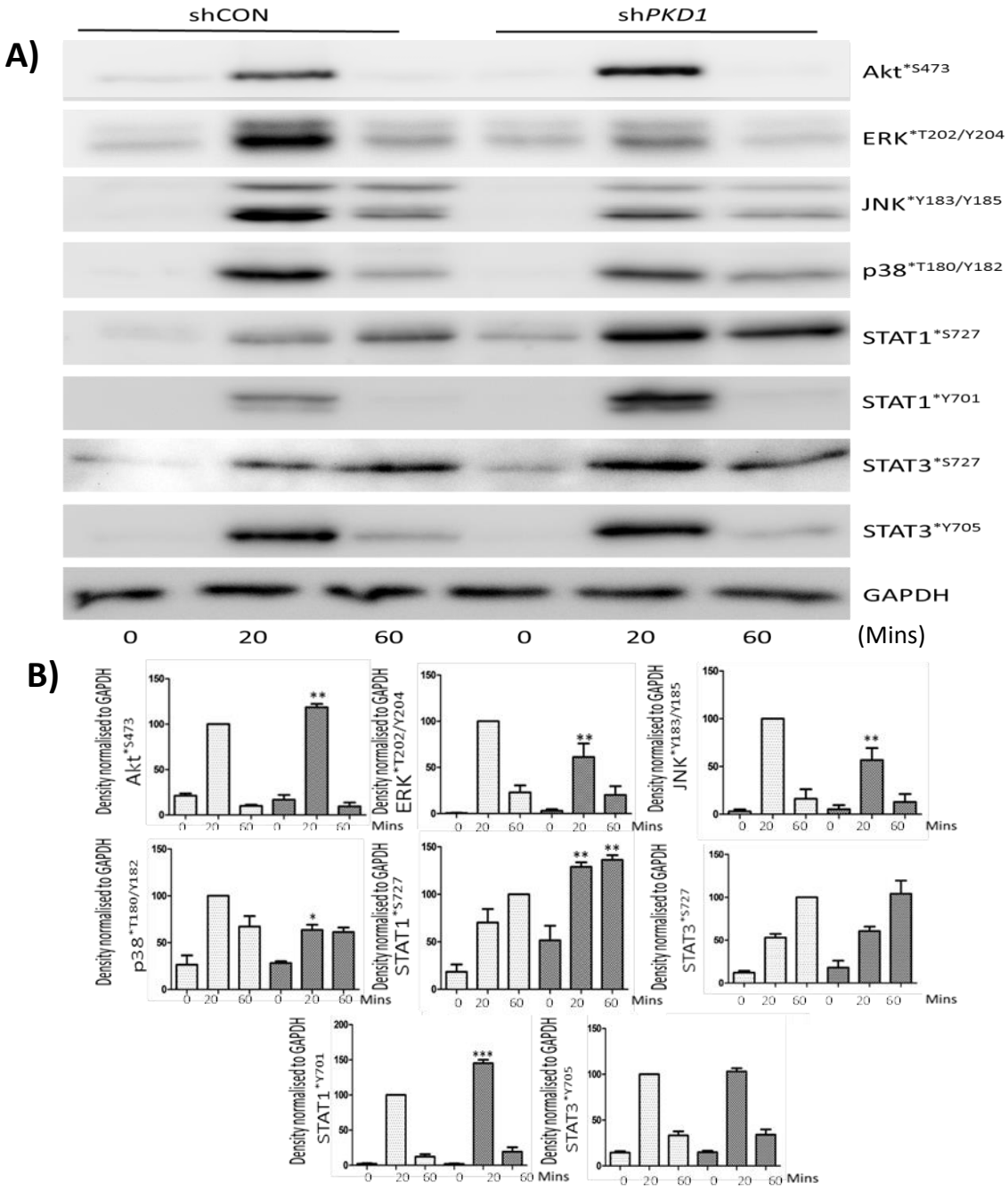


Figure 5.7. The effect of PKD1 gene silencing on the signalling pathways activated by IL-1 in combination with OSM in HAC. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day the medium was removed and replaced with viral supernatant containing either PKD1 shRNA #2 or shCON at a MOI of 30, in a total volume of 1 ml SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH levels, presented as a percentage of the cytokine-induced expression (shCON-transfected).Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) ***, p≤0.001, **, p≤0.01, *, p≤0.05. vs shCON.

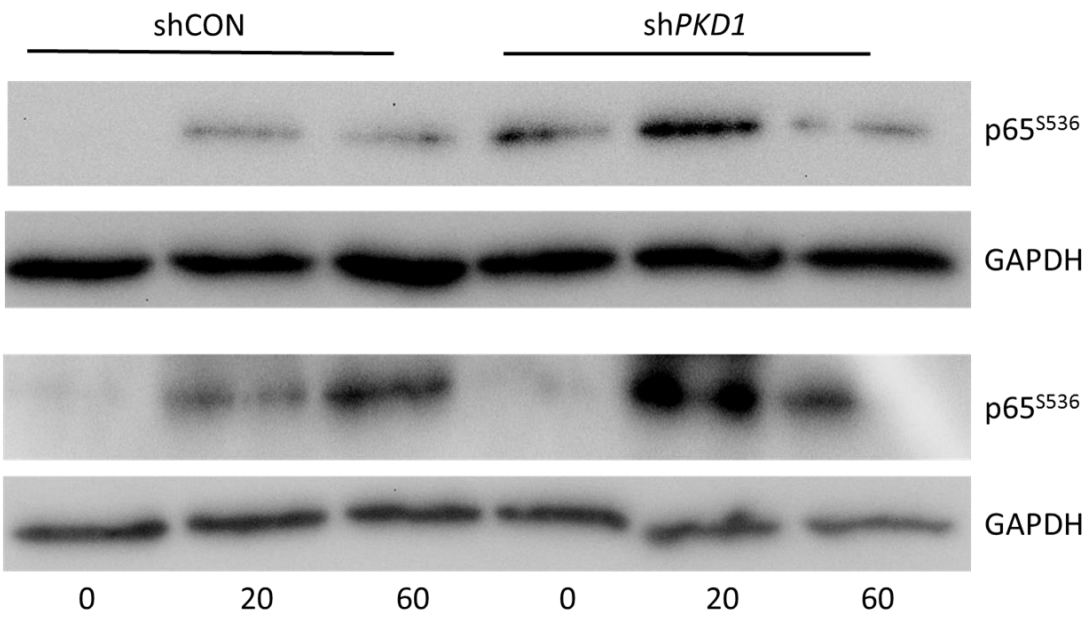


Figure 5.8. The effect of PKD1 gene silencing on p65 phosphorylation when stimulated with IL-1 in combination with OSM in two individual HAC populations. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day the medium was removed and replaced with viral supernatant containing either PKD1 shRNA #2 or shCON at a MOI of 30, in a total volume of 1 ml SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of two separate chondrocyte populations. GAPDH was used as a loading control.

5.3.4.3 The effect of PKD2 gene silencing on IL-1- ± OSM stimulated signalling pathways in HAC

As can be seen in *Figure 5.9*, when PKD2 is silenced in HAC stimulated with IL-1 alone, a reduction in the phosphorylation of ERK, p38 and JNK, STAT-1 and STAT-3 (Ser727) is observed. PKD2 appears to regulate all signalling pathways implicated in collagenase gene expression that are stimulated by IL-1.

When PKD2 is silenced in HAC stimulated with OSM alone, the phosphorylation of Akt is abrogated (*Figure 5.10*). No effect on the phosphorylation of ERK or the tyrosine phosphorylation of STAT-1 and STAT-3 is seen.

Figure 5.11 shows that when PKD2 is silenced in HAC stimulated with IL-1 in combination with OSM, the phosphorylation of STAT-1 and STAT-3 at Ser727 is reduced. Signalling through PKD2 appears to increase the phosphorylation of p38, suggesting this pathway is usually inhibited by PKD2 under this stimulus. PKD2 does not appear to regulate the phosphorylation of Akt, ERK, JNK or the tyrosine phosphorylation of STAT-1 (Tyr701) and STAT-3 (Tyr705).

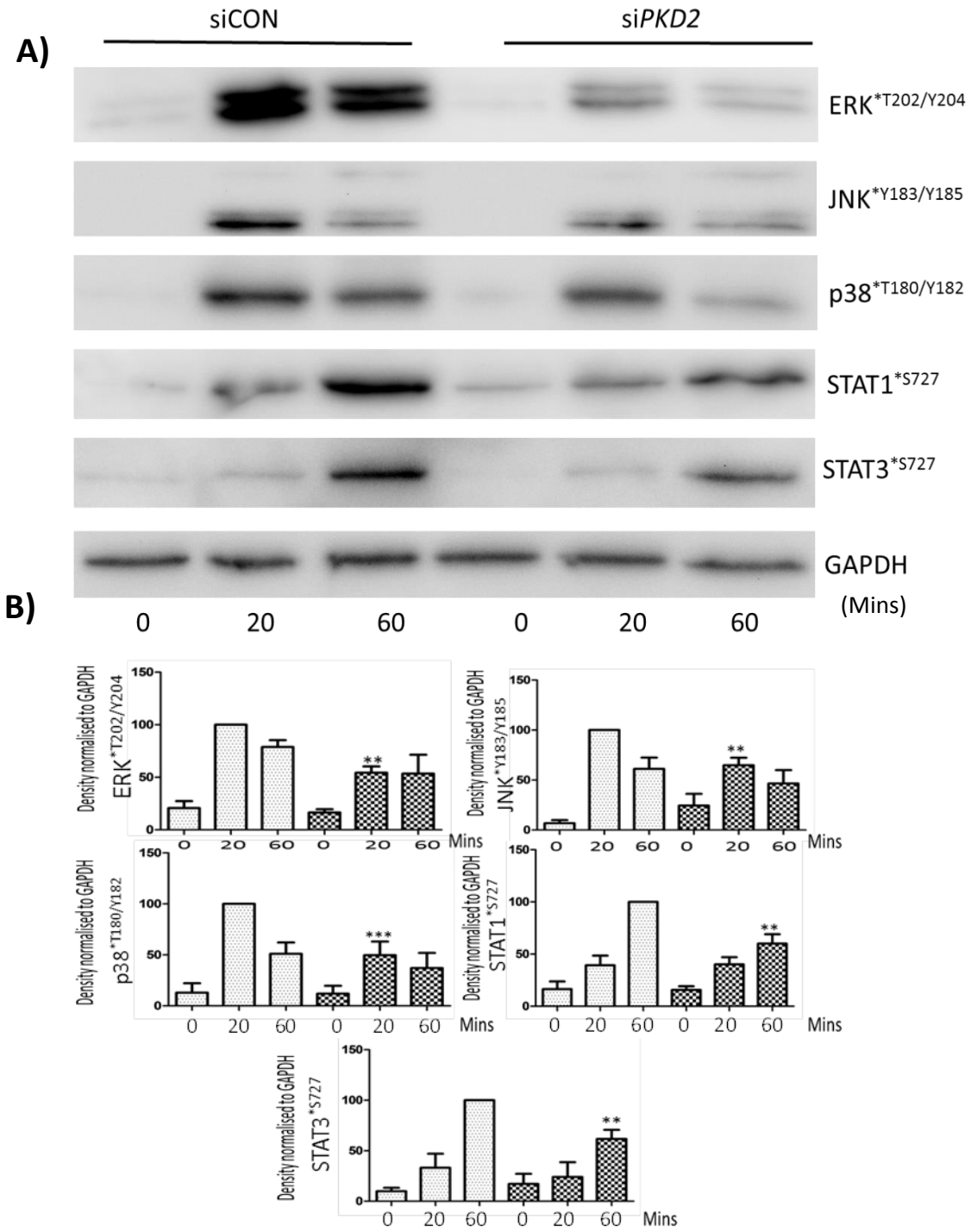


Figure 5.9. The effect of PKD2 gene silencing on IL-1 stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD2 #3 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH, levels presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) ***, p≤0.001, **, p≤0.01, vs siCON.

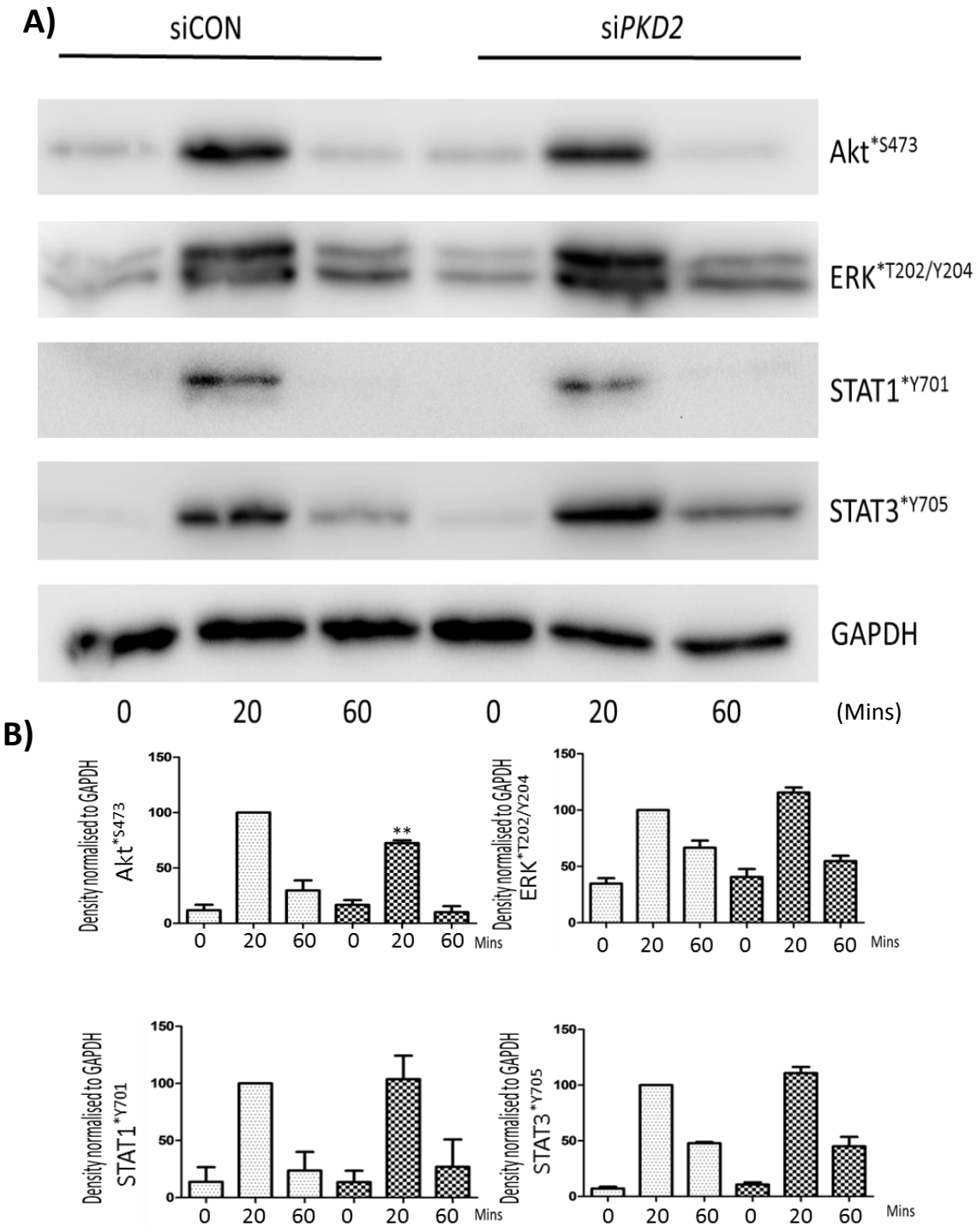


Figure 5.10. The effect of PKD2 gene silencing on OSM stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD2 #3 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH, levels presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) **, p≤0.01, vs siCON.

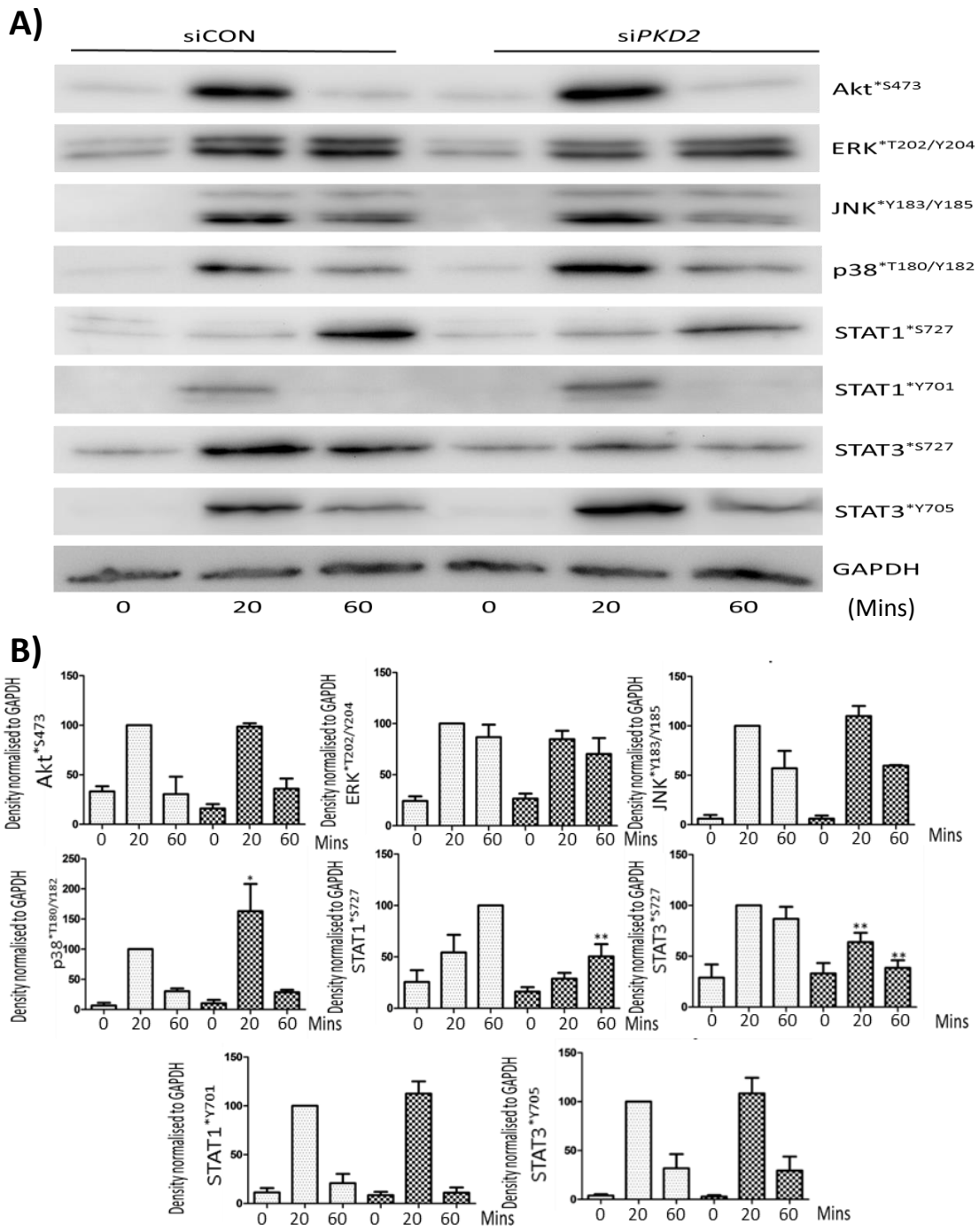


Figure 5.11. The effect of PKD2 gene silencing on IL-1 in combination with OSM stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD2 #3 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH levels, presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) **, p≤0.01, *, p≤0.05 vs siCON.

5.3.4.4 The consequences of PKD3 gene silencing on IL-1- ± OSM stimulated signalling pathways in HAC

PKD3 gene silencing in HAC stimulated with IL-1 alone, led to the reduction of ERK, p38, JNK, STAT-1 and STAT-3 (Ser727) phosphorylation (*Figure 5.12*). PKD3 appears to regulate all signalling pathways implicated in collagenase gene expression which are stimulated by IL-1.

As *Figure 5.13* indicates, PKD3 gene silencing in HAC stimulated with OSM alone had no effect on any of the signalling pathways studied.

As shown in *Figure 5.14*, PKD3 gene silencing in HAC stimulated with IL-1 in combination with OSM led to the abrogation of ERK, p38, JNK, STAT-1 and STAT-3 (Ser727) phosphorylation. PKD3 silencing has no effect on the phosphorylation of Akt, STAT-1 (Tyr701) or STAT-3 (Tyr 705).

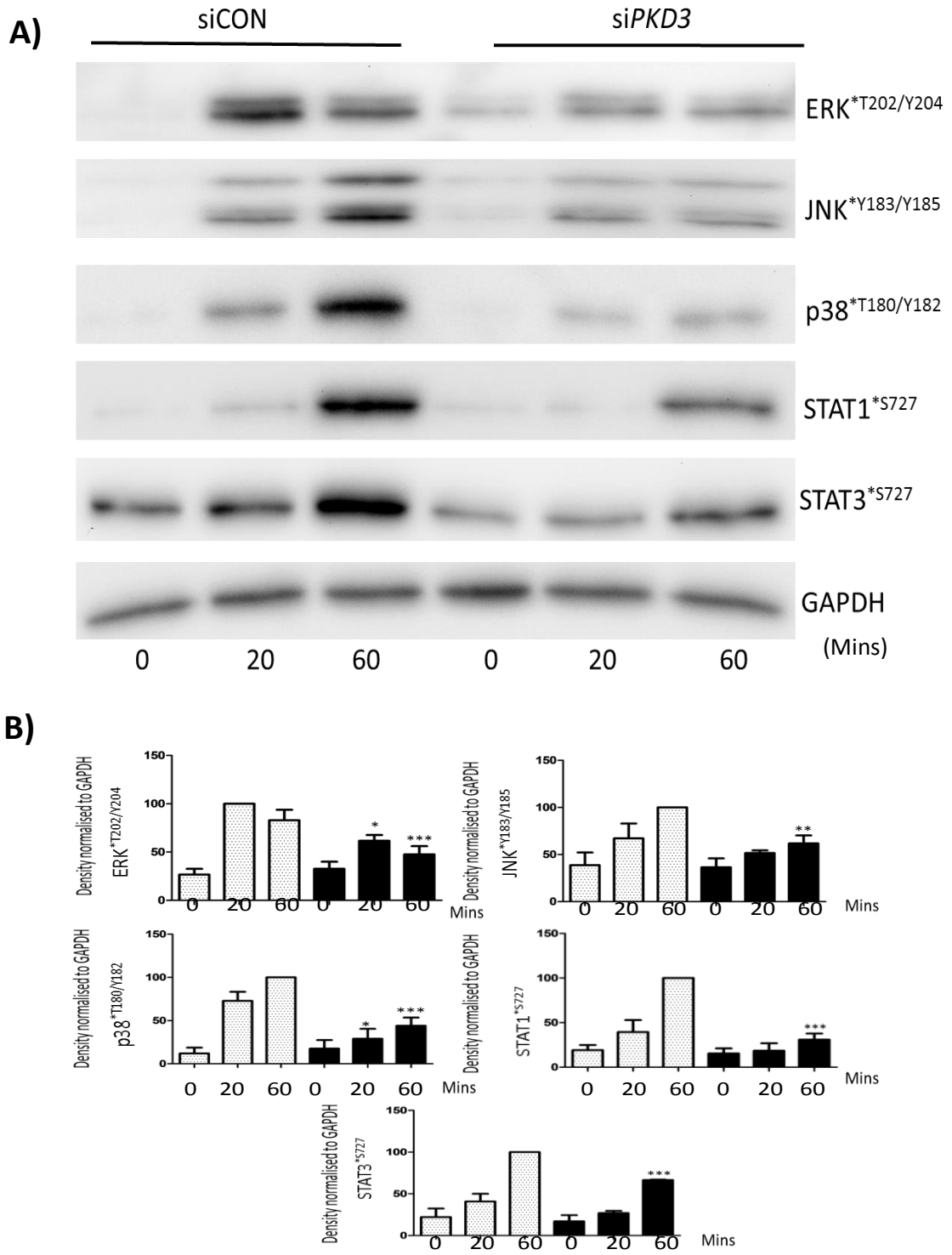


Figure 5.12. The effect of PKD3 gene silencing on IL-1 stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD3 #2 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B) Histograms show quantification of bands normalized to GAPDH levels presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D) ***, p≤0.001, **, p≤0.01, *, p≤0.05 vs siCON.

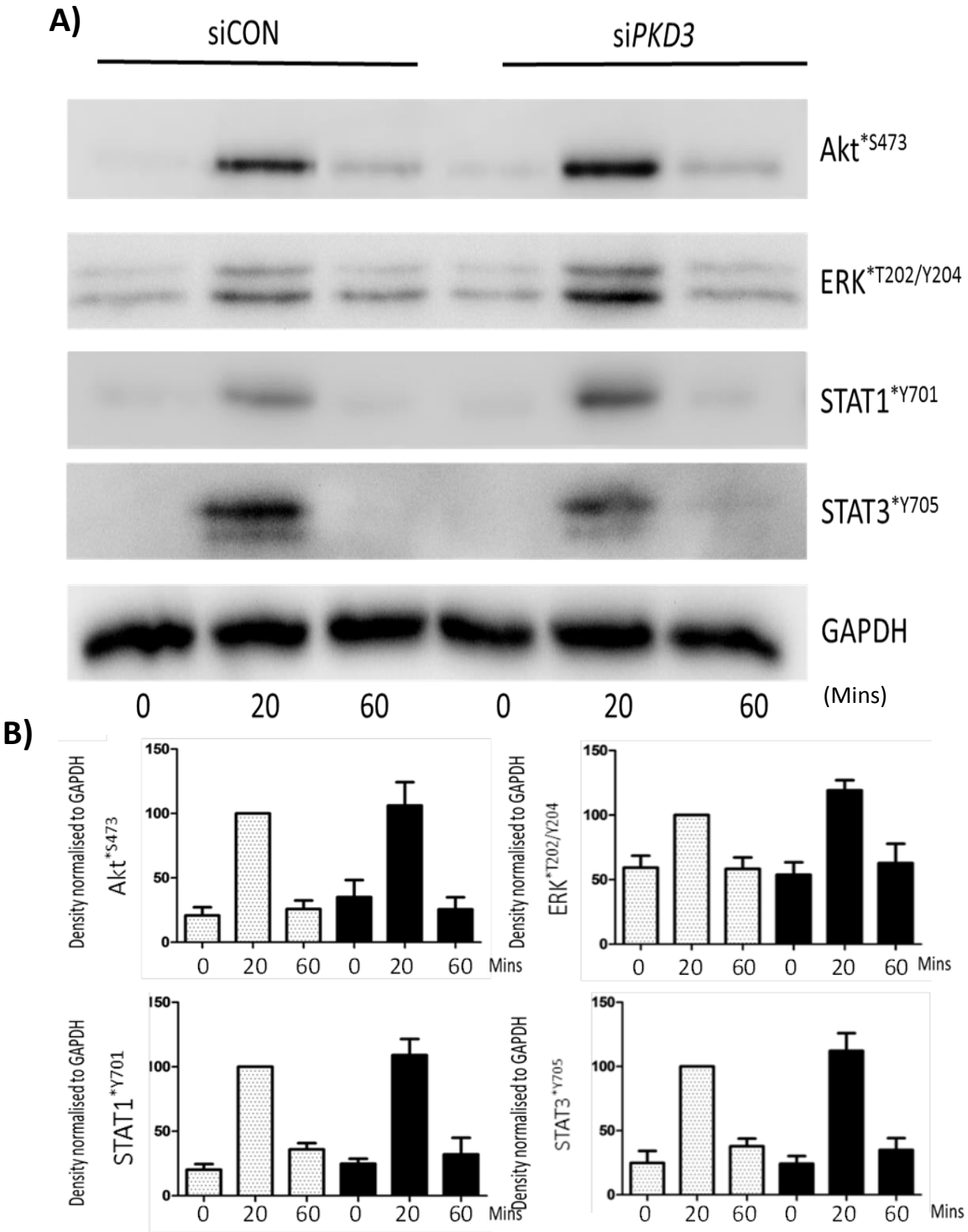


Figure 5.13. The effect of PKD3 gene silencing on OSM stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD3 #2 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with OSM (10 ng/ml) for 20 or 60 minutes. Cells were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B)Histograms show quantification of bands normalized to GAPDH levels presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D)

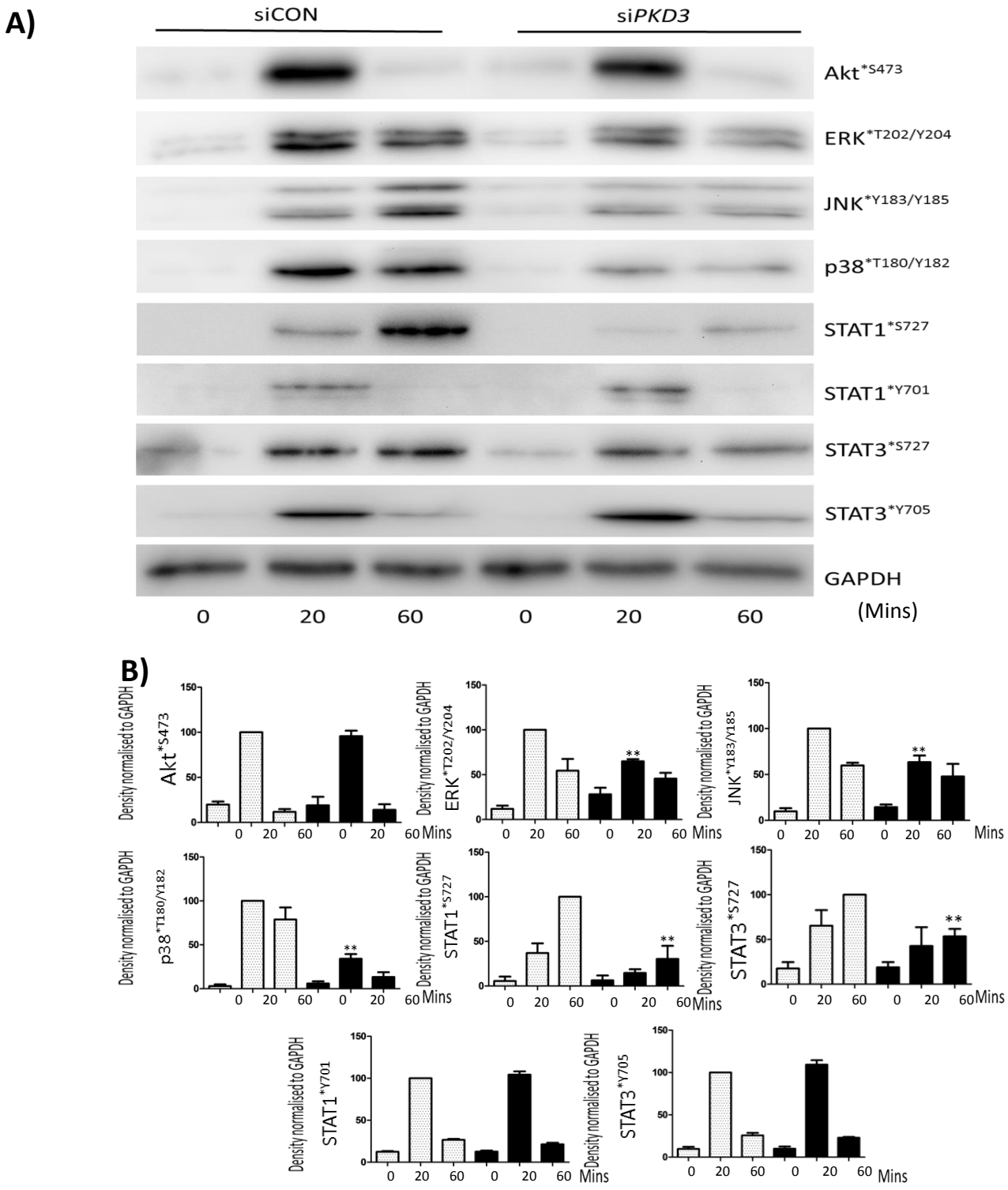


Figure 5.14. The effect of PKD3 gene silencing on IL-1 in combination with OSM stimulated signalling pathways known to regulated collagenase gene expression. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with either siPKD3 #2 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control. (B)Histograms show quantification of bands normalized to GAPDH levels presented as a percentage of the cytokine-induced expression (siCON-transfected). Histograms are cumulative data of at least 3 individual cell populations (Mean ± S.D), **, p≤0.01, vs siCON.

5.3.5 The regulation of STATs in human articular chondrocytes

Once it was established that PKD regulated the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation site, further work was performed to try and understand the regulation of the STAT pathway by PKD. Litherland *et al.,* (2010) had previously shown STAT-3 to be important in the induction of collagenase gene expression under the stimulus of IL-1 in combination with OSM (Litherland et al., 2010). The role of STAT-3 in the regulation of collagenase gene expression was therefore further examined using the STAT-3 inhibitor, S3I-201. As well as this, understanding the mechanism by which PKD may regulate the serine phosphorylation of STAT-1 and STAT-3 was examined.

5.3.5.1 The effect of STAT-3 inhibition on classical collagenase gene expression

The cytotoxicity of the STAT-3 inhibitor S3I-201 in HAC was first assessed. As seen in *Figure 7.5,* no increased cytotoxic effects were seen in chondrocytes treated with the inhibitor at varying concentrations. STAT-3 inhibition in HAC using 100 μM of S3I-201 led to a significant reduction in MMP-1 and MMP-13 gene expression (*Figure 5.15)*. These data further confirmed the role of STAT-3 in the regulation of collagenase gene expression under the pro-inflammatory cytokine stimulus of IL-1 in combination with OSM.

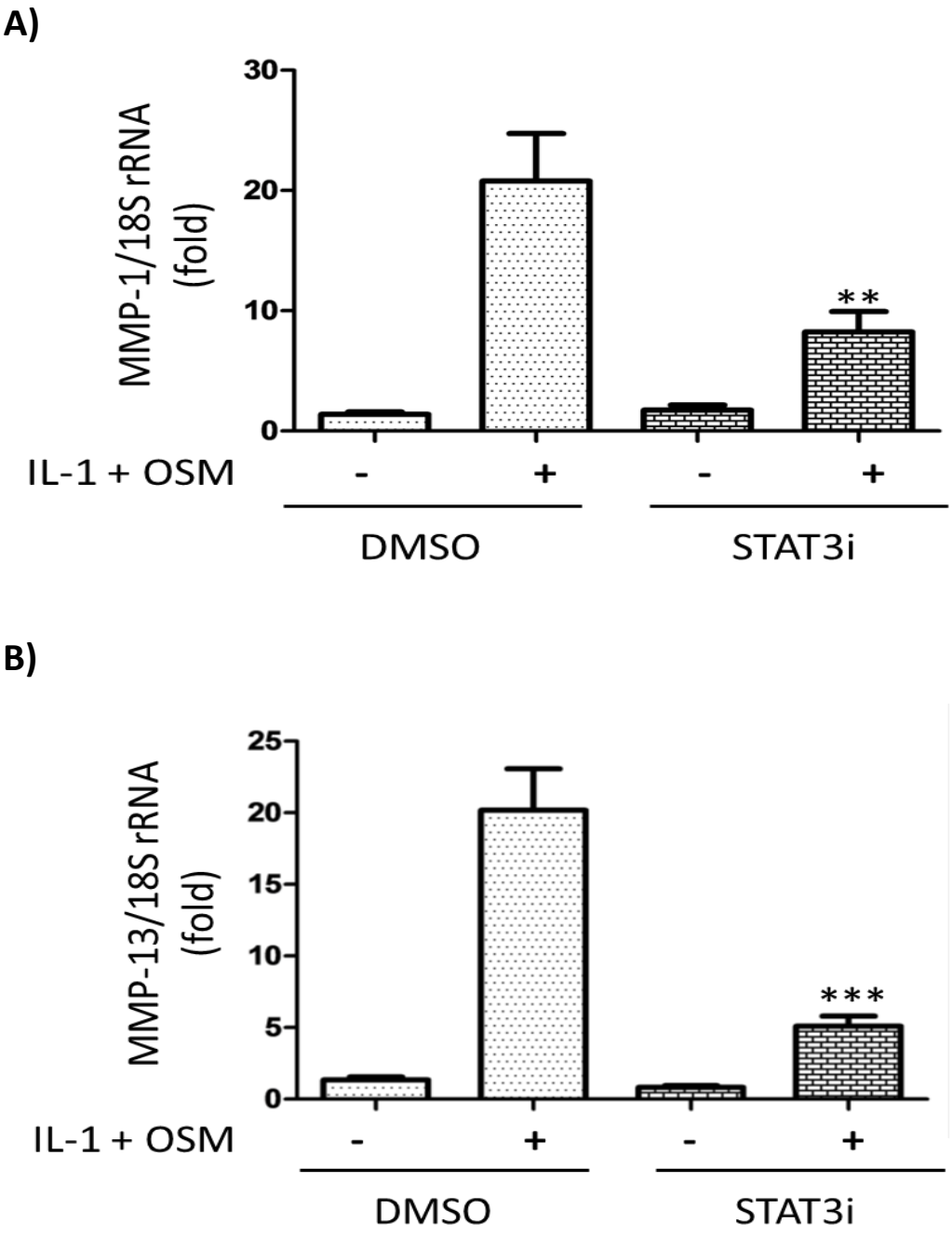


Figure 5.15. The effect of STAT-3 inhibition on collagenase expression in human articular chondrocytes. P0 HAC plated into 96 well plates were grown to 70% confluency and then serum starved overnight. HAC were pre-incubated with 100 μM S3I-201 or DMSO vehicle for 1 hour prior to stimulation with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, as described in the Materials and Methods. Data (mean ± S.E.M.) are representative of at least three combined chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific for PKD inhibitor versus control DMSO treated, where ***, p≤0.001, **, p≤0.01 vs DMSO control.

5.3.5.2 The effect of JNK gene silencing on STAT-1 and STAT-3 serine phosphorylation

PKD has not previously been implicated in the phosphorylation of the STAT-1 or STAT-3 at either phosphorylation site; therefore no mechanistic data were available into how PKD may regulate this phosphorylation. It was therefore unclear whether PKD directly or indirectly phosphorylated STAT-1 and STAT-3 at their serine phosphorylation sites. The MAPKs have previously been implicated in the phosphorylation of this site (Decker and Kovarik, 2000); however, ERK was previously shown not to phosphorylate this site in HAC (Litherland et al., 2010). As PKD regulated the activation of JNK, I assessed the role of this MAPK in the serine phosphorylation of STAT-1 and STAT-3. Following effective silencing of JNK using siRNA against the JNK isoforms JNK1 and JNK2 (*Figure 5.16. A*), a reduction in phosphorylation of STAT-1 and STAT-3 in chondrocytes stimulated with IL-1 in combination with OSM is observed when JNK2 was silenced (*Figure 5.16. B*). No effects on the serine phosphorylation of STAT-1 and STAT-3 were observed when the JNK1 isoform was silenced.

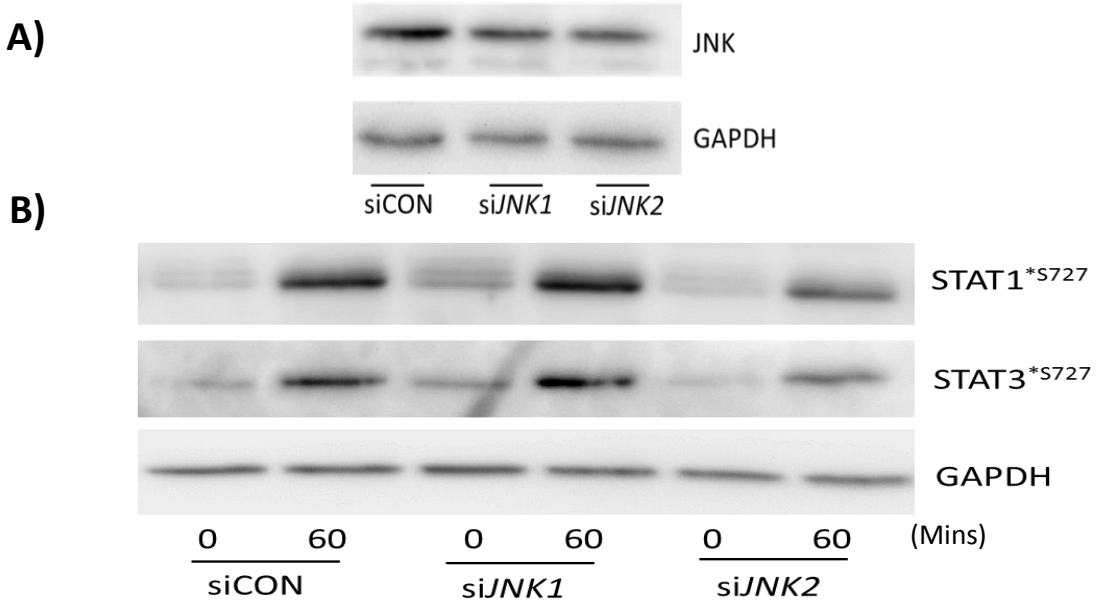


Figure 5.16 The effect of JNK gene silencing on STAT-1 and STAT-3 serine phosphorylation. (A) P0 HAC were trypsinised and plated into 6 well plates and left over-night. The following day cells were treated with siJNK1, siJNK2 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were either lysed or (B) stimulated with IL-1 (0.02 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cell lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. GAPDH was used as a loading control.

5.3.5.3 The effect of PKD3 gene silencing on STAT-1 and STAT-3 nuclear translocation

PKD3 silencing had no effect on the tyrosine phosphorylation of STAT-1 or STAT-3 in whole cell lysates (see *Figure 5.14*); however I set out to establish whether PKD regulated the levels of tyrosine phosphorylated STATs within the nucleus. To assess these effects, lysates in which PKD3 had been silenced were subjected to cytoplasmic and nuclear fractionation. As *Figure 5.17* shows, when PKD3 is silenced the levels of tyrosine phosphorylated STAT-3 within the nucleus are depleted, this is in contrast to STAT-1 in which no changes are observed.

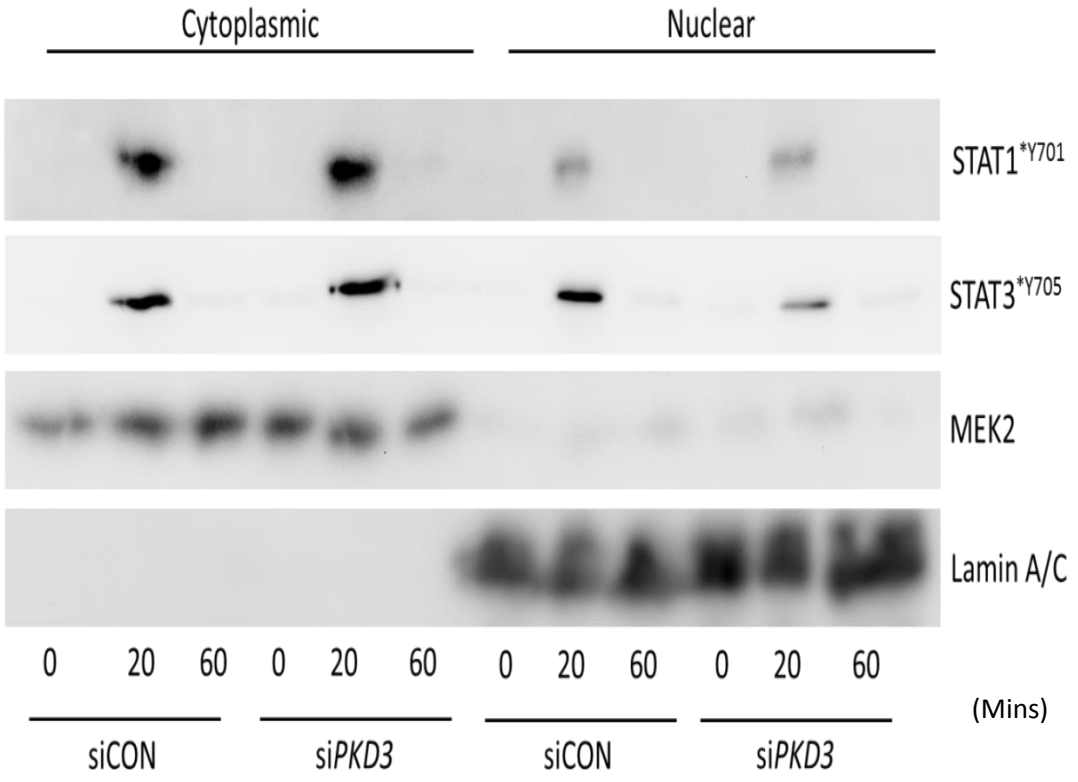


Figure 5.17. The effects of PKD3 gene silencing on the translocation of tyrosine phosphorylated STATs. P0 HAC were trypsinised and plated into 6 cm dishes and left over-night. The following day cells were treated with either siPKD3 #2 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cells were scraped in PBS and subjected to cytoplasmic and nuclear separation. Cytoplasmic and nuclear lysates were then immunoblotted using the antibodies shown. All procedures were followed as described in the Materials and Methods. Data are representative of at least three separate chondrocyte populations. MEK2 was used as a cytoplasmic loading control, whereas Lamin A/C was used as a nuclear loading control.

5.3.6 The regulation of AP-1 transcription factor components by each individual PKD isoform in HAC

The role of the AP-1 transcription factor is well established in the expression of MMPs (Benbow and Brinckerhoff, 1997). The main AP-1 binding site found within the promoters of MMP-1 and MMP-13 bind dimers of Fos and Jun proteins (Vincenti et al., 1998). Of these family members c-*fos* and c-*jun* were shown to be the major components of the AP-1 transcription factor involved in regulating collagenase gene expression (Chamberlain et al., 1993). PKD signalling has been shown to regulate both of these proteins (Sinnett-Smith et al., 2007b, Sinnett-Smith et al., 2004, Bernhart et al., 2013, Yamashita et al., 2010). As data within this chapter showed PKD to regulate many signalling pathways known to induce both the expression and activity of members of AP-1, I sought to understand the isoform specific roles of PKD on the expression of Fos and Jun.

5.3.6.1 The effects of PKD isoform specific gene silencing on c-*fos and* c-*jun mRNA expression in HAC*

Silencing of PKD1 using lentiviral mediated shRNA delivery in HAC stimulated with either IL-1 alone or in combination with OSM had no effects on the mRNA expression of *c-fos* or *c-jun* (*Figure 5.18. A*).

Similarly, PKD2 gene silencing using siRNA in HAC stimulated with either IL-1 alone or in combination with OSM had no effect on the mRNA expression of *c-fos* or *c-jun* (*Figure 5.18. B*).

siRNA mediated silencing of PKD3 in HAC led to a significant decrease in the expression of both c-*fos* and c-*jun* when stimulated with IL-1 alone and in combination with OSM (*Figure 5.18. C*). To further validate these data and identify whether PKD3 also regulated Jun activity and the protein levels of Jun and Fos, PKD3 was silenced and the effects on the phosphorylation of Jun and the protein expression of Fos and Jun examined. As *Figure 5.18. D* shows, a clear reduction in the protein levels of Jun and Fos in the nucleus is observed when PKD3 is silenced in HAC stimulated with IL-1 in combination with OSM. As well as this, a clear reduction in the levels of phosphorylated Jun is seen.

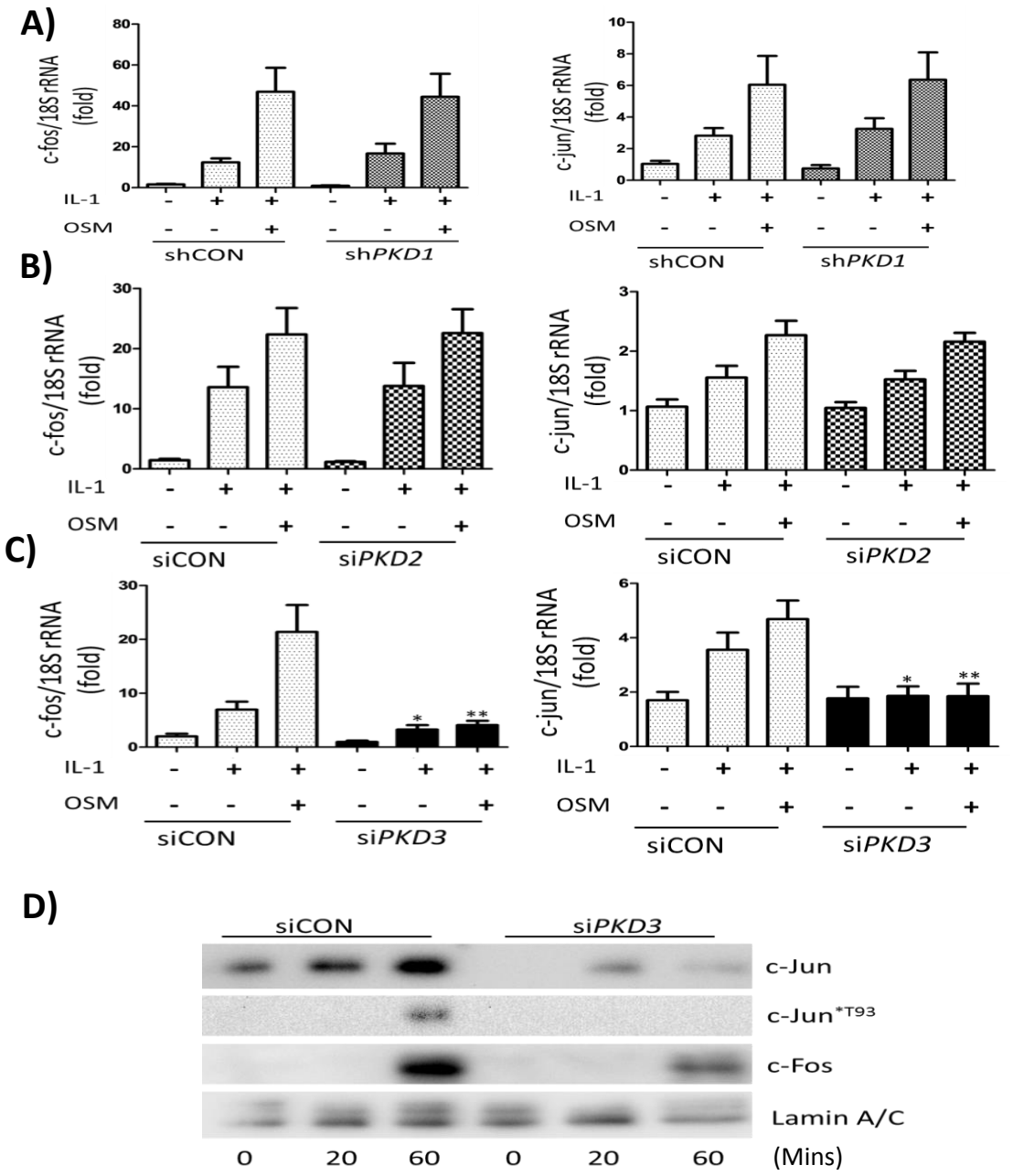


Figure 5.18. The effect of PKD silencing on the key AP-1 transcription factor members c-*fos* and c-*jun*. P0 HAC were trypsinised plated into 96 well plates and left over-night. (A) The following day cells were transduced using lentiviral particles contain either shRNA (MOI = 30) specific to PKD1 or shCON for 72 hours, or transfected with siRNA against (B) PKD2, (C) PKD3 or siCON for 48 hours. Cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 1h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for c-*fos* and *c-jun*, as described in the Materials and Methods. Data plotted are the mean ± S.E.M. data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transduced PKD isoform versus control transduced, where **, p≤0.01, *, p≤0.05 vs siCON. (D) P0 HAC were trypsinised and plated into 6 cm dishes and left over-night. The following day cells were treated with either siPKD3 or siCON (100 nM) for 48 hours. Cells were serum starved over-night. The following days cells were stimulated with IL-1 (0.2 ng/ml) in combination with OSM (10 ng/ml) for 20 or 60 minutes. Cells were scraped in PBS and subjected to cytoplasmic and nuclear separation. Nuclear lysates were then immunoblotted using the antibodies shown. Data are representative of at least three separate chondrocyte populations. Lamin A/C was used as a nuclear loading control.

5.3.7 The effect of PKD3 gene silencing on post-AP-1 transcription factors and cytokine

The maximal expression of c-*fos*, as well as maximal phosphorylation of Jun in HAC stimulated with IL-1 in combination with OSM, is at 1 hour post stimulation (Rowan, personal communication). However, the maximal transcriptional expression of MMP-1 and MMP-13 is observed after 24 hours. It was therefore hypothesised that other post-AP-1 transcription factors must be involved in collagenase gene expression. A DNA microarray on unstimulated and IL-1 in combination with OSM stimulated HAC (performed by C. Macdonald, Newcastle University) identified a number of significantly up-regulated novel transcription factors at a 1 hour 15 mins time point, compared to a 1 hour time point. Once identified, these transcription factors were validated by siRNA and the effects of their silencing on MMP-1 and MMP-13 gene expression studied. This work identified activating transcription factor 3 (ATF3), early growth response 2 (EGR2), nuclear factor of activate T-cells cytoplasmic 1 (NFATc1) and Axin-1 induced gene (AXUD1) as modulators of collagenase gene expression (Rowan, unpublished data). Bone morphogenic protein (BMP-2), although not a transcription factor, was also up-regulated and shown to regulate MMP gene expression. I therefore wanted to assess whether PKD3, as a regulator of Fos and Jun expression, also regulated the expression of these proteins.

5.3.7.1 The role of PKD3 in the expression of post AP-1 transcription factors

To establish the role of PKD3 in the regulation of these post-AP-1 transcription factors and cytokine, the effects of PKD3 gene silencing on the gene expression of these factors was assessed. As *Figure 5.19* shows, a significant reduction in the levels of ATF3, EGR2 and NFATc1 gene expression is observed in cells stimulated with IL-1 and when in combination with OSM. BMP-2 expression is also reduced, but only in HAC stimulated with IL-1 in combination with OSM. No effects on the expression of AXUD1 were observed under any stimulus.

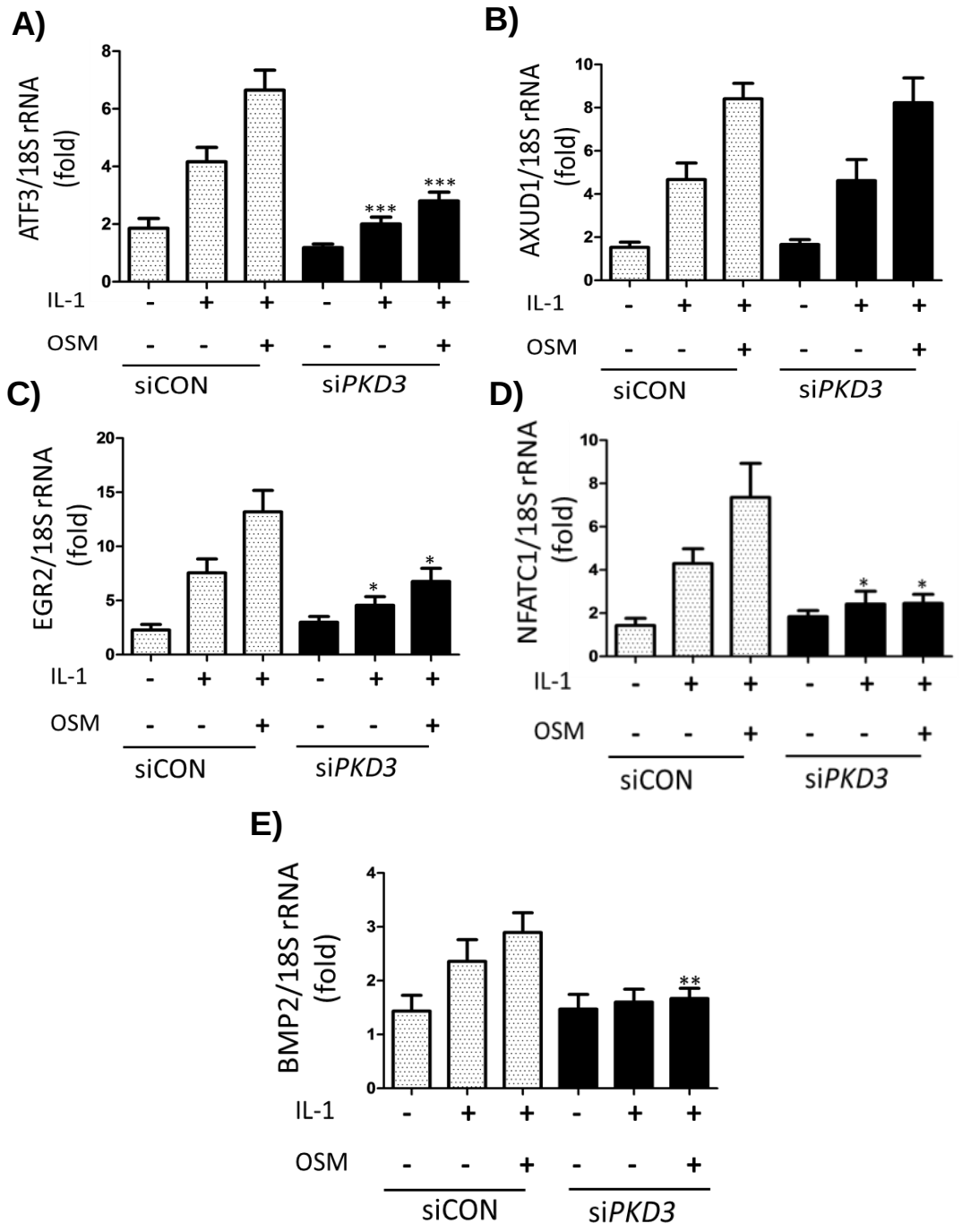


Figure 5.19. The effect of PKD3 gene silencing on post AP-1 transcription factors. P0 HAC were trypsinised and plated into 96 well plates and left over-night. The following day cells were transfected with siRNA specific to PKD3 or siCON (100 nM). 48 hours later cells were serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 1.15 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *ATF3,* (B) *AXUD1,* (C) *EGR2,* (D) *NFATc1* and (E) *BMP-2* 72 h after start of transfection as described in the Materials and Methods. Data plotted are the mean ± S.E.M. data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control transfection, where ***, p≤0.001, **, p≤0.01, *, p≤0.05 vs siCON.

5.4 Discussion

Results from Chapter 4 demonstrated that in HAC stimulated with IL-1 in combination with OSM, PKD1 gene silencing up-regulated MMP-1 and MMP-13 gene expression. Whilst results indicated PKD2 to play no role in this regulation, PKD3 silencing was shown to down-regulate MMP-1 and MMP-13 gene expression. Therefore, to try and elucidate how these structurally similar proteins can have opposing effects on the same genes under the same pro-inflammatory stimulus, each isoform was over-expressed or silenced and the consequences of this on signalling pathways known to regulate collagenase gene expression studied. As well as understanding the signalling cascades which were being regulated, AP-1, one of the key transcription factors involved in MMP gene expression was examined. This was achieved by studying the isoform specific effects of PKD on c-*jun* and c-*fos* gene expression. All of these data would provide further insight into the regulation of collagenase gene expression under this pro-inflammatory stimulus.

5.4.1 The regulation of PKD in chondrocytes

The effect of IL-1 and OSM on the phosphorylation of PKD was assessed. The phosphorylation of PKD by IL-1 was shown to be transient, whereas OSM dependent phosphorylation was shown to be longer in duration. PKD has previously been shown to be phosphorylated and activated under the stimulus of IL-1, implicating PKD downstream of the IL-1 receptor (Fleenor et al., 2003). OSM has not previously been shown to be an activator of PKD. However, OSM is known to activate the PI3K signalling cascade within chondrocytes (Litherland et al., 2008), and this pathway has been directly linked with the activation of PKC and PKD (Lynch et al., 2013), showing a potential mechanism by which OSM stimulation led to PKD phosphorylation.

The subcellular distribution of each isoform of PKD was assessed when cells were stimulated with IL-1 in combination with OSM. Data suggest that stimulation increased the levels of each PKD isoform within the cytoplasm, but had no effects on the shuttling of each isoform to the cell membrane or nucleus. The increase in cytoplasmic PKD was probably due to increased stability of each isoform, due to the stimulation. These data suggest that within SW1353 cells each isoform of PKD shuttles between these compartments in basal

conditions, this is not surprising as translocation has been shown to be independent of activation (Matthews et al., 1999). PKD has been shown to translocate to the cell membrane, here PKD is further activated, this leads to the translocation of PKD to the nucleus where PKD mediated signalling events alter MMP gene expression (Ha et al., 2008). These data show the importance of PKD translocation in altering MMP gene expression. Unfortunately, due to the large quantities of HAC required and reproducibility between patient samples the subcellular distribution of PKD isoforms in HAC was not found.

PKC is a known activator of PKD and has previously been implicated in the regulation of collagenase gene expression in HAC under the stimulus of IL-1 in combination with OSM (Litherland et al., 2010). The effect of PKC inhibition on the phosphorylation of PKD was therefore assessed. PKC was first inhibited by the PKC inhibitor Gö6983. This is a PKC specific inhibitor, that only inhibits PKD when used at high concentrations, allowing any effect observed to be accredited to PKC inhibition and not PKD inhibition (Gschwendt et al., 1996). PKC inhibition led to a loss of PKD phosphorylation at the PKC dependent phosphorylation (Ser744/48) and autophosphorylation (Ser916) sites. These data show PKC to be involved in the direct phosphorylation of PKD in HAC stimulated with IL-1 in combination with OSM and suggest that PKC activates PKD under this stimulus.

Isoform specific roles of PKC were then examined. Gene silencing of PKCι implicated this isoform in the regulation of PKD phosphorylation. Previously to these data, only the nPKCs have been implicated in the regulation of PKD phosphorylation (Zugaza et al., 1996, Bhavanasi et al., 2011, Yuan et al., 2002). When examining the literature, it can be seen that PKCι has not been specifically studied as a regulator of PKD phosphorylation. The aPKC have only been previously discounted in the regulation of PKD phosphorylation as PKCζ was shown not to regulate this phosphorylation (Yuan et al., 2002), in agreement with data presented here. Other Isoforms of PKC may also be involved in the phosphorylation and activation of PKD within HAC. PKCδ has been previously shown to regulate collagenase gene expression in HAC when stimulated with basic fibroblast growth factor (bFGF) (Im et al., 2007). This

isoform has also been shown to phosphorylate and activate PKD (Storz and Toker, 2003). PKCδ may therefore also play a role in the phosphorylation of PKD in HAC, along with other members of the nPKCs. It would have therefore been of interest to examine these isoforms also. However the data presented here, for the first time, implicates PKCι in the regulation of PKD phosphorylation in HAC, suggesting a novel role for PKCι in the phosphorylation of PKD.

5.4.2 PKD modulation of signalling cascades involved in regulating collagenase gene expression

Once it was established that PKD was being activated by both IL-1 and OSM in a PKCι dependent manner, the role of each isoform of PKD in the phosphorylation of downstream signalling pathways known to be involved in collagenase gene expression were examined. To assess PKD1 signalling, whilst the optimisation of PKD1 lentiviral-mediated shRNA gene silencing was being undertaken, the complementary technique of over-expression was used. This work was performed in SW1353 cells because of their increased availability compared to HAC and also their increased reproducibility.

Isoform specific gene silencing of each MAPK in HAC under the stimulation of IL-1 in combination with OSM has shown the importance of each MAPK isoform in the regulation of collagenase gene expression (Litherland and Rowan, unpublished data). These data along with previously published data from within our group (Litherland et al., 2008, Litherland et al., 2010, Catterall et al., 2001) have shown the importance of various signalling pathways in regulating collagenase gene expression.

5.4.2.1 PKD1 modulates signalling cascades involved in collagenase gene expression

5.4.2.1.1 Over-expression of PKD1 in SW1353 cells

To understand how the over-expression of PKD1 down-regulated the expression of MMP-1 and MMP-13, and how PKD1 gene silencing led to increased MMP-1 and MMP-13 gene expression in SW1353 cells (Figure 7.2, supplementary data), the role of PKD1 in the regulation of signalling pathways which regulate collagenase gene expression were examined. In this work the effects of PKD1 on signalling pathways stimulated with IL-1 and OSM alone or

in combination were studied. The work in SW1353 cells aimed to give insights into the potential signalling pathways PKD1 regulated in HAC.

PKD1 over-expression had no effects on the phosphorylation of ERK or STAT-1 and STAT-3 at their tyrosine phosphorylation site (phosphorylation at the serine phosphorylation site could not be detected in these cells), under any stimulus. These data suggest PKD1 does not regulate collagenase gene expression via modulation of these pathways.

However, the over-expression of PKD1 in SW1353 cells stimulated with IL-1 alone or in combination with OSM abrogated the phosphorylation of JNK and p38. Both of these signalling proteins have been implicated in the modulation of collagenase gene expression (Mengshol et al., 2001, Mengshol et al., 2000, Reunanen et al., 2002), through changes in AP-1 family member transcription and post translational modifications (Hess et al., 2004, Cavigelli et al., 1995, Whitmarsh et al., 1997); this could be a possible mechanism by which PKD1 modulates collagenase gene expression in SW1353 cells. Inhibition of these key signalling pathways by PKD1 may reduce the expression and transcription potential of AP-1 family members in SW1353 cells.

PKD1 over-expression also led to a decrease in Akt phosphorylation; however, this was only observed in SW1353 cells stimulated with OSM. In this work phosphorylation at the Ser473 only site was studied, due to poor identification of phosphorylation and reproducibility of results when studying the Thr308 site. These data suggest that the complex stimulus masks the effects of OSM signalling when used alone. The phosphorylation of Akt by IL-1 in combination with OSM is known to be less potent than when stimulated with OSM alone (Litherland et al., 2008), these data could explain the loss of PKD1 effects on this pathway, with PKD1 no longer being able to regulate Akt phosphorylation under this less potent stimulus of Akt.

Taken together these results imply that PKD1 regulates collagenase gene expression in SW1353 cells through the modulation of the JNK and p38 signalling pathways. The consequences of this, may be changes in the transcription of AP-1 transcription factor. These data provide insight into the

mechanism by which PKD1 regulates collagenase gene expression within HAC under the cytokine stimuli used.

5.4.2.1.2 Gene silencing of PKD1 in HAC

To understand how the gene silencing of PKD1 led to an increase in the expression of MMP-1 and MMP-13 (*Chapter 4, Figure 4.3*) and also a down-regulation in collagenase gene expression when over-expressed (*Chapter 4, Figure 4.2*) in HAC, the effect of PKD1 gene silencing on signalling pathways which regulate collagenase gene expression were examined. In this work the effects of PKD1 silencing on signalling pathways stimulate by IL-1 in combination with OSM were only studied, this was due to time constraints due to the time taken to optimise this technique.

PKD1 gene silencing in HAC stimulated with IL-1 in combination with OSM abrogated the phosphorylation of JNK, ERK and p38. These data are contrary to findings in SW1353 cells, in which the phosphorylation of JNK and p38 were reduced when PKD1 was over-expressed. This was an unexpected result, as these pathways are thought to be crucial for the induction of collagenase gene expression. However, PKD1 has previously been implicated in both the activation (Yamamoto et al., 2010, Zhang et al., 2005) and inhibition (Wang et al., 2004) of the JNK pathway, implying this process to be stimulus and cell specific. PKD1 has only been shown previously to activate ERK signalling, with this being through the regulation of upstream MAP2K and MAP3Ks (Sinnett-Smith et al., 2004, Ziegler et al., 2011), as seen in HAC. The role of PKD in the regulation of p38 has been shown to be complex, with PKD being shown to phosphorylate p38 leading to p38 signal transduction (Bernhart et al., 2013, Hao et al., 2012). However, PKD has also been shown to be regulated by p38, with a novel p38 phosphorylation site being identified within the PKD structure. Phosphorylation at this site was shown to inhibit PKD (Sumara et al., 2009). The literature may therefore be suggesting that PKD acts in a feedback loop, with PKD activating p38 leading to its own inhibition by p38, preventing further signal transduction.

The role for increased p38 signalling via PKD1 in the modulation of collagenase gene expression is not clear. As well as being a positive regulator of MMP gene

expression, p38 signalling has been shown to inhibit MMP gene expression. p38 signalling is shown to inhibit the expression of MMP-1 via the down regulation of ERK signalling (Westermarck et al., 2001), with the inhibitory effects of p38 being dependent on the upstream MAP2K which phosphorylates p38. Westermarck *et al.* showed that MKK3/MKK3 activation of p38 lead to decreased MMP gene expression (Westermarck et al., 2001). This work showed p38α to be the important isoform in this regulation, this data may suggest that isoform specific roles of p38 in the regulation of MMP gene expression. As PKD1 has been shown to regulate the phosphorylation of MKK3 (Song et al., 2009), this suggests a potential mechanism by which PKD1 can inhibit MMP gene expression via p38 signalling.

These data suggest that PKD1 signalling in HAC activates all three MAPK pathways, but this does not induce collagenase gene expression. These data therefore suggests that the activation of the MAPK signalling pathways via PKD1 may not induce collagenase gene expression in HAC under the stimulus of IL-1 in combination with OSM. However, if p38 signalling is a negative regulator of collagenase gene expression as proposed, this could explain the effects of PKD1 silencing on this protein.

PKD1 silencing in HAC led to an increase in the phosphorylation of Akt. These data suggest that PKD1 in chondrocytes inhibits Akt signalling under these conditions, implying a potential mechanism by which PKD1 can regulate collagenase gene expression.

The effects of PKD1 silencing on Akt phosphorylation observed in HAC have been supported in the literature. Here two mechanisms have been proposed as to how PKD may inhibit Akt phosphorylation; these mechanisms have the potential to work in tandem to regulate Akt phosphorylation. Ni *et al*. suggested that PKD1 can regulate the phosphorylation of the regulatory subunit of PI3K, p85α. Phosphorylation of p85 led to the binding and activation of Phosphatase and tensin homolog (PTEN) (Ni et al., 2013). PTEN can then dephosphorylate PIP_3, the main upstream signalling molecule of PDK-1. PDK-1 can then no longer phosphorylate and activate Akt. Although PDK-1 is thought to primarily phosphorylate Akt at the Thr308 site, it has been implicated in the

phosphorylation of Ser473; the Rictor-mTOR Complex is also thought to phosphorylate at this site (Sarbassov et al., 2005). As well as this, Hinchliffe *et al.* proposed a mechanism by which PKD phosphorylates a type II PIP kinase, leading to its inactivation. This therefore leads to a reduction in the levels of the PI3K substrate PIP_2 (Hinchliffe and Irvine, 2006). Reduced levels of PIP_2 means PI3K no longer generates PIP_3 and thus reduces Akt phosphorylation. These two mechanisms could therefore be working in tandem, allowing PKD1 control over Akt activation.

NFκB is well established in the regulation of collagenase gene expression in HAC stimulated with IL-1 (Fan et al., 2006). Data presented in this chapter show PKD1 gene silencing to induce the phosphorylation of p65, suggesting PKD1 to usually inhibit the activation of NFκB signalling *(Figure 5.8*). PKD and specifically PKD1 have been shown to regulate the activation and transcriptional activity of this signalling molecule and transcription factor (Storz and Toker, 2003, Chen et al., 2011). These data suggest the opposite to what is seen in this present study. This may therefore further suggest cell specificity in the regulation of signalling pathways modulate by PKD1 in different cells types. As PKD1 is seen to increase the phosphorylation (suggesting increased activity) of NFκB, these data could suggest a signalling and transcriptional mechanism by which PKD1 can inhibit collagenase gene expression when stimulated with IL-1 in combination with OSM. PKD2 and PKD3 were seen to have no effect on phosphorylation of p65 or degradation of IκBα in HAC (data not shown).

Increased levels of STAT-1 phosphorylated at Tyr701 were observed in PKD1 gene silencing studies. These data therefore suggest that PKD1 signalling could potentially inhibit STAT-1 dimerisation and translocation to the nucleus, reducing the transcription potential of STAT-1. The loss of activation of this transcription factor, may explain one of the mechanisms by which PKD1 inhibits collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. Although Litherland *et al*. (2010) did not show a significant effect of STAT1 silencing on collagenase gene expression; this along with the loss of Akt may work in tandem to inhibit collagenase gene expression.

No data within the literature has previously demonstrated any isoform of PKD in the regulation of STAT phosphorylation; therefore no mechanistic data are available to suggest how this may occur. Inhibition of the tyrosine phosphorylation of STATs has been shown to be indirect, with the inhibition of JAKs being shown to be important (Starr and Hilton, 1999). Two protein families are known to be involved in the inhibition of JAK signalling leading to decreased STAT tyrosine phosphorylation; these are SHP-1 and suppressors of cytokine signalling (SOCS) family members (Starr and Hilton, 1999). PKD1 may therefore increase the activity or expression of these proteins leading to decreased tyrosine phosphorylation of STAT-1 by JAKs.

PKD1 silencing in HAC also led to an increase in the levels of STAT-1 serine phosphorylation. This again implies a potential mechanism by which PKD1 could inhibit collagenase gene expression in HAC, with decreased phosphorylation at this site leading to a reduction in the transcriptional activity of STAT-1. How the modulation of STAT-1 via PKD1 regulates collagenase gene expression remains unclear, as PKD1 silencing had no effect on the mRNA expression of c-*fos*. However, this could be due to *c-fos* gene expression already being at maximal capacity, with no increase in expression being induced with the silencing of PKD1. Examining the protein level of Fos may give further insight into the regulation of the STAT pathway by PKD1.

To confirm that the increased tyrosine and serine phosphorylation of STAT-1 were due to PKD1 silencing, the effects of PKD1 silencing on interferon-α and interferon-γ gene expression were examined. This was assessed as viral transduction is known to induce to an interferon response (Kenworthy et al., 2009). Interferons have been shown to directly phosphorylated and activate STAT proteins (Decker and Kovarik, 2000). As *Figure 7.7* (supplementary data) shows, no increase in either interferon was observed, suggesting the increase in phosphorylation to be caused by PKD1 gene silencing.

The conflicting data found between SW1353 cells and HAC shows the importance of studying a disease within the primary cell type of interest. This data shows that the same signalling protein can have opposing effects on the

same signalling pathway but still lead to the same downstream consequence in different cell types.

5.4.2.2 PKD2 modulates signalling cascades involved in collagenase gene expression when stimulated with IL-1 alone or when used in combination with OSM

The signalling consequences of PKD2 silencing in HAC were studied to try and understand why this isoform does not regulate collagenase gene expression. The effect of PKD2 silencing was assessed on signalling pathways stimulated with all three stimuli in HAC.

The effect of PKD2 silencing on the phosphorylation of the MAPK pathways appeared to be stimulus dependent. When PKD2 was silenced and cells were stimulated with IL-1 alone, a reduction in the phosphorylation of all 3 MAPK was seen. However, when cells were stimulated with IL-1 in combination with OSM the reduction in the phosphorylation of ERK or JNK was no longer seen, interestingly p38 phosphorylation increased.

These data suggest that the addition of OSM led to the loss of PKD2 mediated regulation of the MAPKs. One explanation for the loss of PKD2 mediated regulation of the MAPKs could be due to further OSM mediated phosphorylation of these proteins. It would appear that IL-1 stimulation does not maximally phosphorylate JNK, p38 and ERK with the addition of OSM leading to further phosphorylation. The addition of OSM to IL-1 stimulated human gingival fibroblasts has been shown to increase the phosphorylation of JNK (Hosokawa et al., 2010). The addition of OSM may therefore further phosphorylate JNK via a mechanism that is distinct to IL-1 and PKD2 regulation. The loss of JNK phosphorylation via PKD2 in IL-1 only stimulated cells may be counteracted by this increased phosphorylation by a PKD2 independent OSM-mediated pathway. ERK and p38, like JNK, may be further activated by the addition of OSM in a PKD2 independent, OSM driven manner. These data could help explain how PKD2 silencing had no effect on collagenase gene expression, as the JNK, ERK and p38 pathways all are known to be important in inducing gene expression.

The increase in the levels of p38 phosphorylation under the stimulus of IL-1 in combination with OSM could be explained by the dysregulation of a negative feedback loop that PKD and p38 are potentially involved within.

PKD2 silencing in HAC led to a significant reduction in the level of Akt phosphorylation, but only when stimulated with OSM alone. This data suggest that like the MAPK data when the complex stimulus is used the effects of PKD2 on Akt phosphorylation were masked. A further explanation could be down to the fact that stimulation of Akt with IL-1 in combination with OSM does not phosphorylate Akt to the same levels as seen with OSM alone (Litherland et al., 2008), the effects of PKD2 on Akt signalling could therefore be lost.

PKD2 silencing had no significant effect on tyrosine phosphorylation of either STAT-1 or STAT-3, when stimulate with OSM alone or in combination with OSM. However, PKD2 silencing led to a reduction in the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation site under the stimulus of IL-1 alone or in combination with OSM. The loss of STAT-1 and STAT-3 serine phosphorylation does not appear to lead to changes in the transcription of collagenase gene expression suggesting the loss of this regulatory phosphorylation is not itself sufficient to drive collagenase gene expression.

5.4.2.3 PKD3 regulates signalling cascades involved in collagenase gene expression when stimulated with IL-1 alone or when used in combination

To understand how the gene silencing of PKD3 led to a decrease in the expression of MMP-1 and MMP-13 (*Chapter 4, Figure 4.5*), the effect of PKD3 gene silencing on signalling pathways which regulate collagenase gene expression in HAC were examined. In this work the effects of PKD3 silencing on signalling pathways activated by all three stimuli were studied.

PKD3 silencing abrogated the phosphorylation of all three MAPK signalling proteins in HAC stimulated with IL-1 alone or in combination with OSM. These data suggest a potential mechanism by which PKD3 up-regulates collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. PKD3 signalling has been poorly studied with no real mechanistic data being available into how PKD3 regulates the phosphorylation of these MAPKs. PKD3 may

regulate JNK in a similar manner to PKD1, with data suggesting PKD1 to interact directly with JNK (Hurd et al., 2002) as well as phosphorylating the upstream MAP3K, ASK1, with this phosphorylation leading to increased JNK activity (Zhang et al., 2005). The mechanism by which PKD3 phosphorylates ERK still remains unclear, but like PKD1 may occur via MAP3K and MAP2K activation. It has been shown that PKD1 phosphorylates Ras and Rab interactor-1 (RIN1), releasing RAF from its inhibitory binding to RAS, leading to the activation of the RAF-MEK-ERK pathway (Wang et al., 2002). PKD3 may activate p38 leading to the phosphorylation of its downstream target Elk-1, leading to increased stability and enhanced transcriptional activity. The increased transcriptional activity may lead to an increase in the expression of the AP-1 component Fos. (Whitmarsh et al., 1997). MMP gene expression could then be induced by the increased expression of the AP-1 components.

PKD3 silencing in HAC has no effect on Akt phosphorylation under stimulation with OSM alone or when in combination with IL-1. These data suggest that PKD3 does not regulate collagenase gene expression through the modulation of the Akt pathway.

PKD3 silencing did not lead to any significant changes in the tyrosine phosphorylation of either STAT-1 or STAT-3, under any stimuli. Like when PKD1 was over-expressed and PKD2 silenced, small but insignificant increases in levels of phosphorylation were observed. However, decreases in STAT-1 and STAT-3 serine phosphorylation were observed with PKD2 and PKD3 gene silencing. Serine phosphorylation of both STAT-1 and STAT-3 has been shown to decrease tyrosine phosphorylation of both STATs (Chung et al., 1997). Therefore the reduced levels of serine phosphorylation observed with PKD2 and PKD3 gene silencing, could potentially increase in the levels of tyrosine phosphorylation due to the loss of this regulatory mechanism.

PKD3 silencing led to a reduction in the phosphorylation of STAT-1 and STAT-3 at this serine phosphorylation site, priming them for transcription. The increased phosphorylation at this STAT priming event along with the regulation of the MAPKs imply a potential mechanism by which PKD3 could induce the gene expression of MMP-1 and MMP-13,.

5.4.3 PKD and the regulation of STAT-1 and STAT-3

Once it was established that each isoform of PKD regulated the phosphorylation of STAT-1 and/or STAT-3, their role in MMP gene expression was assessed. Furthermore, the potential mechanism by which PKD regulated the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation site was examined.

Frist, to confirm that STAT-3 regulated the expression of MMP-1 and MMP-13, STAT-3 was inhibited using S3I-201 in HAC stimulated with IL-1 in combination with OSM. STAT-3 inhibition led to a significant decrease in MMP-1 and MMP-13 gene expression, confirming STAT-3 regulated collagenase gene expression in HAC.

Gene silencing of all PKD isoforms led to changes in the phosphorylation of either/both STAT-1 and STAT-3 at their serine phosphorylation site. The mechanism by which this may occur in HAC is unclear. The MAPKs are well established as regulators of STAT serine phosphorylation (Decker and Kovarik, 2000). However, the MAPK(s) involved in this phosphorylation has been shown to be both cell and stimulus specific (Kovarik et al., 1999). Much data has focused on ERK as the major regulator of both STAT-1 and STAT-3 serine phosphorylation, however in chondrocytes stimulated with IL-1 in combination with OSM ERK has been shown not to be necessary (Litherland et al., 2010). PKD has not been previously shown to regulate the phosphorylation of STAT-1 or STAT-3 at either site, therefore no data are available to whether PKD directly or indirectly phosphorylates this site. However, each isoform of PKD has been shown to regulate the activation of JNK, a known serine STAT regulator. JNK silencing data showed JNK2 to decrease the levels of STAT-1 and STAT-3 serine phosphorylation. These data suggest that PKD may inhibit JNK signalling leading to the loss of STAT-1 and STAT-3 serine phosphorylation.

Another mechanism by which PKD3 may regulate collagenase gene expression is through increased nuclear translocation of STAT-3. PKD3 silencing is seen to inhibit the translocation of tyrosine phosphorylated STAT-3 into the nucleus. The mechanism by which this occurs is unclear, as no changes in the levels of tyrosine phosphorylated STAT-3 is observed in whole cell lysates. The effects

of PKD3 activity to increase the levels of transcriptionally active STAT-3 within the nucleus may increase the transcription of both MMP-1 and MMP-13.

5.4.4 PKD isoforms and AP-1 gene expression regulation

The induction of the signalling pathways studied is known to modulate the expression and activity of the key collagenase transcription factor, AP-1. The expression of the two major components of AP-1, Fos and Jun, were therefore assessed when each isoform of PKD was silenced in HAC stimulated with IL-1 alone or in combination with OSM.

PKD1 silencing had no effect on the expression of c-*fos* or c-*jun*. Previous data has suggested that through IL-1 stimulation, PKD1 can activate the AP-1 transcription factor and MMP gene expression (Fleenor et al., 2003). If c-*fos* and c-*jun* expression, as described earlier, were at a maximal level no increase in mRNA level of either would be observed. Therefore examining further time points and also the protein expression and phosphorylation of these proteins may have provided further information into whether PKD1 could regulate the AP-1 transcription factor.

PKD2 silencing had no effect on the transcription of either c-*fos* or c-*jun* in HAC under either stimulus. These data implicate a potential mechanistic reason why PKD2 is unable to regulate collagenase gene expression, as these transcription factors are known to induce the MMP-1 and MMP-13 gene expression. As PKD2 was unable to regulate JNK and ERK under the stimulus of IL-1 in combination with OSM, this may explain why unlike previous data PKD2 cannot regulate Jun and Fos in HAC (Sinnett-Smith et al., 2007b, Bernhart et al., 2013).

When examining the effects of PKD3 on the gene expression and protein level of Fos and Jun, it can be seen that PKD3 stimulation via IL-1 in combination with OSM increases the transcription and synthesis of these two transcription factors. As well as increasing the transcription of c-Jun, PKD3 also increases the phosphorylation of Jun, suggesting increased transcriptional activity. The signalling pathway by which PKD3 phosphorylates Jun under this stimulus is not clear. But PKD3, like PKD2 in other cell types, may phosphorylate Jun via JNK (Bernhart et al., 2013), as data presented here shows PKD3 to regulate the

phosphorylation of JNK. The increased expression of both Jun and Fos could also be explained by the regulation of all 3 MAPK pathways, as well as the induction of STAT signalling, as all these pathways have been implicated in the induction of both these transcription factors.

5.4.5 PKD3 and the regulation of post-AP-1 transcription factors and cytokine

To further understand the transcriptional regulation of collagenase gene expression, the effects of PKD3 silencing on the gene expression of ATF3, EGR2, NFATc1 and AXUD1 and BMP-2 was studied. These are newly identified novel collagenase gene modulators. The regulation of MMP-1 and MMP-13 gene expression differ between these factors. ATF3 and EGR2 regulated the expression of MMP-13, whilst AXUD1 and NFATc1 were specific for MMP1 only. BMP-2 was shown to regulate MMP-1 and MMP-13 (Macdonald, 2013). PKD3 silencing of these factors led to a decrease in the expression of ATF3, EGR2, NFATc1 and BMP-2, no effects on AXUD1 expression was observed.

The role of ATF3 in the regulation of MMP gene expression is an area of dispute. ATF3 has been shown to be a negative regulator of MMP gene expression (Stearns et al., 2004, Chen and Wang, 2004, Yan et al., 2002) in work focusing on MMP-2 gene regulation. This report showed that ATF3 inhibited the trans-activation of MMP-2 by interacting and inhibiting cellular tumour antigen p53, a known MMP transcription factor. However, when looking specifically at the collagenases MMP-1 and MMP-13, ATF-3 has been shown to be a positive regulator of both MMPs, with over-expression leading to increased gene expression (Lv et al., 2011, Okamoto et al., 2006). Work showed that under basal conditions ATF3 could bind to the proximal AP-1 site, but with the addition of TGF-β, ATF-3 bound to Runt Domain/ Runt-related transcription factor (RD/RUNX) sites of the human MMP-13 promoter (Kwok et al., 2009). As well as this, ATF3 is known to induce the expression of RUNX2, a known MMP-13 transcription factor (Mengshol et al., 2001) and principal regulator of skeletal formation (Stein et al., 2004). PKD has already been implicated in RUNX2 gene expression, suggesting a mechanism for the induction of this transcription factor (Jensen et al., 2009). PKD3 therefore may induce ATF3 expression through

induced AP-1 mediated transcription or potentially through the modulation of the MAPK pathways (Lu et al., 2007, Hai et al., 1999, Park et al., 2012). The increased expression of ATF3 may induce RUNX2 expression, leading to increased MMP-13 gene expression.

EGR1 expression has previously been shown to be induced by IL-1 in chondrocytes (Goldring et al., 1994) and also shown to induce collagenase expression in RA synoviocytes (Trabandt et al., 1992). EGR2 may therefore regulate MMP gene expression in a similar manner under the stimulation of IL-1 in combination with OSM. EGR2 over-expression is also known to induce collagen I expression, suggesting a further role in joint homeostasis (Fang et al., 2011). PKD3 silencing decreased the expression of EGR2, suggesting a further mechanism by which PKD3 modulates collagenase gene expression. PKD has not been previously implicated in the regulation of EGR2 expression, so no mechanistic data are available as to how PKD3 regulates the changes in expression. However, data does suggest that the expression of EGR2 is regulated by MAPK stimulation, suggesting a potential mechanism by which PKD3 could regulate the expression of EGR2 in HAC (Fang et al., 2011).

Previous work has implicated NFATc1 in arthritis progression and cartilage degradation. NFATc1 is shown to be expressed in the synovium of RA patients (Pessler et al., 2006) and NFAT inhibition has also been shown to decrease IL-1-mediated collagenase gene expression (Yoo et al., 2007). PKD3 has been linked with the nuclear accumulation and gene induction of NFATc4 (Li et al., 2011). This work also showed NFATc1 to induce the expression of PKD3. These data, along with my own, suggest PKD3 may lie in a regulatory loop, in which PKD3 could potentially induce the expression of NFATc1 as well as being stimulated itself by NFATc1. PKD3 may therefore regulate the levels and transcriptional activity of this protein leading to the induction of MMP gene expression.

PKD has been shown to be phosphorylated and activated by BMP-2. BMP-2 is known to be important in the differentiation of osteoblast into bone and has been shown to induce the expression of both MMP-13 and RUNX2 (Caron et al., 2013). BMP-2 is shown to activate PKD, leading to JNK and p38 signalling

inducing RUNX2 (Jensen et al., 2009). Stimulation of HAC with IL-1 in combination with OSM therefore has the potential to stimulate PKD3, leading to the induction of BMP-2, which induces further activation of PKD3 driving RUNX2 expression, which in turn induce collagenase gene expression.

Data presented in this chapter have shown the potential signalling consequences of each isoform of PKD in HAC stimulation with pro-inflammatory cytokines seen to be elevated in arthritic diseases. These data suggest potential mechanisms by which each isoform may regulate collagenase gene expression in HAC under these stimuli.

5.4.6 Summary

Studies in this chapter have shown:

- PKD1 regulates signalling pathways known to induce collagenase gene expression in a cell dependent manner.
- PKD1 activates the MAPK signalling pathways but inhibits the phosphorylation of Akt, STAT-1 at both phosphorylation sites and STAT-3 at its serine phosphorylation site in HAC
- PKD2 no longer regulates MAPK pathway regulation when OSM is in combination with IL-1; serine phosphorylation of STAT-1 and STAT-3 still occurs.
- PKD3 phosphorylates all three MAPKs and also phosphorylates STAT-1 and STAT-3 at their serine phosphorylation site
- STAT-3 is crucial in the induction of collagenase gene expression
- JNK2 is responsible for the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation site
- PKD3 gene silencing inhibits the levels of tyrosine phosphorylated STATs in the nucleus

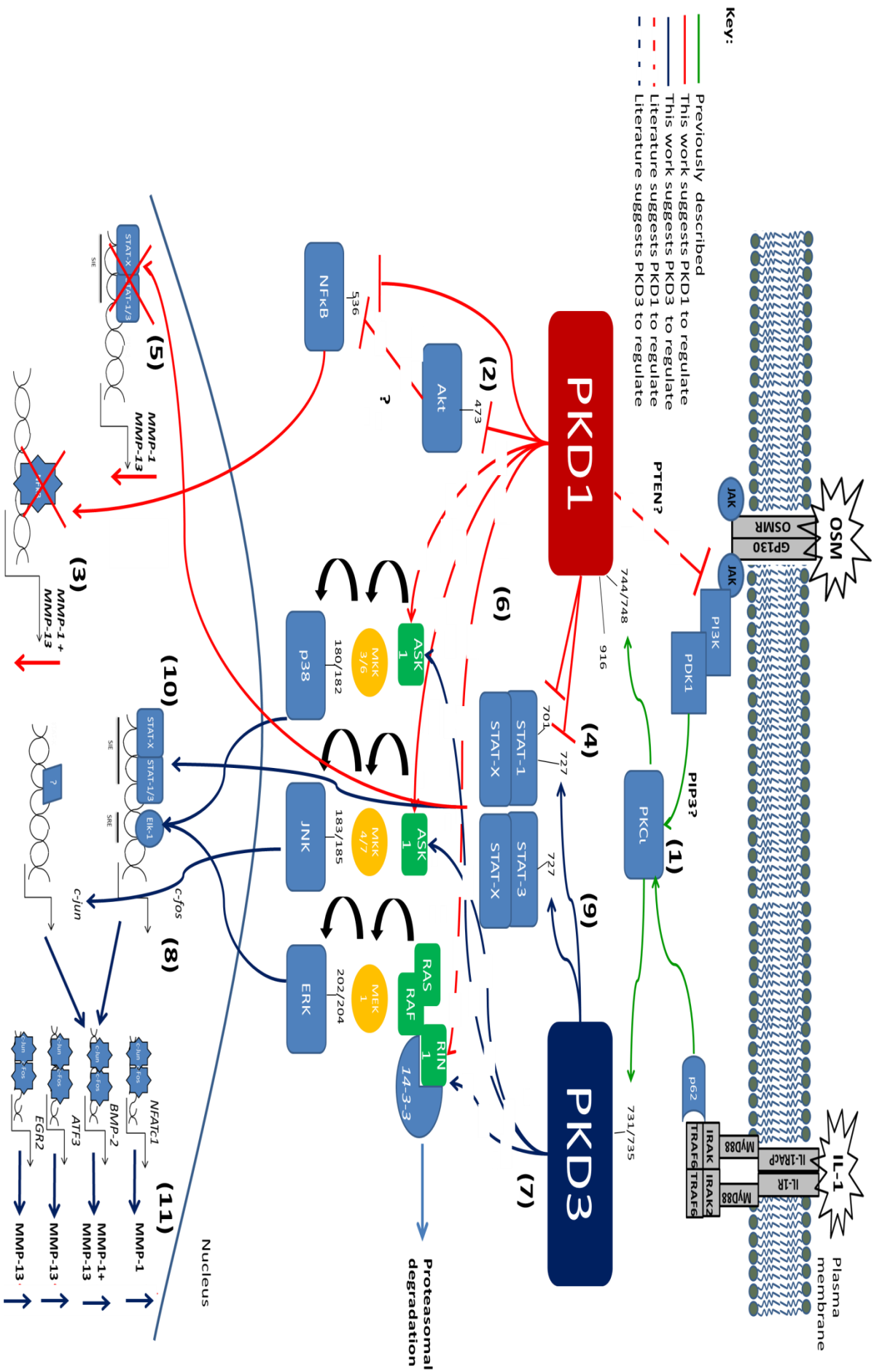

Key:
Previously described
This work suggests PKD1 to regulate
This work suggests PKD3 to regulate
Literature suggests PKD1 to regulate
Literature suggests PKD3 to regulate
OSM
JAK
GP130
OSMR
JAK
PI3K
PDK1
PIP3?
PTEN?
PKD1
PKD3
PKCι
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
Akt
473
?
NFκB
536
744/748
916
701
727
727
731/735
STAT-1
STAT-X
STAT-3
STAT-X
ASK 1
MKK 3/6
p38
180/182
ASK 1
MKK 4/7
JNK
183/185
RAS
RAF
MEK 1
ERK
202/204
RIN 1
14-3-3
p62
IRAK
TRAF6
MyD88
IL-1RAcP
IRAK2
TRAF6
MyD88
IL-1R
IL-1
Plasma membrane
Nucleus
Proteasomal degradation
STAT-X
STAT-1/3
SIE
Elk-1
SRE
c-fos
c-jun
MMP-1
MMP-13
MMP-1 + MMP-13
NFATc1
BMP-2
ATF3
EGR2
MMP-1
MMP-1+ MMP-13
MMP-13
MMP-13

Figure 5.20 Signalling diagram of the potential mechanisms by which PKD1 and PKD3 regulate collagenase gene expression in HAC stimulated with IL-1 in combination with OSM. 1) Stimulation of the IL-1 and OSM receptors has previously been shown to phosphorylate and activate PKCι (Litherland et al., 2010); this is suggested to occur via PIP_3 and PDK1, as well as p62 activation. PKCι is known to phosphorylate PKD at serine 744/748, activating the protein kinase. **(2)** Activation of PKD1 leads to the loss of Akt phosphorylation at its serine 473 site. Currently, it is unclear whether this is by direct inhibition of Akt or indirectly via PKD1 inhibition of PI3K signalling via activation of PTEN and/or other PIP phosphatases (Ni et al., 2013, Ittner et al., 2012). Phosphorylation of the p65 subunit of NFκB, a known Akt downstream target, is also reduced. PKD1 has the potential to regulate this phosphorylation via Akt, however PKD1 is also reported to inhibit IKK, a known upstream activator of p65 (Storz and Toker, 2003). **(3)** The loss of p65 phosphorylation negates its nuclear translocation, thus preventing transcription of *MMP-1* and *MMP-13*. **(4)** PKD1 –also inhibits phosphorylation of STAT-1 at its serine and tyrosine phosphorylation sites. **(5)** The consequences of this are not fully clear; the STATs have been shown to regulate *FOS* expression leading to the regulation of MMP gene expression (Catterall et al., 2001). However, the data presented here show no change in *FOS* expression when PKD1 is silenced. STAT1 may therefore directly bind the *MMP-1* and *MMP-13* promoters (El Mabrouk et al., 2007, Korzus et al., 1997), with PKD1 preventing this from occurring by inhibiting STAT phosphorylation and hence reducing collagenase gene expression. **(6)** PKD1 is seen to phosphorylate and activate JNK, ERK and p38. However, the activation of these pathways, although important in the regulation of collagenase gene expression, does not appear to alter collagenase gene expression under the regulation of PKD1. The effect of PKD1 on NFκB, Akt and STAT-1 appears to 'over-ride' the activation of these MAPKs. In chondrocytes, the regulation of MAPKs at the MAPKK and MAPKKK levels remains unclear. However data suggest PKD family members regulate the MAPKKK, ASK-1, a known upstream activator of JNK and p38 (Zhang et al., 2005). PKD1 is also seen to phosphorylate the MAPKKK, RIN-1, leading to the binding of *14-3-3* to the protein, inducing proteasomal degradation. This leads to the phosphorylation of RAF and activation of ERK signalling (Ziegler et al., 2011). **(7)** PKD3 also phosphorylates and activates JNK, ERK and p38. Again the mechanism by which this occurs is not fully characterised, but like PKD1, may occur through the phosphorylation of MAPKKKs and MAPKKs. **(8)** Activation of the MAPK pathways is known to lead to an induction in the expression of FOS and JUN; two key members of the AP-1 heterodimer**.** These proteins are known to be crucial in the induction of *MMP-1* and *MMP-13* expression in HAC stimulated with IL-1 in combination with OSM (Litherland et al., 2010, Catterall et al., 2001). JNK is known to phosphorylate and induce the stability of JUN (Leppa et al., 1998), whilst ERK and p38 are known to activate elk-1 inducing *FOS* expression (Hodge et al., 1998, Whitmarsh et al., 1997). **(9)** PKD3 also phosphorylates STAT-1 and STAT-3 at their serine phosphorylation site, priming both for transcription. **(10)** STAT-1 and STAT-3 bind to the *c-fos* promoter, inducing its expression (Catterall et al., 2001). **(11)** Induction of *c-fos* and *c-jun* in chondrocytes stimulated with IL-1 in combination with OSM has recently been shown to induce the expression of multiple post-AP-1 transcription factors, which have been demonstrated to be important in the regulation of collagenase gene expression (Macdonald, 2013). PKD3 signalling via the regulation of the key signalling pathways highlighted, and through the modulation of *c-fos* and *c-jun*, regulates the expression of these transcription factors leading to the induction of *MMP-1* and *MMP-13* expression under this pro-inflammatory cytokine stimulus. The other isoform of the PKD family, PKD2, was shown not to regulate the expression of *MMP-1* or *MMP-13* although it does appear to regulate the serine phosphorylation of the STATs and the phosphorylation of p38. Together, this regulation appears insufficient to lead to downstream transcriptional changes of the collagenases. This may be due to the lower expression of *PRKD2* in chondrocytes compared to the other two isoforms (D A Young, personal communication).

6 Chapter 6: General discussion

Cartilage degradation due to the expression of proteolytic enzymes is a key pathological process in arthritic disease. This stage in disease is characterised by irreversible collagenolysis. Of the enzymes capable of cleaving native collagen fibrils, MMP-1 is described as the key collagenase in RA, whereas MMP-13 is thought to be the major collagenolytic enzyme in OA. In disease, an increase in the expression of pro-inflammatory cytokines is observed, with elevated levels of IL-1 and OSM being detected (Hui et al., 1997, Fontana et al., 1982). These two pro-inflammatory cytokines have been shown to synergise, leading to a robust induction of collagenase gene expression, protein synthesis and subsequent collagenolysis of cartilage collagen, all being induced within the chondrocyte (Rowan et al., 2003, Rowan et al., 2001, Koshy et al., 2002b, Cawston et al., 1995b, Barksby et al., 2006). Although used as a model of inflammatory arthritis, this stimulus has been shown to be an important model in understanding the complex signalling cascades and cross-talk that initiates MMP gene expression and cartilage degradation (Barksby et al., 2006, Litherland et al., 2008, Litherland et al., 2010). The induction of subsequent signalling events involved in collagenase gene expression have not been well examined, and only a few previous studies have examined the signalling events stimulated by IL-1 in combination with OSM which lead to collagenase gene expression in HAC (Litherland et al., 2008, Litherland et al., 2010, Catterall et al., 2001). The aim of this study was to further understand these signalling events, examining the signalling pathways regulated by the individual isoforms of PKD and known to modulate of collagenase gene expression.

The *in vitro* model of arthritis used to investigate the signalling consequences which lead to the induction of collagenase gene expression in HAC within a pro-inflammatory setting, is well-established and seen as a robust model for disease progression (Rowan et al., 2003). *In vitro* models have limitations, as do *in vivo,* and are only ever used as an indicator of the potential signalling events which occur within the chondrocytes of patients suffering from arthritis. *In vitro* data

does have relevance and can be used as an indicator of the roles of proteins within disease, further increasing our knowledge of disease progression. The use of primary HAC, over cell lines, further validates the data found within this thesis, as they are the cell type involved in disease.

OSM is not the only pro-inflammatory cytokine which has been shown to synergise with IL-1, and the addition of the pro-inflammatory cytokine IL-6 with its receptor (IL-6 receptor) and IL-17, both synergise with IL-1 to induce collagenase gene expression in HAC (Rowan et al., 2001, Koshy et al., 2002a). The complex milieu of pro-inflammatory cytokines released during disease progression is seen to activate many of the signalling cascades studied within this thesis. These data show the model used, although simplified, is a relevant model for understanding the signalling events involved in the induction of collagenase gene expression that occurs in disease progression. Many have been shown, like IL-1 in combination with OSM to signal and activate MAPK signalling leading to the induction of AP-1 gene expression (Chakraborti et al., 2003, Yan and Boyd, 2007). This *in vitro* model therefore demonstrates the activation of signalling events believed to occur within the chondrocytes during disease.

During this thesis the cell line SW1353 was used to gain further insight into the regulation of collagenase gene expression within chondrocytes. This cell line was used to optimise many experiments and understand the regulation of collagenase gene expression. This cell line, although a good model for understanding the role of each isoform of PKD within chondrocytes, did have its limitations. Differences in the regulation of the MAPK signalling pathways under PKD1 regulation were observed in SW1353 cells and HAC. These differences did not alter the effect PKD1 had on collagenase gene expression, suggesting that different signalling events were involved in the modulation of collagenase gene expression within the two cell types. Also, PKD2 was shown to regulate collagenase gene expression in SW1353 cells, whereas this did not occur in HAC. These data show the importance of using primary cells, as although sometimes difficult to acquire and manipulate, they can give a greater understanding of the signalling events and gene induction that occur within disease.

6.1 The importance of developing and optimising tools capable of silencing each isoform of PKD specifically

In this thesis, the role of each isoform of PKD was studied to understand their individual roles in modulating the expression of MMPs. Most current studies have examined PKD as an 'entity', with work focusing on the family as one kinase or examining the isoform PKD1 only. When I began this work, no data were published on the roles of each isoform of PKD within the same cell type; I therefore set out to understand the individual roles of each isoform of PKD in chondrocytes.

To assess each PKD isoform, establishing robust and specific biological tools to silence and/or over-express these isoforms of PKD was crucial; siRNA-mediated gene silencing was first used. siRNA was chosen over biological inhibitors due to the lack of specificity between isoforms, with similar IC_{50} values being found for each isoform of PKD for all PKD inhibitors available (Harikumar et al., 2010, George et al., 2011). As well as this, the specificities of most of these newly synthesised PKD inhibitors have not been fully elucidated, i.e., with kinome scans, and therefore off-target effects cannot be exluded. To study the effects of each PKD isoform on collagenase gene expression it was crucial to ensure that each PKD isoform was being specifically silenced.

Lentiviral-mediated delivery of shRNA was also used in this work. This method of shRNA delivery has many advantages over transient transfection, with constant expression of the shRNA occurring. This increased the chances of gene and protein silencing, as increasing numbers of shRNA are present throughout the experiment. This is thought to be the explanation for the ability of shRNA to decrease the protein level of PKD1 compared to the transient delivery of siRNA, as siRNA half-life was no longer problematic. PKD1 over-expression was also optimised during the time taken to develop lentiviral-mediated delivery of shRNA, and work showed that the use of ionic polymers was best to achieve over-expression of PKD1. All of the time invested to develop specific and robust gene silencing or over-expression methods for the isoforms of PKD ensured that I could be confident that any effects observed on collagenase gene expression and signalling pathways could be accounted for by the individual isoforms of PKD.

6.2 Collagenase gene expression is regulated in an Isoform specific manner

Once the tools enabling the silencing and over-expression of PKD isoforms were established, the effects of each individual isoform on MMP-1 and MMP-13 gene expression were assessed. PKD has not been previously studied in chondrocytes or in the regulation of collagenase gene expression within the context of arthritis. However, PKD has been extensively studied in relation to MMP gene regulation and cancer progression. MMPs are known to be involved in cancer metastasis and cell mobility (Eiseler et al., 2009, Wille et al., 2013), with work focussing on the role of PKD1 in the expression of MMPs in both prostate and breast cancer cells. Data have suggested opposing roles for PKD1 in the regulation of MMP gene expression, with this being dependent on cell type (Eiseler et al., 2009, Biswas et al., 2010). Data are now beginning to emerge on the roles of PKD2 and PKD3 in the regulation of MMP gene expression within these cell types (Zou et al., 2012, Bernhart et al., 2013). Interestingly opposing roles for PKD1 compared to PKD2 and PKD3 are being observed, even within the same cell type (Borges and Storz, 2013, Wille et al., 2013).

When examining the effects of PKD in relation to collagenase gene expression in HAC stimulated with IL-1 in combination with OSM, opposing roles between all three isoform of PKD are seen. These data show that three closely related isoforms can have distinct roles in the regulation of the expression of the same genes under the same stimulus. These data suggest a mechanism by which each isoform counteracts the other in chondrocytes under an inflammatory stimulus, demonstrating how within chondrocytes these closely related kinases attempt to counteract the effects of an influx of inflammatory cytokines into the synovial joint and prevent ECM remodelling.

As well as the regulation of the gene expression of the classical collagenases (MMP-1 and MMP-13), other matrix degrading MMPs known to be crucial in the degradation of the ECM of cartilage were studied. Differences within the proximal promoters of each MMP have been seen (Rowan and Young, 2007, Vincenti et al., 1998); this could account for the differential effects PKD1 and PKD3 had on the expression of these MMPs. Results presented demonstrate

PKD3 silencing to reduce MMP-8 expression, whereas PKD1 silencing had no effect on the expression of this MMP. MMP-8 is seen to have an Erythroid-specific transcription factor binding site (NF-E1); this transcription binding site is not found in the promoter any of the other MMPs studied. PKD3 may therefore have the capability of regulating this protein, inducing the expression of MMP-8 in healthy chondrocytes. EGR1 is also only found within the promoter of MMP-14 compared to the MMPs studied, PKD1 may therefore only regulate the expression of this transcription factor leading to modulation in MMP-14 transcription, all of this is only speculation with no data to back up these theories. These data provide further understanding into the global consequences of individual PKD isoform inhibition on total MMP gene expression. If any isoform of PKD were to be examined as a potential therapeutic for arthritis, the consequence of their inhibition on MMP gene expression as whole would need to be fully understood.

MMP-1 and MMP-13 are not solely expressed within chondrocytes, with each enzyme being expressed within other cell types found within the joint, as well as the invading immune cells. MMP-13 expression is mainly limited to chondrocytes and bone, but fibroblasts as well as the invading macrophages have been demonstrated to express MMP-13 (Cawston and Wilson, 2006). MMP-1 is ubiquitously expressed within many cell types within the joint including; macrophages, fibroblast, endothelial and epithelial cells (Cawston and Wilson, 2006, Vincenti and Brinckerhoff, 2002). Inhibition of the signalling events in chondrocytes cannot therefore reduce complete expression of these ECM degrading enzymes, but has the potential to significantly down regulate the levels of these cartilage degrading enzymes at the site of cartilage degradation. Understanding the regulation of collagenase gene expression modulated by PKD in other tissues of the joint would provide further information into the wider consequences of PKD inhibition within the joint, if ever taken further as a potential arthritis therapeutic. These data are of even more importance as cell specific regulation of MMP gene expression has been observed (Eiseler et al., 2009, Biswas et al., 2010).

6.3 PKD signalling regulation

Once observations of differential regulation of MMPs by specific PKD isoforms had been demonstrated, I next sought to understand the signalling cascades each regulated. The MAPK signalling pathways have been shown to be important in the induction of collagenase gene expression within chondrocytes, but not fully understood (Rowan and Young, 2007). Signalling via JNK and ERK pathways activated by IL-1 leads to the phosphorylation and activation of the AP-1 family member Jun, inducing dimerization with Fos, which then drives the transcription of various MMP genes, including MMP-1 and -13 (Vincenti and Brinckerhoff, 2002). p38 is also seen to recruit the AP-1 complex to the promoter of MMP-13, leading to increased transcription (Mengshol et al., 2001). The consequences of PKD gene silencing on these signalling pathways is therefore crucial in understanding how each isoform of PKD regulates MMP-1 and MMP-13 gene expression.

PKD1 silencing data suggested that the MAPK signalling pathways were not sufficient in the regulation of collagenase gene expression in HAC when phosphorylated by this isoform. Data showed MAPK signalling to be inhibited by this isoform, but signalling events still led to an increase in collagenase gene expression. These data suggested that other signalling consequences may be necessary in addition to the MAPK pathway signalling events when regulated by PKD1. However, PKD3 silencing data clearly showed the importance of the MAPK pathway in regulating collagenase gene expression, implicating MAPK regulation as a key process in PKD3 driven induction of collagenase gene expression. This data suggests that other signalling pathways may be involved in the induction of collagenase gene expression than just MAPK regulation, with the total signal transduced by each isoform needed to be taken into account, rather than looking at one specific signalling cascade.

PKD2 data showed this isoform only regulated the phosphorylation of the MAPK pathways when stimulated within IL-1 alone, whilst the addition of OSM appeared to mask these effects. When examining the consequences of PKD2 silencing on collagenase gene expression under the stimulus of IL-1 alone, no effect on collagenase gene expression is observed, even with MAPK signalling being down-regulated. These data suggest that PKD2 has the potential to

regulate collagenase gene expression under this stimulus of IL-1 alone via MAPK regulation. One explanation for the modulation of the MAPK pathways but lack of downstream gene expression could be due to threshold levels of phosphorylation. The activation of signalling pathways is dependent on a threshold level of phosphorylation being met, with this level being exceeded for signal transduction to occur (Ferrell, 1996). The MAPKs pathways contain levels of complexity due to their three tiered system of activation. This system allows the action of this pathway to have numerous different cellular outcomes, dependent on stimuli. Here, it can be thought that the resting state of the MAPK could be well below the threshold level of phosphorylation needed to reach signal transduction. Therefore a small change in the levels of phosphorylation may lead to no signal transduction, with this being filtered out as noise (Ferrell, 1996). This could be one explanation for how PKD2 can regulate the phosphorylation of JNK, ERK and p38 under the stimulation of IL-1, but have no downstream signalling effects, with the changes in phosphorylation observed being unable to exceed the threshold level needed for signal transduction.

As well as the MAPKs, the PI3K signalling cascade has been implicated in the regulation of collagenase gene expression. The downstream substrate of the PI3K signalling pathway, Akt, has also been shown to modulate collagenase gene expression (Litherland et al., 2008). The downstream events of these pathways however remained unclear. Akt has been shown to regulate numerous downstream signalling cascades (Fresno Vara et al., 2004) and therefore understanding the downstream signalling consequences of PI3K and Akt may help to understand their role in the regulation collagenase gene expression

PKD1 gene silencing in HAC showed a marked increase in the phosphorylation of Akt, the downstream signalling consequences of this however remained unclear, with Litherland *et al*, 2008 not showing any downstream signalling consequences. One known transcription factor, downstream of Akt, is NFκB. PKD1 modulation of Akt signalling may therefore decrease the activity of the NFκB signalling pathway, a known modulator of collagenase gene expression (Fan et al., 2006, Radwan et al., 2013, Mengshol et al., 2000). Data presented in this thesis show PKD1 silencing in HAC stimulated with IL-1 in combination

with OSM to increase p65 phosphorylation at its serine 536 phosphorylation site (*Figure 5.8)*. This data opposes data presented by (Storz and Toker, 2003). PKD1 signalling in HAC stimulated with IL-1 in combination with OSM may therfore inhibit Akt, preventing its phosphorylation of IKK, inhibiting IκBα dissociation, leading to a reduction in the phosphorylation of p65, preventing its translocation to the nucleus and induction of MMP gene expression. These data suggest a potential negative feedback mechanism by which PKD1 can modulate transcriptional changes in collagenase gene expression. This mechanism does however have the potential to by Akt independent with IKK activation being seen to be directly modulated by IL-1, suggesting a further level of regulation of NFκB signalling by PKD1 under this stimulus.

PKD2 and PKD3 silencing in HAC stimulated with IL-1 in combination with OSM had no effect on the phosphorylation of Akt, suggesting these isoforms do not signal through this pathway. No effect of NFκB signalling was seen either (data not shown).

As well as activating the MAPK and PI3K signalling pathways, the pro-inflammatory cytokine stimulus of IL-1 in combination with OSM can also regulate the activation of the STAT pathway. OSM mediated activation of the JAK/STAT pathway has been implicated in the induction of collagenase gene expression (Litherland et al., 2010, Catterall et al., 2001, Rowan et al., 2001) and may contribute to the pathogenic progression of OA and RA. The addition of IL-1 induces further activation of this pathway via serine phosphorylation, showing a point of cross talk between the IL-1 and OSM signalling pathways. The STAT pathway has been clearly implicated in the progression of RA, with the over-expression of the JAK inhibiting protein, SOCS-3, in antigen-induced or collagen-induced arthritis leading to a marked reduction in the phosphorylation of STAT-3 and reduced synovitis (Shouda et al., 2001) in mice. The importance of STAT signalling in arthritis is further confirmed by the development of JAK inhibitors, such as the drug Tofacitinib, a JAK3 inhibitor which is clinically available to treat RA (Rakieh and Conaghan, 2013). The data presented in this thesis suggest that PKD can regulate the activation of this pathway, leading to potential changes in collagenase gene expression. The opposing roles of PKD1 and PKD3 in the serine phosphorylation of STAT-1 and STAT-3 suggest a

possible explanation for the opposing effects these two proteins have on collagenase gene expression.

How each isoform of PKD regulates the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation site remains unclear. This site is known to be phosphorylated by members of the MAPK family (Decker and Kovarik, 2000), but data presented in this thesis suggest that although JNK may play a role in the regulation of serine phosphorylation, PKD1 and PKD2 regulate this phosphorylation through an unknown, independent mechanism. When examining the literature, no data are evident linking PKD isoforms with STAT phosphorylation. However, stable isotope labelling of amino acids (SILAC) quantitative phosphoproteomic analysis was performed to detect phosphorylation events dependent on PKD1 activity in HEK293T cells; these data showed STAT-1 or STAT-3 were not detected as possible targets of PKD1 (Franz-Wachtel et al., 2012). These data suggest that this regulation is not via direct phosphorylation. The mechanism by which each isoform of PKD regulates the phosphorylation of this site therefore still remains unclear.

6.4 PKD and the transcriptional regulation of collagenase gene expression

The AP-1 transcription factor is well established as a regulator of collagenase gene expression in HAC following pro-inflammatory cytokine stimulation (Vincenti and Brinckerhoff, 2002), including when stimulated with IL-1 in combination with OSM (Catterall et al., 2001). Fos and Jun are the two major components of this transcription factor in HAC (Catterall et al., 2001). Many of the signalling pathways studied have been shown to either induce the expression, or phosphorylate and stabilise the protein expression of both these transcription factors (Kok et al., 2009, Litherland et al., 2010, Catterall et al., 2001, Gille et al., 1995, Wang et al., 2004, Cavigelli et al., 1995). The effects of isoform specific gene silencing on the mRNA expression of c-*fos* and c-*jun* were therefore examined. These data showed only PKD3 regulated their expression, suggesting a potential mechanism by which PKD3 signalling can lead to transcriptional changes in the mRNA expression of MMP-1 and MMP-13. The changes in expression of c-*fos* and c-*jun* as well as the phosphorylation of Jun may be regulated by PKD3 through the downstream activation of the MAPK and

STAT pathways, observed in this study. In hindsight it would have been interesting to have examined the effect of PKD1 silencing on the protein levels of Fos and Jun. As hypothesised, the expression of these may have already been maximally induced by IL-1 in combination with OSM, with gene silencing of PKD1 not further increasing mRNA expression. Increases in the duration of expression as well as the phosphorylation and stabilisation of these transcription factors may have occurred.

A temporal lag between the maximal expression of Fos, the peak of Jun activation, and MMP gene expression occurs in HAC stimulated with IL-1 in combination with OSM. At 1 hour this maximal expression and activation of c-*fos* and c-*jun* is seen, where as it takes 24 hours for peak MMP gene expression to occur. To understand this, the expression of factors which were induced as a direct consequence of AP-1 transcription were identified (Macdonald, 2013). The effect of PKD3 silencing on the expression of these factors was assessed. PKD3 was shown to regulate the expression of EGR2, BMP-2, NFATc1 and ATF3. This may be a direct result of AP-1-mediated transcription, but also a consequence of transcriptional changes modulated by the regulation of signalling pathways shown to be activated by PKD3 (Rockel et al., 2009, De Croos et al., 2007, Lu et al., 2007). As the expression of these factors occurred at an early time, further transcription factors may be expressed subsequently. One such example is RUNX2, which is known to regulate MMP-13 gene expression (Mengshol et al., 2001) and has been shown to be regulated by PKD (Jensen et al., 2009). RUNX2 is known to be induced by ATF3 and BMP-2 suggesting this as a possible mechanism (James et al., 2006, Jensen et al., 2009).

6.5 PKD as a potential therapeutic target

The role of the MMP family in disease is contradictory, with opposing roles in the progression and inhibition of different diseases reported. As well as their importance in disease, MMPs are shown to be important in normal physiology. They are shown to be crucial in development, with remodelling of ECM needed for bone deposition (Johansson et al., 1997) and also in wound healing (Ravanti and Kahari, 2000). As a consequence, broad-spectrum MMP inhibition is not thought to be a viable therapeutic option for the treatment of arthritis. Such

inhibitors were shown to be ineffective, with many drugs causing musculoskeletal side-effects (Clark and Parker, 2003). Therefore, understanding the upstream signalling events which lead to the induction of these catabolic enzymes, under a pro-inflammatory context similar to that observed in disease, may lead to the discovery of potential novel therapeutic targets. Understanding the global signalling consequences activated during disease will give further insight into the possible outcomes of drug inhibitors, and will help provide specificity of the drug, leading to reduced side effects.

There are currently no treatments that inhibit the cartilage destruction observed in OA, and treatments for RA can be expensive and seen to be ineffective in many patients. With an ageing population and increased disease burden, the development of new therapies to treat age related disease is crucial. The success of current biological therapies suggests that targeting key signalling mediators is a viable therapeutic strategy. Data presented in this thesis have demonstrated that PKD3 is able to specifically inhibit MMP-1 and MMP-13 expression, suggesting this kinase to be a possible therapeutic target for both OA and RA. However, a better understanding of the mechanisms by which PKD3 mediates collagenase gene expression is needed. For PKD3 to become a viable therapeutic target the development of isoform-specific inhibitors are needed. Isoform specific inhibitors are thought as potential therapeutics for the treatment of cancer patients, with the need for PKD3 specific inhibitors also required, as results show that PKD1, like in chondrocytes, PKD1 down regulates MMP gene expression (Borges and Storz, 2013). Once such inhibitors are available they may be even more effective in combination with other currently used therapeutics.

The current study further highlights the complexity that underpins MMP gene regulation following an inflammatory stimulus, with multiple points for cross-talk. A detailed understanding of these signalling events activated during disease progression will help to understand the processes involved in the induction of proteolytic enzymes that induce cartilage degradation. IL-1 and OSM, although not the only pro-inflammatory cytokines involved in disease progression, activate a multitude of signalling pathways known to be stimulated by other proinflammatory cytokines during disease. The understanding of signalling

kinases activated by many of the inflammatory mediators expressed during disease provides a more specific and targetable approach to treating disease, compared targeting one of the cytokines elevated during disease.

6.6 Future work

The work in this thesis highlights the importance of the individual isoforms of PKD in the regulation of collagenase gene expression. A number of points are raised into the possible mechanism by which each isoform is capable of achieving this regulation. Future work is therefore required to address these questions as well as confirming some of the conclusions made in this study.

- To further implicate PKD in MMP regulation and cartilage degradation, the effects of pan PKD inhibition, using the PKD specific inhibitor kb NB 142-70, on collagen release could be assessed. Using the bovine nasal hydroxy proline assay, bovine nasal cartilage treated with IL-1 in combination with OSM with or without the inhibitor could be used to assess the effects of PKD on collagen release. This assay could also be performed on bovine or human cartilage, although the latter is known to be difficult due to poor cartilage breakdown when stimulated with IL-1 in combination with OSM. This will provide information into the effect of pan PKD inhibition in cartilage degradation.
- Further use of the PKD inhibitor in HAC may give further clues into the dominant isoform of PKD in HAC. Using this inhibitor to study the effects of PKD inhibition on further MMPs may show a similar pattern to gene silencing studies.
- The effects of PKD1 gene silencing on signalling pathways activated by IL-1 and OSM alone would provide further insight into the mechanism by which PKD1 regulates collagenase gene expression in HAC.
- The effect of PKD2 silencing on the expression of other MMP family members could also be studied. This would give further insight into the specificity of this isoform in the regulation of MMP gene expression.
- To gain further insight into the regulation and phosphorylation of PKD, further gene silencing studies of all PKC isoforms could be performed. This would provide further information into the regulation of PKD activity via IL-1 and OSM stimulation in HAC.

- Each isoform of PKD was shown to regulate the phosphorylation of the MAPK pathways. The level at which each isoform of PKD regulates this phosphorylation still remains unclear within HAC. Phospho-MAP2K and phospho-MAP3K antibodies could be used to identify the level at which PKD regulates the MAPK pathways.
- The effect of PKD1 gene silencing on the nuclear translocation of NFκB could be studied. This would give a greater understanding of the role of this transcription factor in the regulation of collagenase gene expression via PKD1 induction.
- The effects of PKD1 silencing on post-AP-1 transcription factors could also be studied. If, as speculated, PKD1 modulates Jun expression, it would be interesting to understand whether PKD1 also modulates the expression of these transcription factors.
- As previously mentioned ATF3 has been shown to induce the expression of RUNX2, a known MMP-13 gene expression regulator. PKD has also been implicated in regulation of RUNX2 gene expression. This suggests a potential mechanism by which PKD3 could modulate the expression of RUNX2 through inducing ATF3. The effects of PKD3 gene silencing on the expression of RUNX2 could therefore be studied. The time point at which RUNX2 expression occurred would also be of interest. If as expected the expression of this transcription factor is dependent on ATF3, this could help explain the temporal lag between AP-1 gene transcription and MMP gene expression.
- To further confirm the data observed in HAC stimulated with IL-1 in combination with OSM and PKD, further pro-inflammatory cytokines seen to be elevated in arthritic disease, could be used to stimulate HAC and the assess the effects of PKD signalling. This would give further insight into the role of these proteins in the regulation of collagenase gene expression within a further arthritic context. These data would also give further insight into the usefulness of IL-1 in combination with OSM as a model to study the signalling pathways activated during inflammation during arthritic disease.
- To fully understand the role of PKD in the regulation of collagenase gene expression within the joint, the role of each isoform of PKD in the

modulation of MMP gene expression could be studied in further cell types found within the joint. As cell-specific roles of PKD in the induction of MMPs are seen, this may indicate the impact of isoform specific inhibition of each isoform of PKD within the whole joint.

6.7 Summary

This study has demonstrated the individual roles of each isoform of PKD in the regulation of collagenase gene expression under the pro-inflammatory cytokine stimulus of IL-1 in combination with OSM in HAC. Each isoform has been shown to differentially regulate collagenase gene expression, with PKD1 inhibiting MMP-1 and MMP-13 gene expression, PKD2 not being involved in their regulation and PKD3 promoting expression. Data have shown PKCι to regulate the phosphorylation of PKD in HAC stimulated with IL-1 in combination with OSM. This, to my knowledge, is the first example of this isoform of PKC regulating this phosphorylation.

Using gene silencing the signalling events involved in the induction of collagenase gene expression were shown to be differentially regulated by each isoform of PKD under this pro-inflammatory stimulus. Data imply PKD1 inhibits the expression of MMP-1 and MMP-13 through the inhibition of STAT-1 phosphorylation. This loss of phosphorylation may prevent the transcriptional activity of STAT-1. PKD1 also inhibits Akt signalling, which is a known regulator of collagenase gene expression under this stimulus; NFκB has the potential to be a downstream transcription factor involved in this process. Although PKD2 regulated the serine phosphorylation of STAT-1 and STAT-3, the lack of modulation of the MAPKs, in IL-1 in combination with OSM stimulate cells, appears to prevent this isoform from regulating collagenase gene expression. PKD3 on the other hand induces collagenase gene expression through the induction of all three MAPK pathways, as well as priming STAT-1 and STAT-3 for transcription through serine phosphorylation. These differences in the signalling pathways regulated may explain why only PKD3 regulated the expression of the key collagenase transcription factor AP-1, with the modulation of both the MAPKs and STAT pathway needed for significant changes in expression to be observed.

The potential mechanisms by which each isoform of PKD modulate MMP gene expression can be seen in *Figure 6.1, Figure 6.2* and *Figure 6.3.* Overall, I have shown the importance of studying the individual isoforms of a kinase family within one cell type, with opposing effects being observed on gene expression under the same stimulus via closely related proteins.

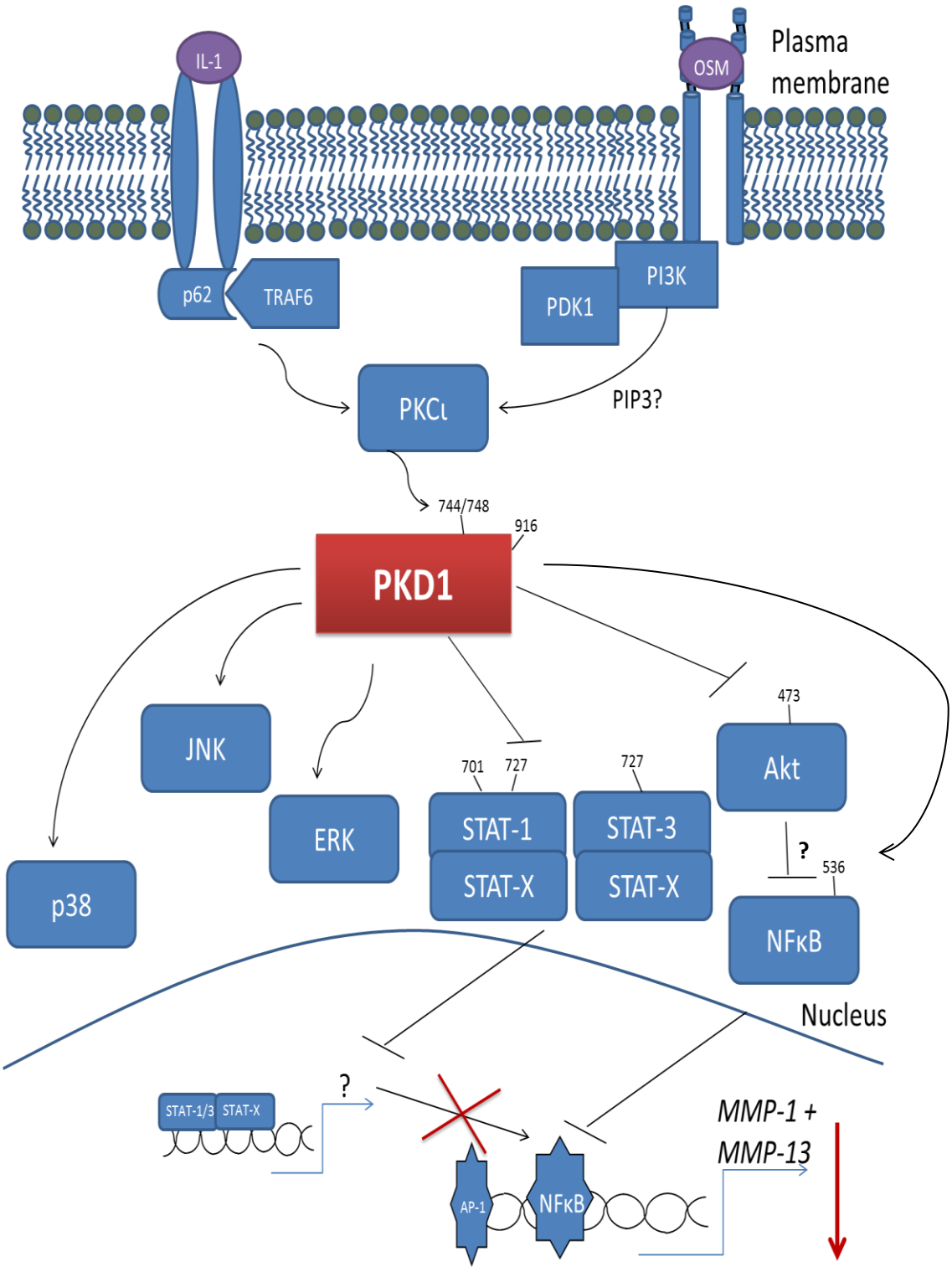


Figure 6.1. Diagram summarising the potential signalling consequences of PKD1 in HAC stimulate with IL-1 in combination with OSM. PKD1 phosphorylates all three MAPK pathways, but this appears not to regulate collagenase gene expression. PKD1 signalling inhibits the phosphorylation of STAT-1 at its tyrosine and serine sites, while phosphorylating STAT-3 only at its serine site. PKD1 also inhibits Akt signalling, which may modulate collagenase gene expression via NFκB.

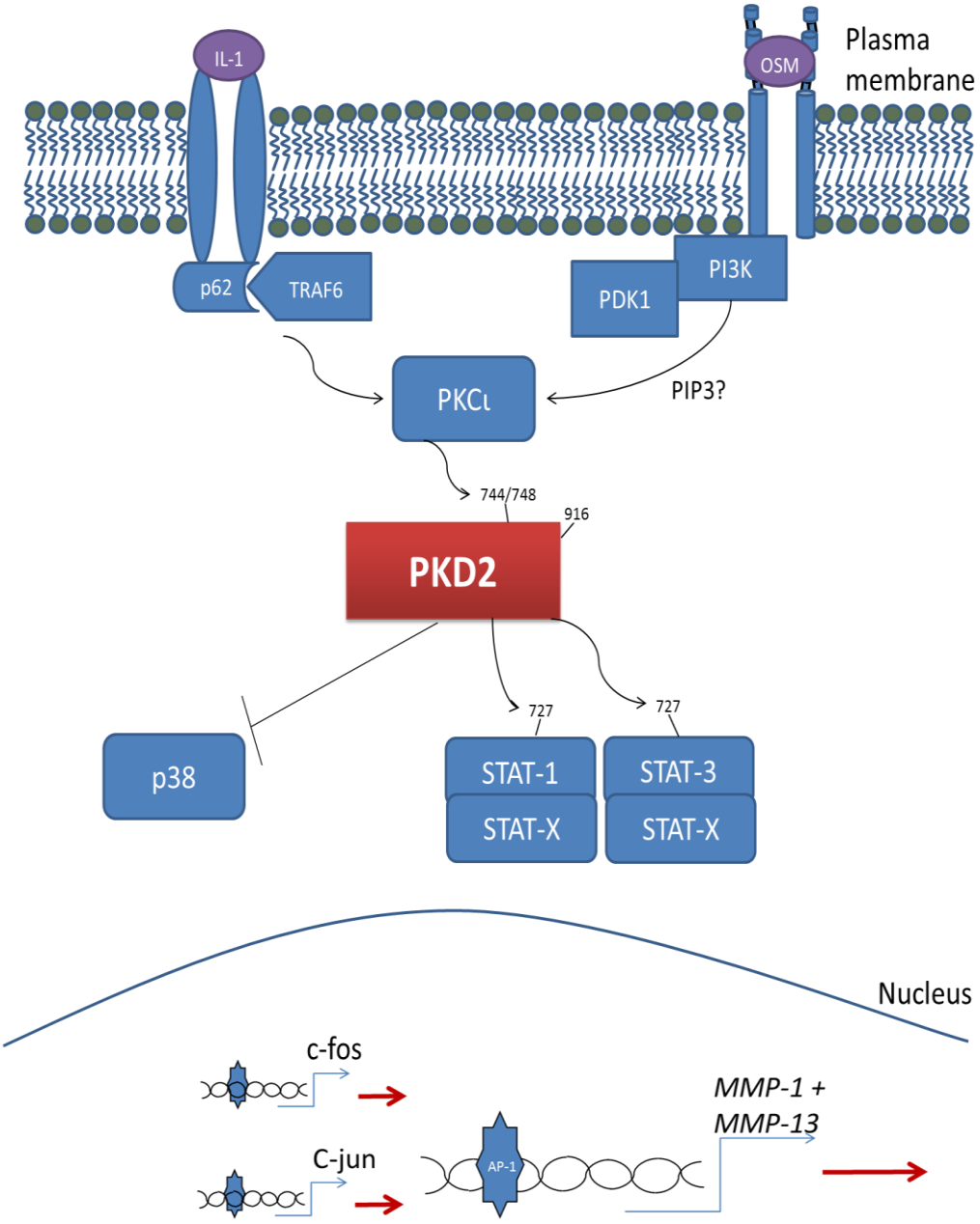


Figure 6.2. Diagram summarising the potential signalling consequences of PKD2 in HAC stimulate with IL-1 in combination with OSM. PKD2 signalling inhibits the phosphorylation of p38 but no other MAPK; this inhibition appears to have no effect on collagenase gene expression. PKD2 signalling leads to the phosphorylation of STAT-1 and STAT-3 at their serine phosphorylation sites. This phosphorylation does not have any downstream transcriptional consequences in relation to MMP-1 or MMP-13.

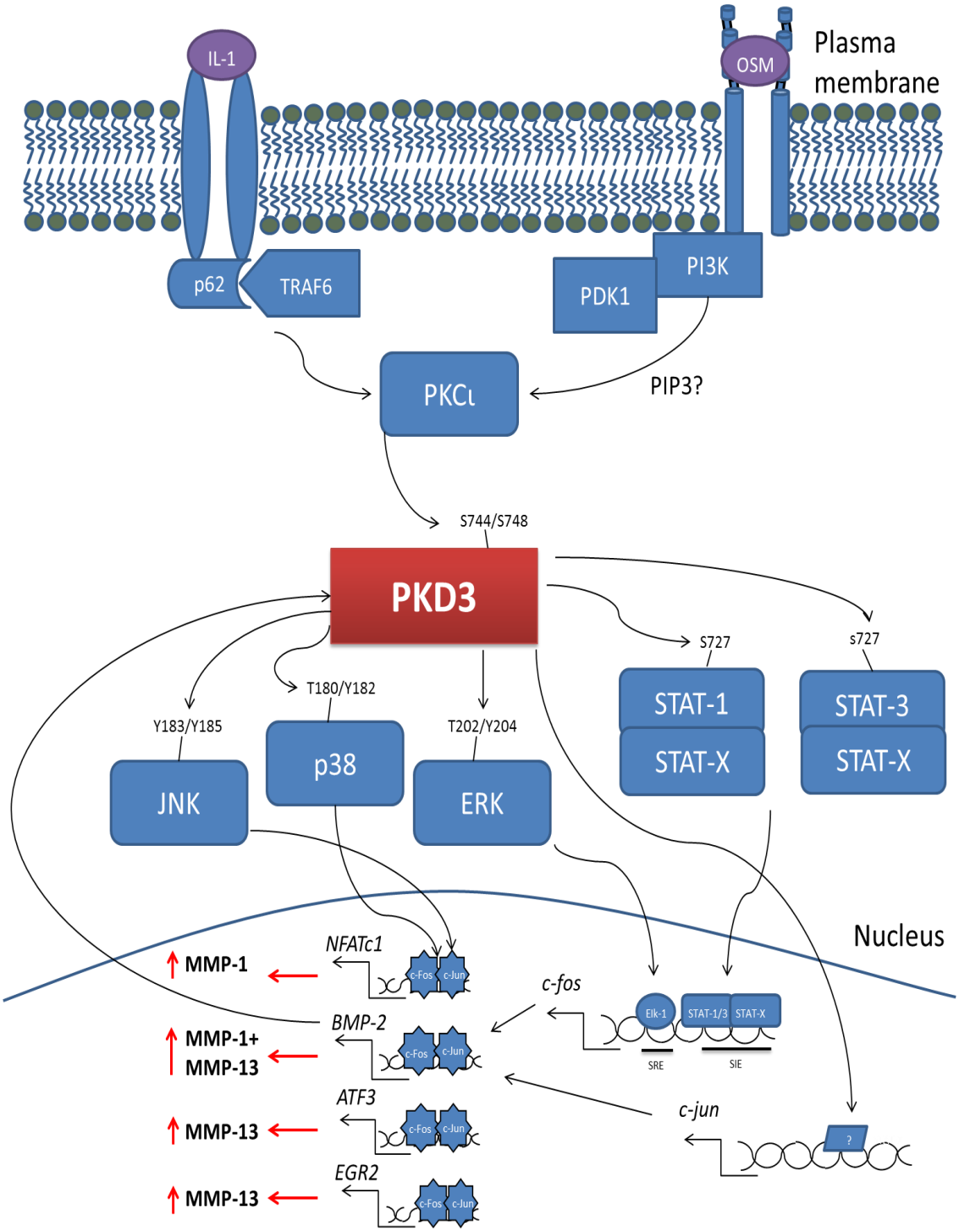


Figure 6.3. Diagram summarising the potential signalling consequences of PKD3 in HAC stimulate with IL-1 in combination with OSM. PKD3 signalling phosphorylates all three MAPK, this has the potential to lead to the induction of c-*fos* and c-*jun* gene expression as well as increase the transcriptional activity of both c-Fos and c-Jun via their phosphorylation. PKD3 also phosphorylates STAT-1 and STAT-3 at their serine phosphorylation sites. This phosphorylation may lead to the induction of c-*fos* gene expression via binding to the SIE element of the c-*fos* promoter. The induction of Fos and Jun may also increase the expression of many post-AP-1 transcription factors and cytokine, which can then induce the expression of MMP-1 and MMP-13.

7 Appendices

7.1 The effect of the PKD inhibitor kb NB 142-70 on cell death in HAC

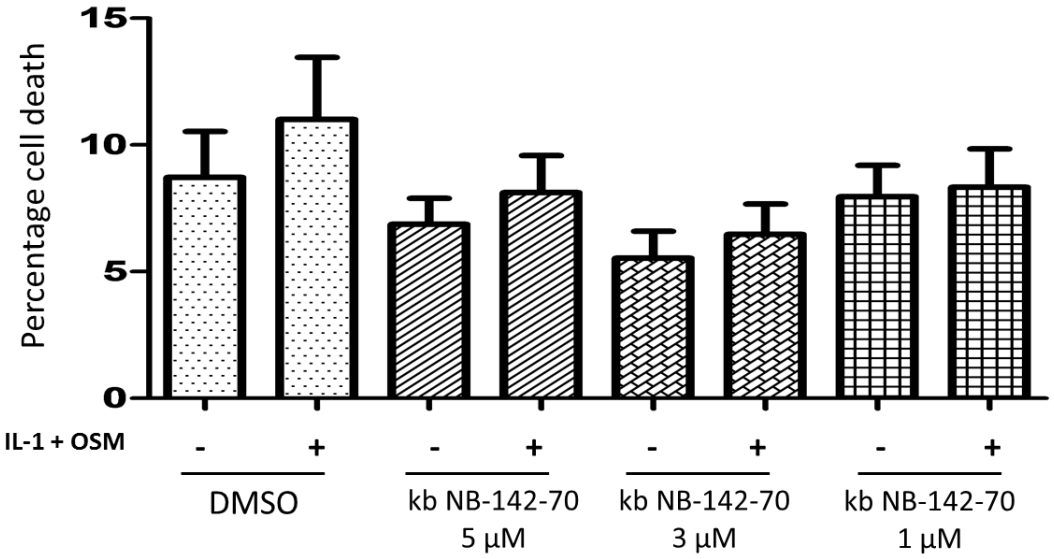


Figure 7.1. The cytotoxic effects of the PKD inhibitor kb NB 142-70 on cell death in HAC. HAC plated into 96 well plates were grown to 70% confluency and then serum starved overnight. The following day, 1 hour prior to stimulation, cells were treated with either DMSO, 1, 3, 5 µM of kb NB142-70. Cells were then stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24h. The following day half the media was extracted and the cells were freeze thawed 3 times to ensure complete lysis. The extracted and freeze thawed medium were then used in the toxi-light assay as described in the Materials and Methods. Percentage of cell death of HAC treated with the inhibitor can then be calculated. These data are accumulative of two separate chondrocyte populations (each assayed in hextuplicate).

7.2 The effect of PKD1 over-expression in SW1353 cells on collagenase gene expression

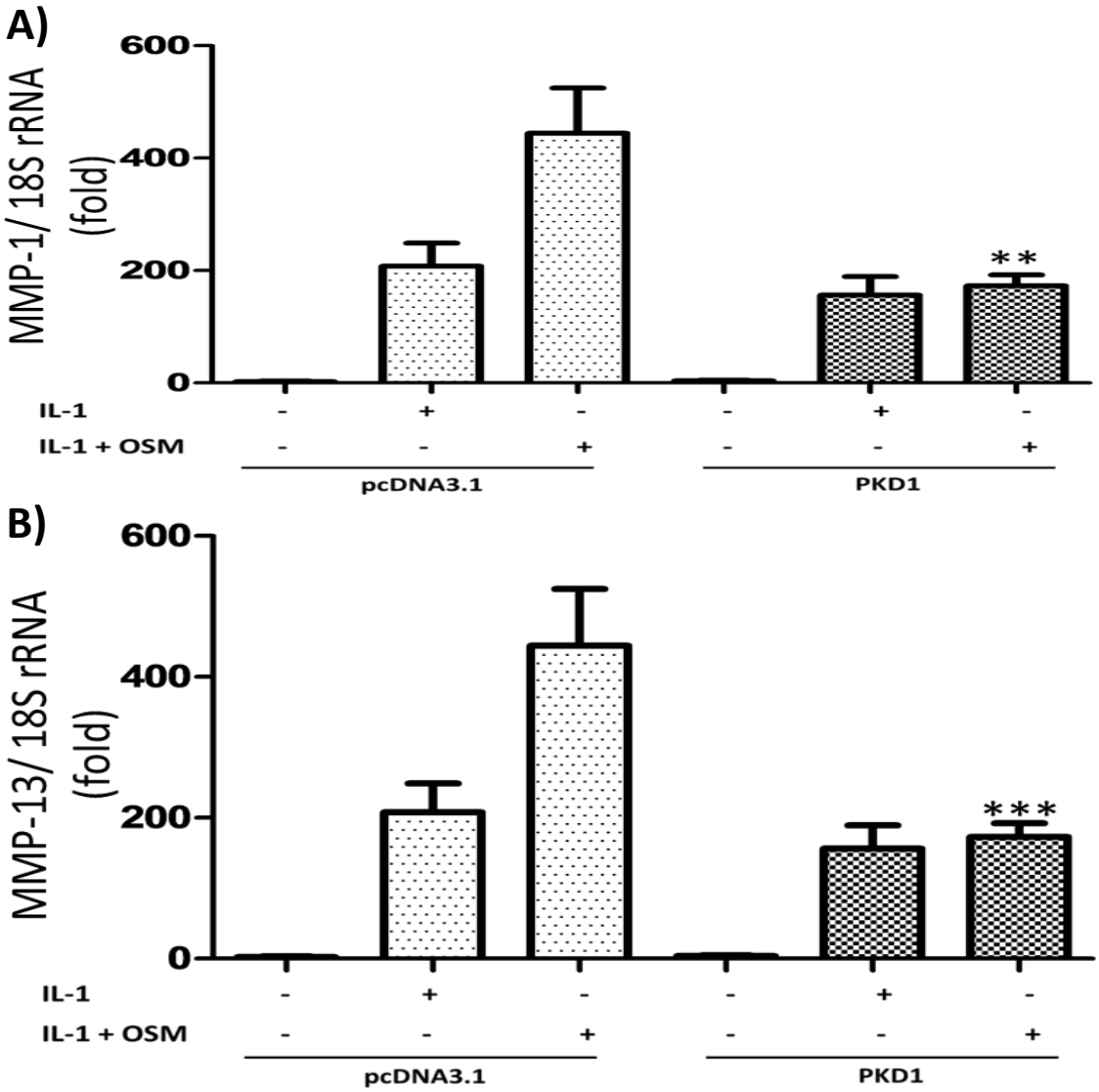


Figure 7.2. The **effect of PKD1 over-expression on collagenase expression in SW1353 cells** SW1353 cells in 96 well plates were transfected with pcDNA3-PKD1 or pcDNA3.1, and then serum starved overnight. The following day cells were stimulated with IL-1 (0.5 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 48 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control over-expression, where **, p≤0.01, ***, p≤0.001, vs transfected vector.

7.3 The effect of PKD1 gene silencing in SW1353 cells on collagenase gene expression

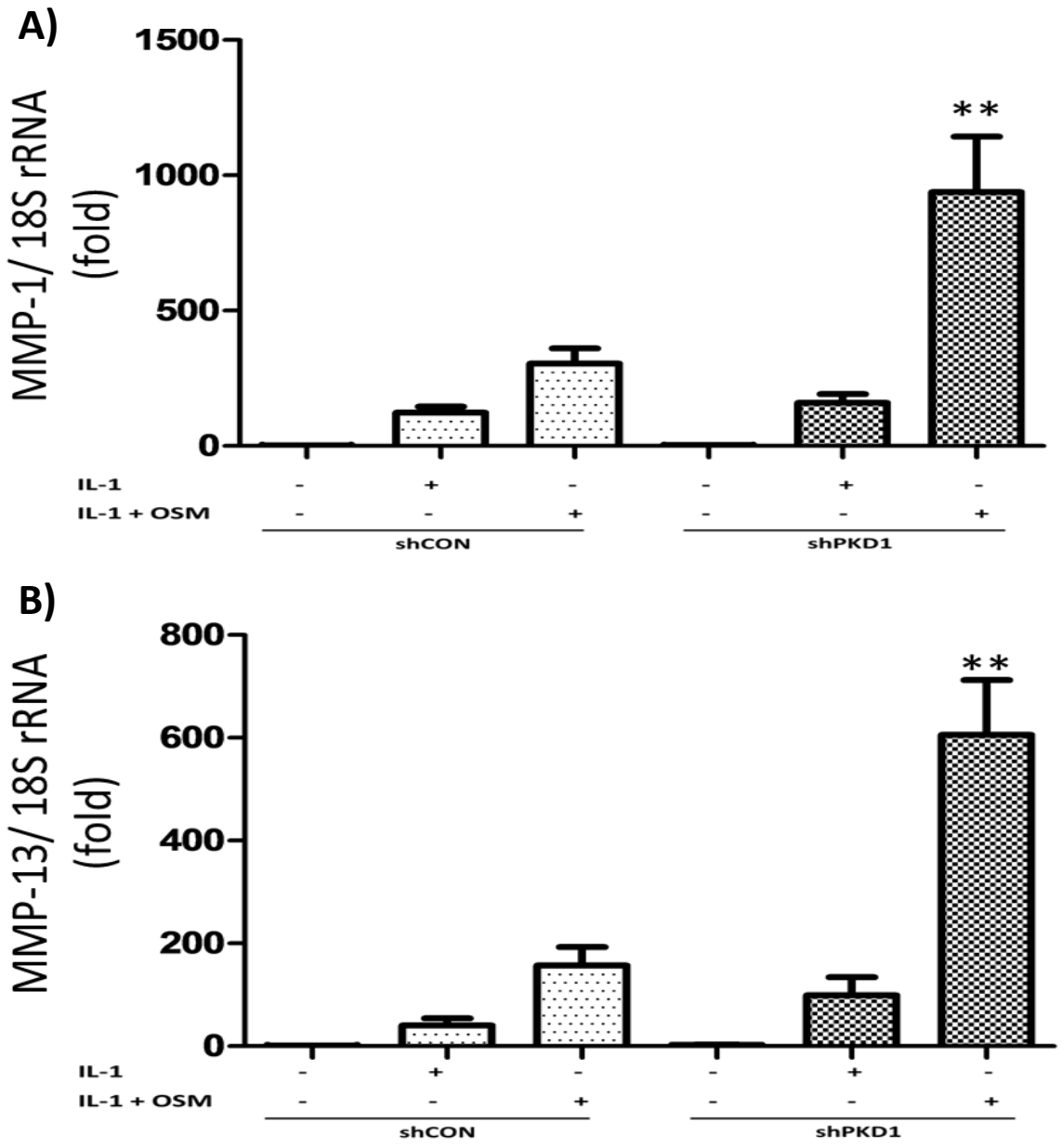


Figure 7.3. The effect of PKD1 gene silencing on collagenase expression in SW1353 cells. SW1353 cells were plated into 96 well plates and left over-night. The following day the medium was removed and replaced with viral supernatant containing either a shCON or PKD1 shRNA #2 at a MOI of 30, in a total volume of 1 ml SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.5 ng/ml) in combination with OSM (10 ng/ml) for 24 hours. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 96 h after start of transduction as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transduced PKD isoform versus control transduced, where **, p≤0.01 vs shCON.

7.4 The effect of PKD2 gene silencing in SW1353 cells on collagenase gene expression

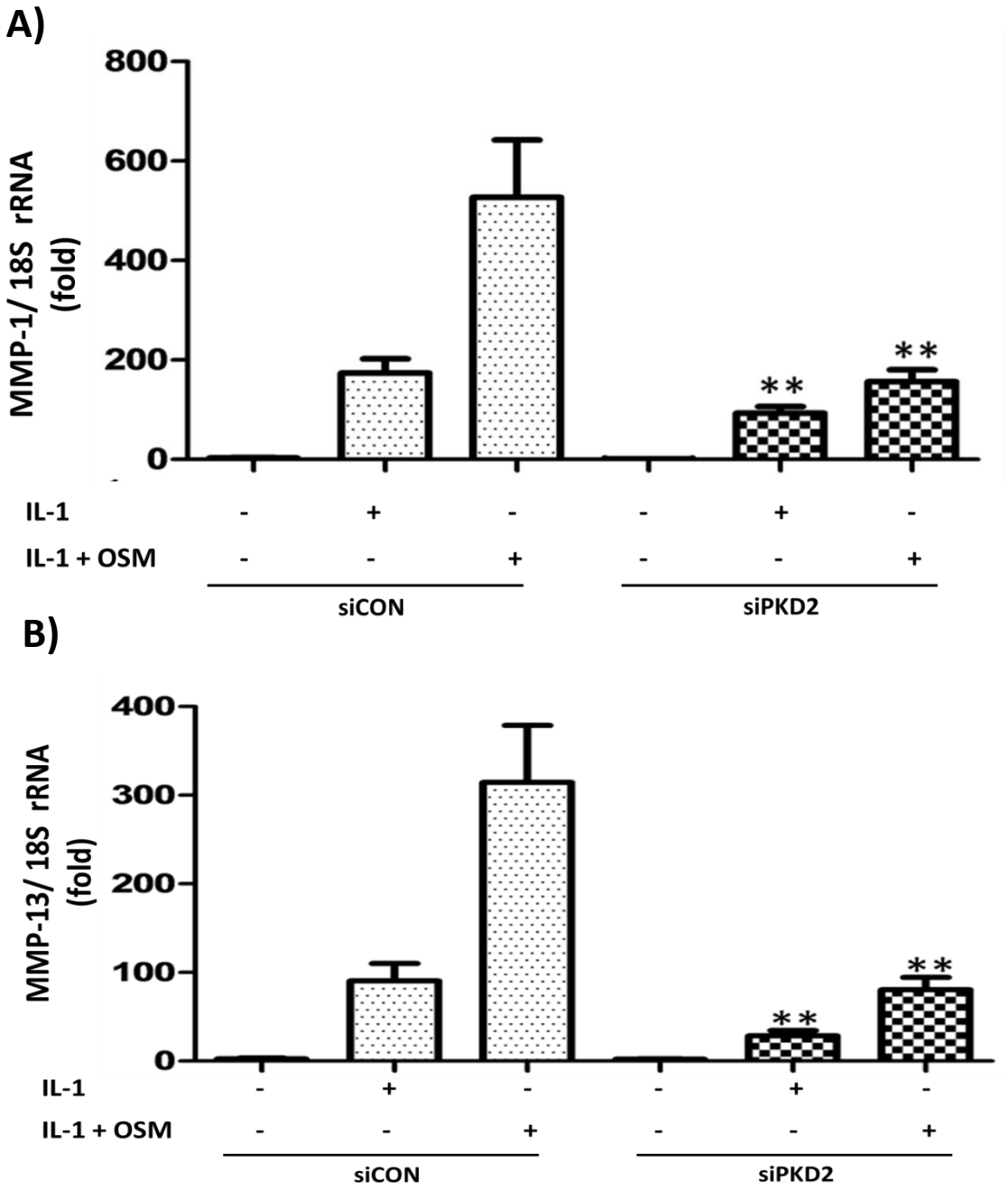


Figure 7.4. The effect of PKD2 gene silencing on collagenase expression in SW1353 cell line. Following transfection with siRNA specific to PKD2 or non-targeting siCON (100 nM) SW1353 cells were stimulated with IL-1 (0.5 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *MMP-1* and (B) *MMP-13*, 72 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control transfection, where **, p≤0.01 vs siCON.

7.5 The effect of the STAT-3 inhibitor S3I-201 on cell death in HAC

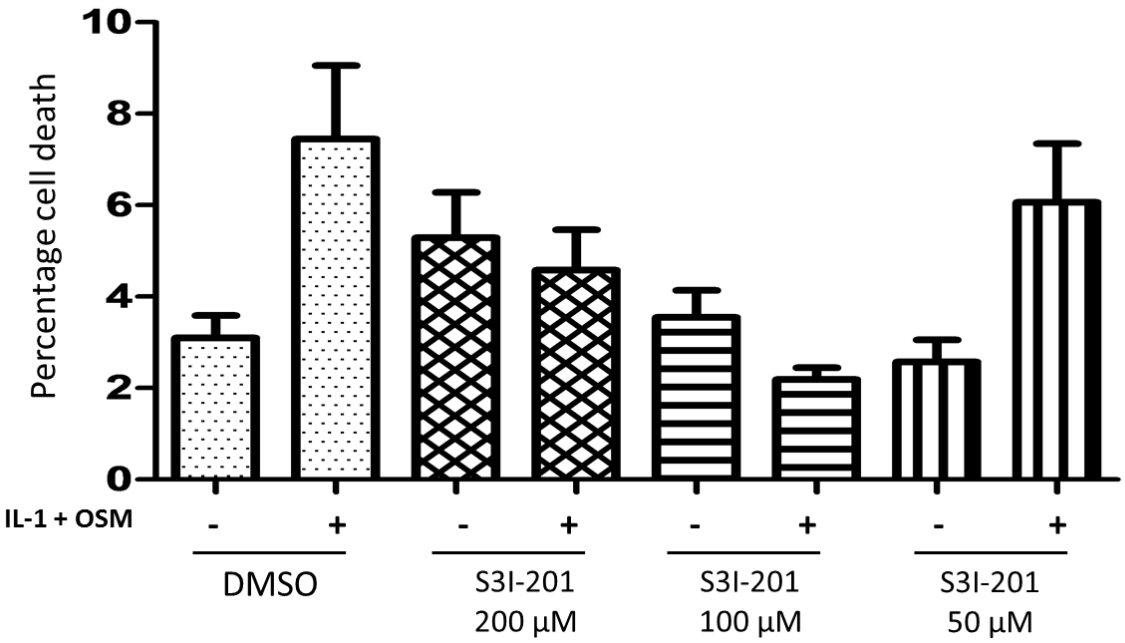


Figure 7.5. The cytotoxic effects of the STAT-3 inhibitor, S3I-201, on cell death in HAC. HAC plated into 96 well plates were grown to 70% confluency and then serum starved overnight. The following day, 1 hour prior to stimulation, cells were treated with either DMSO, 200, 100, 50 µM of S3I-201. Cells were then stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24h. The following day half the media was extracted and the cells were freeze thawed 3 times to ensure complete lysis. The extracted and freeze thawed medium were then used in the toxi-light assay as described in the Materials and Methods. Percentage of cell death of HAC treated with the inhibitor can then be calculated. These data are accumulative of two separate chondrocyte populations (each assayed in hextuplicate).

7.6 The effect of PKD3 silencing on MMP-9 gene expression

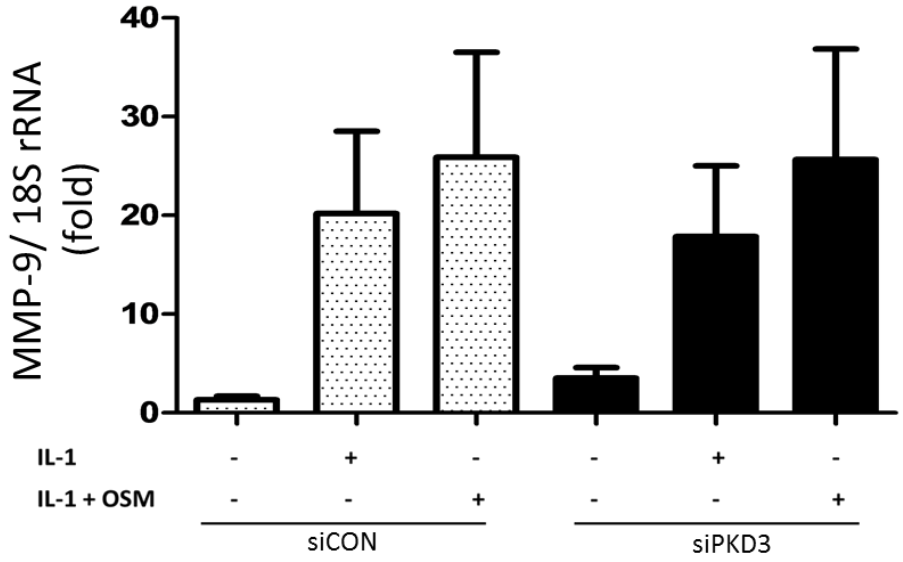


Figure 7.6 The effect of PKD3 gene silencing on MMP-9 gene expression in human chondrocytes. P0 HAC were trypsinised and plated into 96 well plates and left over-night. The following day cells were transfected with siRNA specific to PKD3 or siCON (100 nM). 48 hours later cells were serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) ± OSM (10 ng/ml) for 24 h. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for *MMP-9*, 72 h after start of transfection as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transfected PKD isoform versus control transfection, where ***, p≤0.001, **, p≤0.01, *, p≤0.05 vs siCON.

7.7 The effect of PKD1 shRNA mediated gene silencing on interferon gene expression in HAC

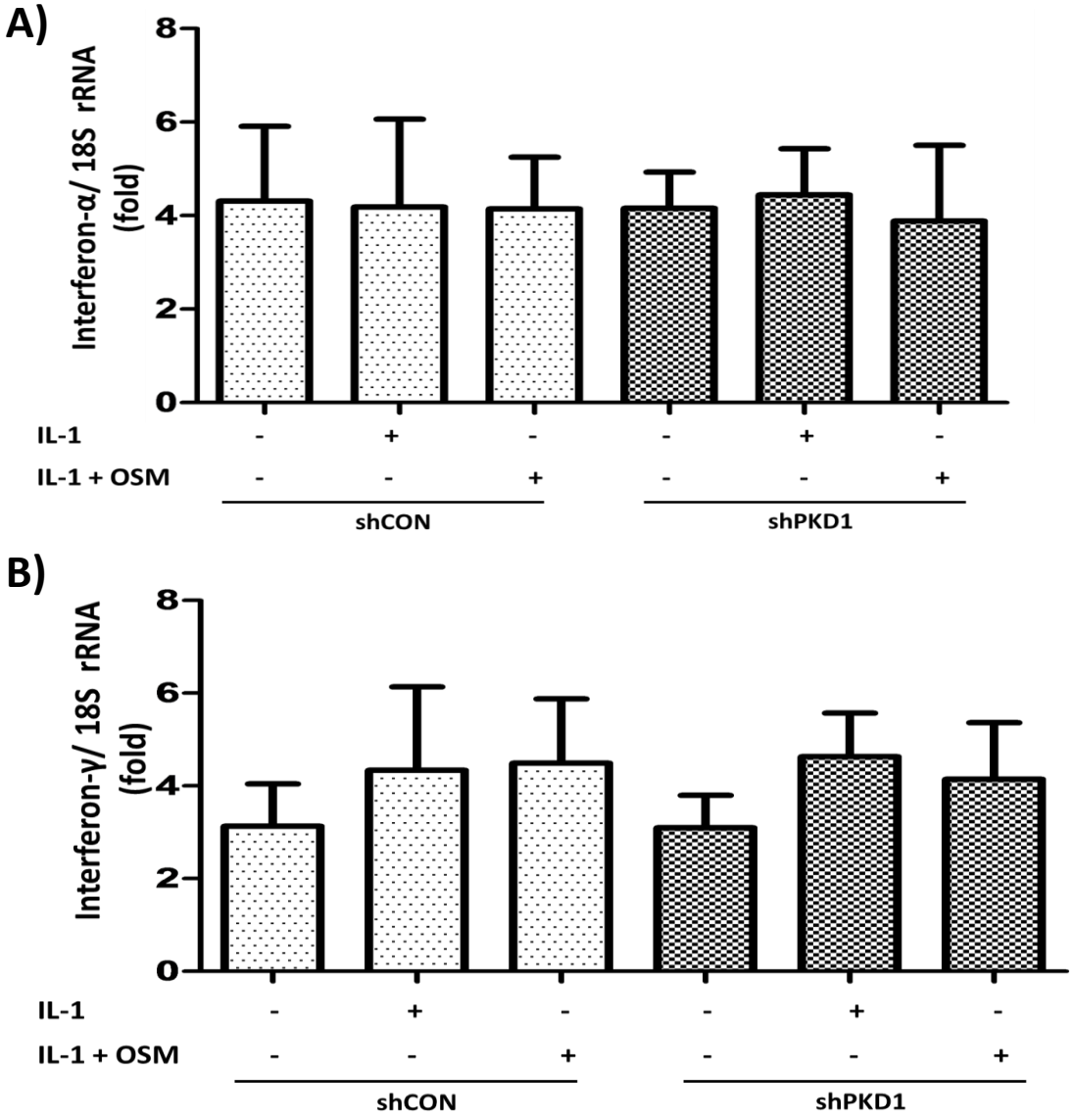


Figure 7.7. The effect of PKD1 gene silencing on interferon expression in HAC. P0 HAC were trypsinised and plated into 96 well plates and left over-night. The following day the medium was removed and replaced with viral supernatant containing either a shCON or PKD1 shRNA #2 at a MOI of 30, in a total volume of 1 ml SFM. Polybrene at a concentration of 8 µg/ml was also added. Supernatant was replenished with fresh serum containing medium after 24 hours. Cells were then visualised 72 hours post transduction to confirm shRNA expression. Cells were then serum starved over-night. The following day cells were stimulated with IL-1 (0.05 ng/ml) in combination with OSM (10 ng/ml) for 24 hours. Cells were lysed and lysates reversed transcribed to cDNA. Real-time PCR was performed for (A) *interferon-α* and (B) interferon-γ, 96 h after start of transduction as described in the Materials and Methods. Data (mean ± S.E.M.) are accumulative data of at least three separate chondrocyte populations (each assayed in hextuplicate) presented as relative expression levels normalised to 18S rRNA housekeeping gene. Comparisons shown are specific transduced PKD isoform versus control transduced, where **, p≤0.01 vs shCON.

7.8 An example of a primer dissociation curve for the primers used for the quantification of lentiviral RNA

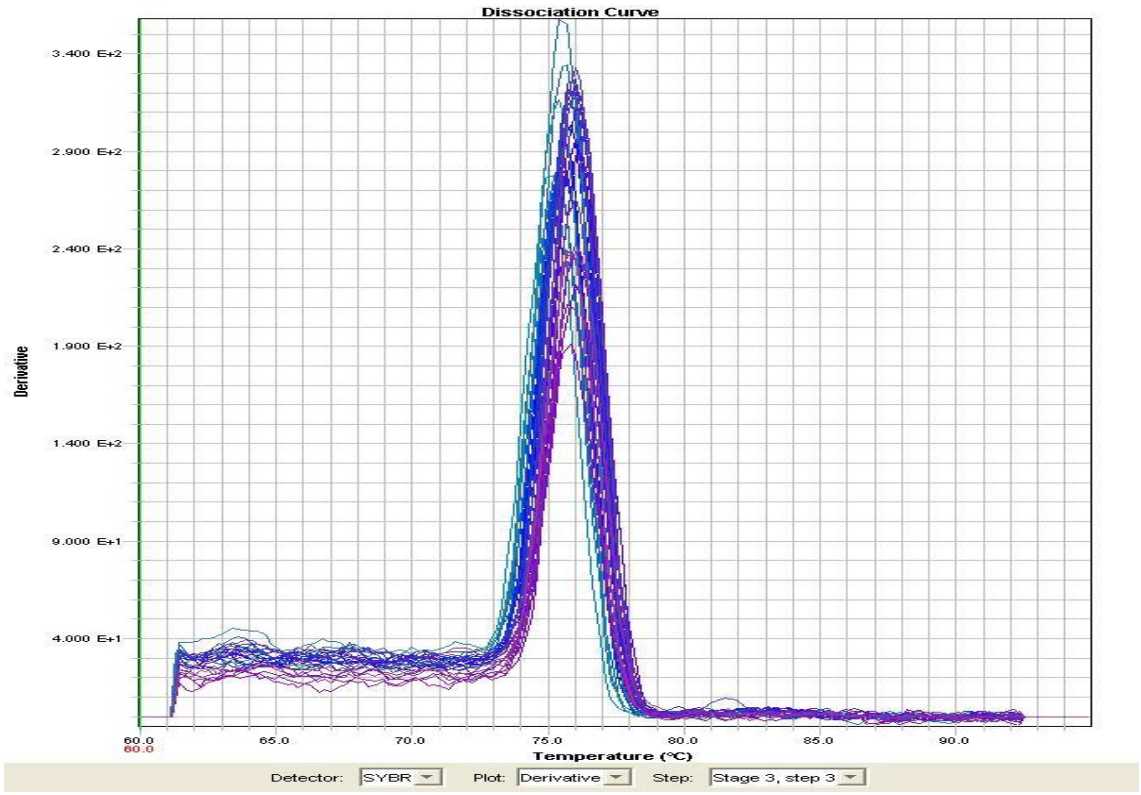
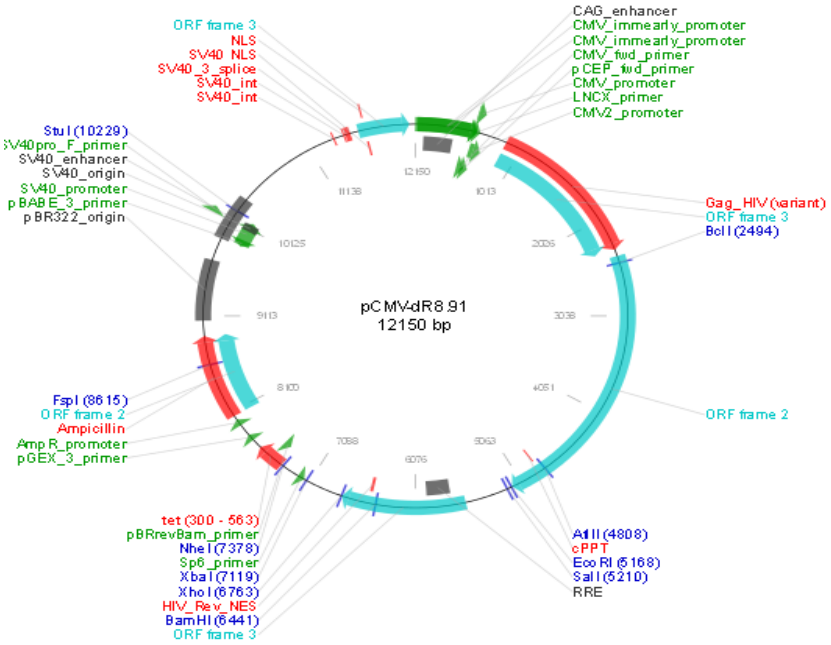


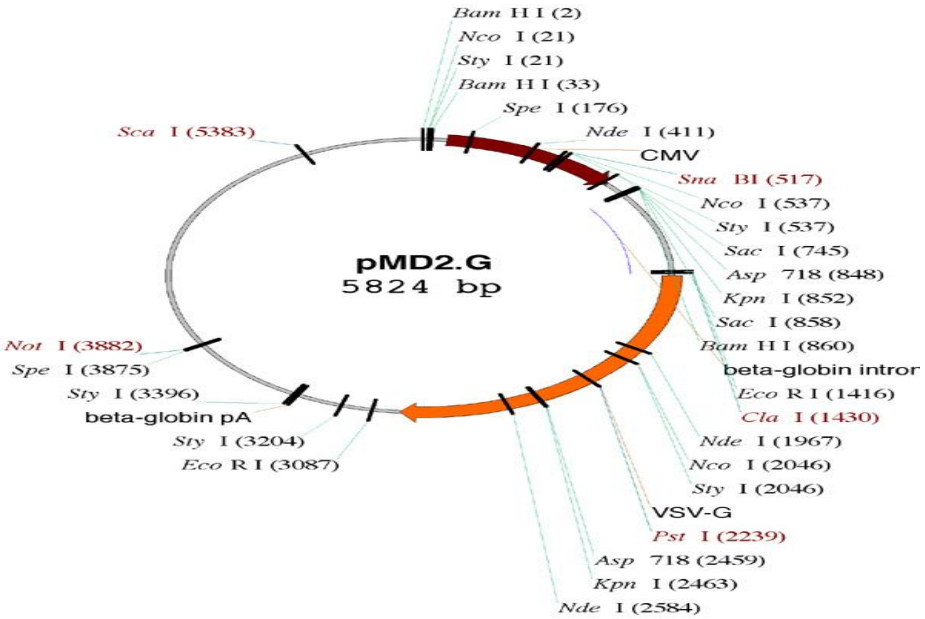
Figure 7.8. Primer dissociation curve. An example of the dissociation curves observed for the primers used for the quantification of lentiviral RNA. The One peak with no smaller peaks either side indicates that there were no primer dimers or secondary non-specific PCR artefacts.

7.9 Lentiviral packaging, envelope, PKD1 over-expression and PKD1 shRNA plasmid maps

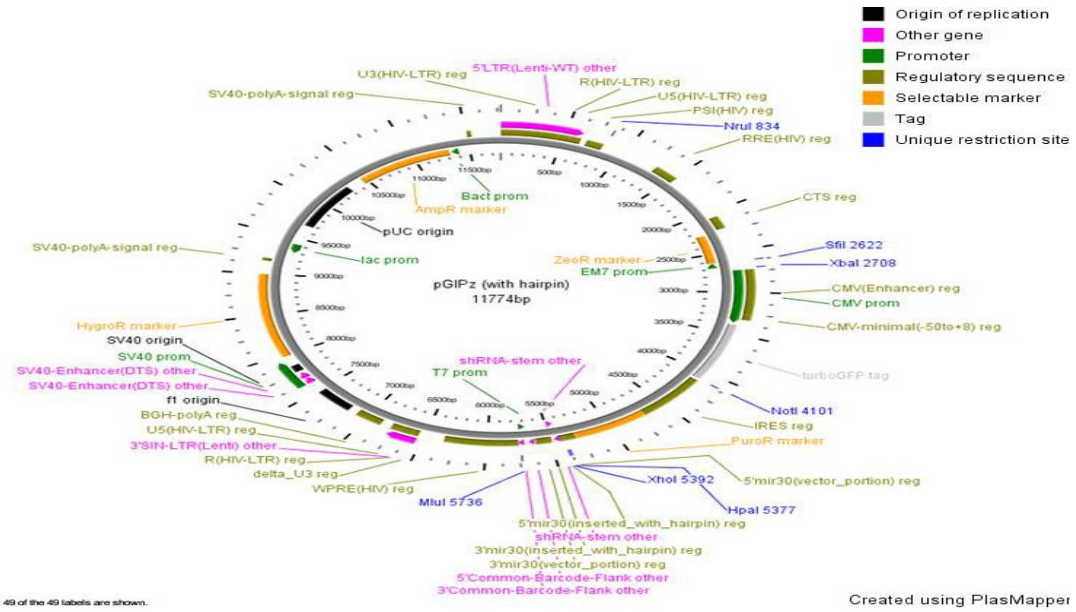
pCMV-dR8.91 packaging plasmid



pMD2.G envelope plasmid

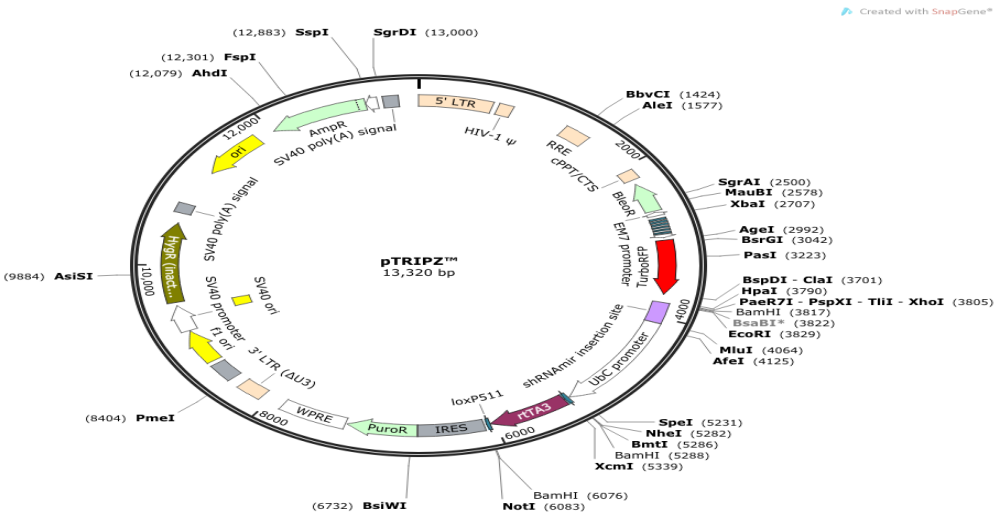


GIPZ shRNA plasmid



Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboGFP	Marker to track shRNAmir expression
IRES-puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E. coli*
SIN-LTR	3' self inactivating long terminal repeat (Shimada, et al. 1995)
RRE	Rev response element
Zeo resistance	Bacterial selectable marker

TRIPZ shRNA plasmid



Vector Element	Utility
TRE-minCMV promoter	Tetracycline responsive RNA Polymerase II promoter
UBC promoter	Drives expression of rtTA3 and IRES-puro
rtTA3	Reverse tetracycline transactivator
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
TurboRFP	Marker to track shRNAmir expression
IRES-Puro resistance	Mammalian selectable marker
Amp resistance	Ampicillin (carbenicillin) bacterial selectable marker.
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in *E.coli*
SIN-LTR	Self inactivating long terminal repeat (Shimada, et al. 1995)
RRE	Rev response element
Zeo resistance	Bacterial selectable marker

HA-tagged PKD1 over-expression plasmid

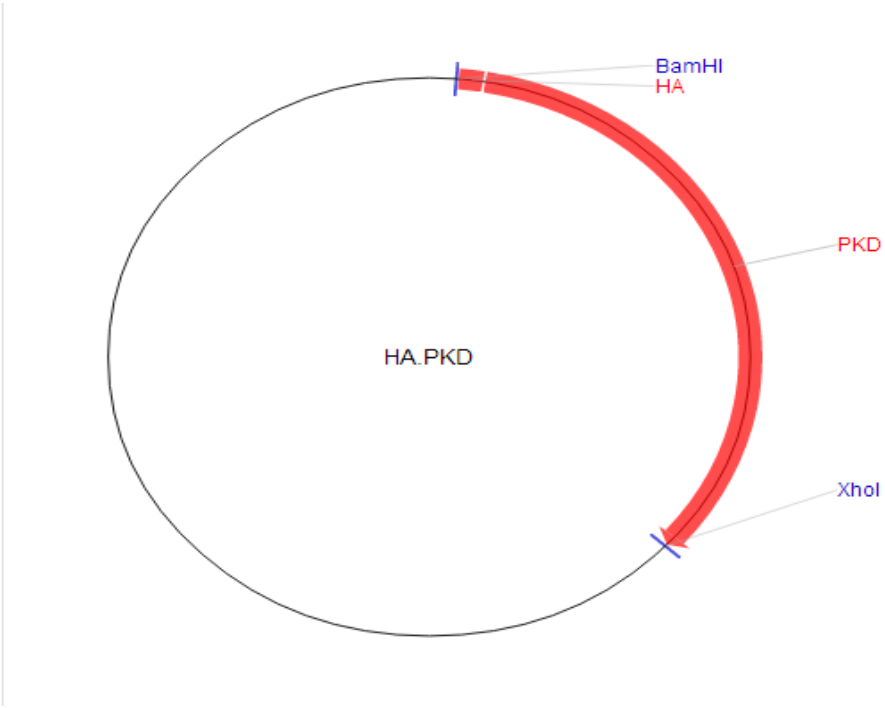


Figure 7.9 Plasmid maps for all plasmids used in this thesis. Plasmid maps for pCMV-dR8.91, pMD.2G and HA.PKD were all taken from www.addgene.org, where further information on these plasmids can be found. Plasmid maps for TRIPZ and GIPZ plasmids were taken from http://dharmacon.gelifesciences.com/shrna/tripz-lentiviral-shrna-libraries/ for TRIPZ plasmids http://dharmacon.gelifesciences.com/shrna/gipz-lentiviral-shrna-libraries/ for GIPZ plasmids, where further information on these plasmids can be found. All plasmids were transfected into cells using JetPEI transfection reagent as described in Material and methods.

8 References

ABRAMSON, S. B. & ATTUR, M. 2009. Developments in the scientific understanding of osteoarthritis. *Arthritis Res Ther,* 11**,** 227.

AHRENS, D., KOCH, A. E., POPE, R. M., STEIN-PICARELLA, M. & NIEDBALA, M. J. 1996. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. *Arthritis Rheum,* 39**,** 1576-87.

AIGNER, T., REICHENBERGER, E., BERTLING, W., KIRSCH, T., STOSS, H. & VON DER MARK, K. 1993. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. *Virchows Arch B Cell Pathol Incl Mol Pathol,* 63**,** 205-11.

AIMES, R. T. & QUIGLEY, J. P. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem,* 270**,** 5872-6.

ALEMAN, L. M., DOENCH, J. & SHARP, P. A. 2007. Comparison of siRNA-induced off-target RNA and protein effects. *RNA,* 13**,** 385-95.

ARUK. 2008. *Arthritis in the UK – key facts* [Online]. [Accessed 27.05.14 2014].

ARUK. 2011a. *Data and statistics on osteoarthritis* [Online]. Available: http://www.arthritisresearchuk.org/arthritis-information/data-and-statistics/osteoarthritis.aspx [Accessed 20/11 2013].

ARUK. 2011b. *Rheumatoid arthritis* [Online]. Available: http://www.arthritisresearchuk.org/arthritis-information/data-and-statistics/rheumatoid-arthritis.aspx [Accessed 20/11 2013].

AVKIRAN, M., ROWLAND, A. J., CUELLO, F. & HAWORTH, R. S. 2008. Protein kinase d in the cardiovascular system: emerging roles in health and disease. *Circ Res,* 102**,** 157-63.

BADGER, A. M., BRADBEER, J. N., VOTTA, B., LEE, J. C., ADAMS, J. L. & GRISWOLD, D. E. 1996. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J Pharmacol Exp Ther,* 279**,** 1453-61.

BAKIRI, L., MATSUO, K., WISNIEWSKA, M., WAGNER, E. F. & YANIV, M. 2002. Promoter specificity and biological activity of tethered AP-1 dimers. *Mol Cell Biol,* 22**,** 4952-64.

BARKSBY, H. E., HUI, W., WAPPLER, I., PETERS, H. H., MILNER, J. M., RICHARDS, C. D., CAWSTON, T. E. & ROWAN, A. D. 2006. Interleukin-1 in combination with oncostatin M up-regulates multiple genes in chondrocytes: implications for cartilage destruction and repair. *Arthritis Rheum,* 54**,** 540-50.

BELIVEAU, A., MOTT, J. D., LO, A., CHEN, E. I., KOLLER, A. A., YASWEN, P., MUSCHLER, J. & BISSELL, M. J. 2010. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. *Genes Dev,* 24**,** 2800-11.

BENBOW, U. & BRINCKERHOFF, C. E. 1997. The AP-1 site and MMP gene regulation: what is all the fuss about? *Matrix Biol,* 15**,** 519-26.

BERNHART, E., DAMM, S., WINTERSPERGER, A., DEVANEY, T., ZIMMER, A., RAYNHAM, T., IRESON, C. & SATTLER, W. 2013. Protein kinase D2 regulates migration and invasion of U87MG glioblastoma cells in vitro. *Exp Cell Res,* 319**,** 2037-48.

BHAVANASI, D., KIM, S., GOLDFINGER, L. E. & KUNAPULI, S. P. 2011. Protein kinase Cdelta mediates the activation of protein kinase D2 in platelets. *Biochem Pharmacol,* 82**,** 720-7.

BIGG, H. F., MORRISON, C. J., BUTLER, G. S., BOGOYEVITCH, M. A., WANG, Z., SOLOWAY, P. D. & OVERALL, C. M. 2001. Tissue inhibitor of metalloproteinases-4 inhibits but does not

support the activation of gelatinase A via efficient inhibition of membrane type 1-matrix metalloproteinase. *Cancer Res,* 61**,** 3610-8.

BIRKEDAL-HANSEN, H., TAYLOR, R. E., BHOWN, A. S., KATZ, J., LIN, H. Y. & WELLS, B. R. 1985. Cleavage of bovine skin type III collagen by proteolytic enzymes. Relative resistance of the fibrillar form. *J Biol Chem,* 260**,** 16411-7.

BIRMINGHAM, A., ANDERSON, E. M., REYNOLDS, A., ILSLEY-TYREE, D., LEAKE, D., FEDOROV, Y., BASKERVILLE, S., MAKSIMOVA, E., ROBINSON, K., KARPILOW, J., MARSHALL, W. S. & KHVOROVA, A. 2006. 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods,* 3**,** 199-204.

BISWAS, M. H., DU, C., ZHANG, C., STRAUBHAAR, J., LANGUINO, L. R. & BALAJI, K. C. 2010. Protein kinase D1 inhibits cell proliferation through matrix metalloproteinase-2 and matrix metalloproteinase-9 secretion in prostate cancer. *Cancer Res,* 70**,** 2095-104.

BLACK, R. A., KRONHEIM, S. R., CANTRELL, M., DEELEY, M. C., MARCH, C. J., PRICKETT, K. S., WIGNALL, J., CONLON, P. J., COSMAN, D., HOPP, T. P. & ET AL. 1988. Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. *J Biol Chem,* 263**,** 9437-42.

BODE, W., GOMIS-RUTH, F. X. & STOCKLER, W. 1993. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett,* 331**,** 134-40.

BODEN, D., PUSCH, O., SILBERMANN, R., LEE, F., TUCKER, L. & RAMRATNAM, B. 2004. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. *Nucleic Acids Res,* 32**,** 1154-8.

BOKAREWA, M., DAHLBERG, L. & TARKOWSKI, A. 2005. Expression and functional properties of antibodies to tissue inhibitors of metalloproteinases (TIMPs) in rheumatoid arthritis. *Arthritis Res Ther,* 7**,** R1014-22.

BONIZZI, G. & KARIN, M. 2004. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol,* 25**,** 280-8.

BORGES, S. & STORZ, P. 2013. Protein kinase D isoforms: new targets for therapy in invasive breast cancers? *Expert Rev Anticancer Ther,* 13**,** 895-8.

BRANDLIN, I., HUBNER, S., EISELER, T., MARTINEZ-MOYA, M., HORSCHINEK, A., HAUSSER, A., LINK, G., RUPP, S., STORZ, P., PFIZENMAIER, K. & JOHANNES, F. J. 2002. Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways. *J Biol Chem,* 277**,** 6490-6.

BRAVO-ALTAMIRANO, K., GEORGE, K. M., FRANTZ, M. C., LAVALLE, C. R., TANDON, M., LEIMGRUBER, S., SHARLOW, E. R., LAZO, J. S., WANG, Q. J. & WIPF, P. 2011. Synthesis and Structure-Activity Relationships of Benzothienothiazepinone Inhibitors of Protein Kinase D. *ACS Med Chem Lett,* 2**,** 154-159.

BROWN, M. D. & SACKS, D. B. 2009. Protein scaffolds in MAP kinase signalling. *Cell Signal,* 21**,** 462-9.

BRUMMELKAMP, T. R., BERNARDS, R. & AGAMI, R. 2002. Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell,* 2**,** 243-7.

BUCHSCHACHER, G. L., JR. & WONG-STAAL, F. 2000. Development of lentiviral vectors for gene therapy for human diseases. *Blood,* 95**,** 2499-504.

BUCKLEY, C. D. 1997. Science, medicine, and the future. Treatment of rheumatoid arthritis. *BMJ,* 315**,** 236-8.

BUCKWALTER, J. A., MANKIN, H. J. & GRODZINSKY, A. J. 2005. Articular cartilage and osteoarthritis. *Instr Course Lect,* 54**,** 465-80.

BUCKWALTER, J. A., MOW, V. C. & RATCLIFFE, A. 1994. Restoration of Injured or Degenerated Articular Cartilage. *J Am Acad Orthop Surg,* 2**,** 192-201.

BUI, C., BARTER, M. J., SCOTT, J. L., XU, Y., GALLER, M., REYNARD, L. N., ROWAN, A. D. & YOUNG, D. A. 2012. cAMP response element-binding (CREB) recruitment following a

specific CpG demethylation leads to the elevated expression of the matrix metalloproteinase 13 in human articular chondrocytes and osteoarthritis. *FASEB J,* 26**,** 3000-11.

BURNS, J. C., FRIEDMANN, T., DRIEVER, W., BURRASCANO, M. & YEE, J. K. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A,* 90**,** 8033-7.

BURRAGE, P. S., MIX, K. S. & BRINCKERHOFF, C. E. 2006. Matrix metalloproteinases: role in arthritis. *Front Biosci,* 11**,** 529-43.

BUSTIN, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology,* 25**,** 169-193.

CARON, M. M., EMANS, P. J., CREMERS, A., SURTEL, D. A., COOLSEN, M. M., VAN RHIJN, L. W. & WELTING, T. J. 2013. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthritis Cartilage,* 21**,** 604-13.

CATTERALL, J. B., CARRERE, S., KOSHY, P. J., DEGNAN, B. A., SHINGLETON, W. D., BRINCKERHOFF, C. E., RUTTER, J., CAWSTON, T. E. & ROWAN, A. D. 2001. Synergistic induction of matrix metalloproteinase 1 by interleukin-1alpha and oncostatin M in human chondrocytes involves signal transducer and activator of transcription and activator protein 1 transcription factors via a novel mechanism. *Arthritis Rheum,* 44**,** 2296-310.

CAVIGELLI, M., DOLFI, F., CLARET, F. X. & KARIN, M. 1995. Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO J,* 14**,** 5957-64.

CAWSTON, T. E., CURRY, V. A. & SUMMERS, C. A. 1998a. The role of oncostatin M in animal and human connective tissue collagen turnover and its localisation within the rheumatoid joint *Arthritis and Rheumatism,* 41**,** 1760-1771.

CAWSTON, T. E., CURRY, V. A., SUMMERS, C. A., CLARK, I. M., RILEY, G. P., LIFE, P. F., SPAULL, J. R., GOLDRING, M. B., KOSHY, P. J., ROWAN, A. D. & SHINGLETON, W. D. 1998b. The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint. *Arthritis Rheum,* 41**,** 1760-71.

CAWSTON, T. E., CURRY, V. A., SUMMERS, C. A., CLARK, I. M., RILEY, G. P., LIFE, P. F., SPAULL, J. R., GOLDRING, M. B., KOSHY, P. J. T., ROWAN, A. D. & SHINGLETON, W. D. 1998c. The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint. *Arthritis & Rheumatism,* 41**,** 1760-1771.

CAWSTON, T. E., ELLIS, A. J., HUMIN, G. & LEAN, E. 1995a. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine cartilage treated with cytokines. *Biochim Biophys Res Commun,* 215**,** 377-85.

CAWSTON, T. E., ELLIS, A. J., HUMM, G., LEAN, E., WARD, D. & CURRY, V. 1995b. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture. *Biochem Biophys Res Commun,* 215**,** 377-85.

CAWSTON, T. E. & MERCER, E. 1986. Preferential binding of collagenase to alpha 2-macroglobulin in the presence of the tissue inhibitor of metalloproteinases. *FEBS Lett,* 209**,** 9-12.

CAWSTON, T. E., MILNER, J. M., CATTERALL, J. B. & ROWAN, A. D. 2003. Cytokine synergy, collagenases and cartilage collagen breakdown. *Biochem Soc Symp***,** 125-33.

CAWSTON, T. E. & WILSON, A. J. 2006. Understanding the role of tissue degrading enzymes and their inhibitors in development and disease. *Best Pract Res Clin Rheumatol,* 20**,** 983-1002.

CAWSTON, T. E. & YOUNG, D. A. 2010. Proteinases involved in matrix turnover during cartilage and bone breakdown. *Cell Tissue Res,* 339**,** 221-35.

CHAKRABORTI, S., MANDAL, M., DAS, S., MANDAL, A. & CHAKRABORTI, T. 2003. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem,* 253**,** 269-85.

CHAMBERLAIN, S. H., HEMMER, R. M. & BRINCKERHOFF, C. E. 1993. Novel phorbol ester response region in the collagenase promoter binds Fos and Jun. *J Cell Biochem,* 52**,** 337-51.

CHAND, S., MEHTA, N., BAHIA, M. S., DIXIT, A. & SILAKARI, O. 2012. Protein kinase C-theta inhibitors: a novel therapy for inflammatory disorders. *Curr Pharm Des,* 18**,** 4725-46.

CHANG, S. H., OH, C. D., YANG, M. S., KANG, S. S., LEE, Y. S., SONN, J. K. & CHUN, J. S. 1998. Protein kinase C regulates chondrogenesis of mesenchymes via mitogen-activated protein kinase signaling. *J Biol Chem,* 273**,** 19213-9.

CHEN, A., GUPTE, C., AKHTAR, K., SMITH, P. & COBB, J. 2012. The Global Economic Cost of Osteoarthritis: How the UK Compares. *Arthritis,* 2012**,** 698709.

CHEN, F. H., ROUSCHE, K. T. & TUAN, R. S. 2006. Technology Insight: adult stem cells in cartilage regeneration and tissue engineering. *Nat Clin Pract Rheumatol,* 2**,** 373-82.

CHEN, H. H. & WANG, D. L. 2004. Nitric oxide inhibits matrix metalloproteinase-2 expression via the induction of activating transcription factor 3 in endothelial cells. *Mol Pharmacol,* 65**,** 1130-40.

CHEN, J., CRAWFORD, R. & XIAO, Y. 2013. Vertical inhibition of the PI3K/Akt/mTOR pathway for the treatment of osteoarthritis. *J Cell Biochem,* 114**,** 245-9.

CHEN, J., GIRIDHAR, K. V., ZHANG, L., XU, S. & WANG, Q. J. 2011. A protein kinase C/protein kinase D pathway protects LNCaP prostate cancer cells from phorbol ester-induced apoptosis by promoting ERK1/2 and NF-{kappa}B activities. *Carcinogenesis,* 32**,** 1198-206.

CHEN, R. H., ABATE, C. & BLENIS, J. 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc Natl Acad Sci U S A,* 90**,** 10952-6.

CHIU, T. T., LEUNG, W. Y., MOYER, M. P., STRIETER, R. M. & ROZENGURT, E. 2007. Protein kinase D2 mediates lysophosphatidic acid-induced interleukin 8 production in nontransformed human colonic epithelial cells through NF-kappaB. *Am J Physiol Cell Physiol,* 292**,** C767-77.

CHOI, S. I. & BRAHN, E. 2010. Rheumatoid arthritis therapy: advances from bench to bedside. *Autoimmunity,* 43**,** 478-92.

CHUNG, J., UCHIDA, E., GRAMMER, T. C. & BLENIS, J. 1997. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol,* 17**,** 6508-16.

CHUNG, L., DINAKARPANDIAN, D., YOSHIDA, N., LAUER-FIELDS, J. L., FIELDS, G. B., VISSE, R. & NAGASE, H. 2004. Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. *EMBO J,* 23**,** 3020-30.

CLARK, I. M. & PARKER, A. E. 2003. Metalloproteinases: their role in arthritis and potential as therapeutic targets. *Expert Opin Ther Targets,* 7**,** 19-34.

CLARK, I. M., SWINGLER, T. E., SAMPIERI, C. L. & EDWARDS, D. R. 2008. The regulation of matrix metalloproteinases and their inhibitors. *Int J Biochem Cell Biol,* 40**,** 1362-78.

COELEN, R. J., JOSE, D. G. & MAY, J. T. 1983. The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV. *Arch Virol,* 75**,** 307-11.

CREEMERS, L. B., JANSEN, I. D., DOCHERTY, A. J., REYNOLDS, J. J., BEERTSEN, W. & EVERTS, V. 1998. Gelatinase A (MMP-2) and cysteine proteinases are essential for the degradation of collagen in soft connective tissue. *Matrix Biol,* 17**,** 35-46.

CUADRADO, A. & NEBREDA, A. R. 2010. Mechanisms and functions of p38 MAPK signalling. *Biochem J,* 429**,** 403-17.

CURCI, J. A., MAO, D., BOHNER, D. G., ALLEN, B. T., RUBIN, B. G., REILLY, J. M., SICARD, G. A. & THOMPSON, R. W. 2000. Preoperative treatment with doxycycline reduces aortic wall expression and activation of matrix metalloproteinases in patients with abdominal aortic aneurysms. *J Vasc Surg,* 31**,** 325-42.

D'ORTHO, M. P., WILL, H., ATKINSON, S., BUTLER, G., MESSENT, A., GAVRILOVIC, J., SMITH, B., TIMPL, R., ZARDI, L. & MURPHY, G. 1997. Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. *Eur J Biochem,* 250**,** 751-7.

DANI, C., PIECHACZYK, M., AUDIGIER, Y., EL SABOUTY, S., CATHALA, G., MARTY, L., FORT, P., BLANCHARD, J. M. & JEANTEUR, P. 1984. Characterization of the transcription products of glyceraldehyde 3-phosphate-dehydrogenase gene in HeLa cells. *Eur J Biochem,* 145**,** 299-304.

DAUN, J. M. & FENTON, M. J. 2000. Interleukin-1/Toll receptor family members: receptor structure and signal transduction pathways. *J Interferon Cytokine Res,* 20**,** 843-55.

DAVIDSON, R. K., WATERS, J. G., KEVORKIAN, L., DARRAH, C., COOPER, A., DONELL, S. T. & CLARK, I. M. 2006. Expression profiling of metalloproteinases and their inhibitors in synovium and cartilage. *Arthritis Res Ther,* 8**,** R124.

DAVIES, S. P., REDDY, H., CAIVANO, M. & COHEN, P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J,* 351**,** 95-105.

DAVIS, H. E., ROSINSKI, M., MORGAN, J. R. & YARMUSH, M. L. 2004. Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys J,* 86**,** 1234-42.

DE CROOS, J. N., JANG, B., DHALIWAL, S. S., GRYNPAS, M. D., PILLIAR, R. M. & KANDEL, R. A. 2007. Membrane type-1 matrix metalloproteinase is induced following cyclic compression of in vitro grown bovine chondrocytes. *Osteoarthritis Cartilage,* 15**,** 1301-10.

DECKER, T. & KOVARIK, P. 2000. Serine phosphorylation of STATs. *Oncogene,* 19**,** 2628-37.

DEPOLO, N. J., REED, J. D., SHERIDAN, P. L., TOWNSEND, K., SAUTER, S. L., JOLLY, D. J. & DUBENSKY, T. W., JR. 2000. VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Mol Ther,* 2**,** 218-22.

DIDONATO, J. A., HAYAKAWA, M., ROTHWARF, D. M., ZANDI, E. & KARIN, M. 1997. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature,* 388**,** 548-54.

DINARELLO, C. A. 1996. Biologic basis for interleukin-1 in disease. *Blood,* 87**,** 2095-147.

DINARELLO, C. A. 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood,* 117**,** 3720-32.

DINGLE, J. T., PAGE THOMAS, D. P. & HAZLEMAN, B. 1987. The role of cytokines in arthritic diseases: in vitro and in vivo measurements of cartilage degradation. *Int J Tissue React,* 9**,** 349-54.

DOHERTY, P. J., ZHANG, H., TREMBLAY, L., MANOLOPOULOS, V. & MARSHALL, K. W. 1998. Resurfacing of articular cartilage explants with genetically-modified human chondrocytes in vitro. *Osteoarthritis Cartilage,* 6**,** 153-9.

DOPPLER, H., STORZ, P., LI, J., COMB, M. J. & TOKER, A. 2005. A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J Biol Chem,* 280**,** 15013-9.

DORSETT, Y. & TUSCHL, T. 2004. siRNAs: applications in functional genomics and potential as therapeutics. *Nat Rev Drug Discov,* 3**,** 318-29.

EASTGATE, J. A., SYMONS, J. A., WOOD, N. C., GRINLINTON, F. M., DI GIOVINE, F. S. & DUFF, G. W. 1988. Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis. *Lancet,* 2**,** 706-9.

EDWARDS, J. C., SZCZEPANSKI, L., SZECHINSKI, J., FILIPOWICZ-SOSNOWSKA, A., EMERY, P., CLOSE, D. R., STEVENS, R. M. & SHAW, T. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med,* 350**,** 2572-81.

EECKHOUT, Y. & VAES, G. 1977. Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Effects of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. *Biochem J,* 166**,** 21-31.

EISELER, T., DOPPLER, H., YAN, I. K., GOODISON, S. & STORZ, P. 2009. Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. *Breast Cancer Res,* 11**,** R13.

EL MABROUK, M., SYLVESTER, J. & ZAFARULLAH, M. 2007. Signaling pathways implicated in oncostatin M-induced aggrecanase-1 and matrix metalloproteinase-13 expression in human articular chondrocytes. *Biochim Biophys Acta,* 1773**,** 309-20.

ELBASHIR, S. M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K. & TUSCHL, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature,* 411**,** 494-8.

ELLIS, A. J., CURRY, V. A., POWELL, E. K. & CAWSTON, T. E. 1994. The prevention of collagen breakdown in bovine nasal cartilage by TIMP, TIMP-2 and a low molecular weight synthetic inhibitor. *Biochem Biophys Res Commun,* 201**,** 94-101.

ELLWANGER, K., PFIZENMAIER, K., LUTZ, S. & HAUSSER, A. 2008. Expression patterns of protein kinase D 3 during mouse development. *BMC Dev Biol,* 8**,** 47.

FAN, Z., YANG, H., BAU, B., SODER, S. & AIGNER, T. 2006. Role of mitogen-activated protein kinases and NFkappaB on IL-1beta-induced effects on collagen type II, MMP-1 and 13 mRNA expression in normal articular human chondrocytes. *Rheumatol Int,* 26**,** 900-3.

FANG, F., OOKA, K., BHATTACHARYYA, S., WEI, J., WU, M., DU, P., LIN, S., DEL GALDO, F., FEGHALI-BOSTWICK, C. A. & VARGA, J. 2011. The early growth response gene Egr2 (Alias Krox20) is a novel transcriptional target of transforming growth factor-beta that is up-regulated in systemic sclerosis and mediates profibrotic responses. *Am J Pathol,* 178**,** 2077-90.

FEARON, U., MULLAN, R., MARKHAM, T., CONNOLLY, M., SULLIVAN, S., POOLE, A. R., FITZGERALD, O., BRESNIHAN, B. & VEALE, D. J. 2006. Oncostatin M induces angiogenesis and cartilage degradation in rheumatoid arthritis synovial tissue and human cartilage cocultures. *Arthritis Rheum,* 54**,** 3152-62.

FERRELL, J. E., JR. 1996. Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem Sci,* 21**,** 460-6.

FIELITZ, J., KIM, M. S., SHELTON, J. M., QI, X., HILL, J. A., RICHARDSON, J. A., BASSEL-DUBY, R. & OLSON, E. N. 2008. Requirement of protein kinase D1 for pathological cardiac remodeling. *Proc Natl Acad Sci U S A,* 105**,** 3059-63.

FIRESTEIN, G. S. 2003. Evolving concepts of rheumatoid arthritis. *Nature,* 423**,** 356-61.

FITZGERALD, J. B., JIN, M., DEAN, D., WOOD, D. J., ZHENG, M. H. & GRODZINSKY, A. J. 2004. Mechanical compression of cartilage explants induces multiple time-dependent gene expression patterns and involves intracellular calcium and cyclic AMP. *J Biol Chem,* 279**,** 19502-11.

FLEENOR, D. L., PANG, I. H. & CLARK, A. F. 2003. Involvement of AP-1 in interleukin-1alpha-stimulated MMP-3 expression in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci,* 44**,** 3494-501.

FONTANA, A., HENGARTNER, H., WEBER, E., FEHR, K., GROB, P. J. & COHEN, G. 1982. Interleukin 1 activity in the synovial fluid of patients with rheumatoid arthritis. *Rheumatol Int,* 2**,** 49-53.

FOURNIER, M. L., PAULSON, A., PAVELKA, N., MOSLEY, A. L., GAUDENZ, K., BRADFORD, W. D., GLYNN, E., LI, H., SARDIU, M. E., FLEHARTY, B., SEIDEL, C., FLORENS, L. & WASHBURN, M. P. 2010. Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin. *Mol Cell Proteomics,* 9**,** 271-84.

FRANCH, H. A., SOOPARB, S., DU, J. & BROWN, N. S. 2001. A mechanism regulating proteolysis of specific proteins during renal tubular cell growth. *J Biol Chem,* 276**,** 19126-31.

FRANZ-WACHTEL, M., EISLER, S. A., KRUG, K., WAHL, S., CARPY, A., NORDHEIM, A., PFIZENMAIER, K., HAUSSER, A. & MACEK, B. 2012. Global detection of protein kinase

D-dependent phosphorylation events in nocodazole-treated human cells. *Mol Cell Proteomics,* 11**,** 160-70.

FREED, E. O. & MARTIN, M. A. 1994. HIV-1 infection of non-dividing cells. *Nature,* 369**,** 107-8.

FRESNO VARA, J. A., CASADO, E., DE CASTRO, J., CEJAS, P., BELDA-INIESTA, C. & GONZALEZ-BARON, M. 2004. PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev,* 30**,** 193-204.

FRIDMAN, R., TOTH, M., PENA, D. & MOBASHERY, S. 1995. Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Cancer Res,* 55**,** 2548-55.

FUKAMI, K., INANOBE, S., KANEMARU, K. & NAKAMURA, Y. 2010. Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Prog Lipid Res,* 49**,** 429-37.

GEBAUER, M., SAAS, J., SOHLER, F., HAAG, J., SODER, S., PIEPER, M., BARTNIK, E., BENINGA, J., ZIMMER, R. & AIGNER, T. 2005. Comparison of the chondrosarcoma cell line SW1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to IL-1beta. *Osteoarthritis Cartilage,* 13**,** 697-708.

GEETHA, T. & WOOTEN, M. W. 2002. Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett,* 512**,** 19-24.

GENG, Y., VALBRACHT, J. & LOTZ, M. 1996. Selective activation of the mitogen-activated protein kinase subgroups c-Jun NH2 terminal kinase and p38 by IL-1 and TNF in human articular chondrocytes. *J Clin Invest,* 98**,** 2425-30.

GEORGE, K. M., FRANTZ, M. C., BRAVO-ALTAMIRANO, K., LAVALLE, C. R., TANDON, M., LEIMGRUBER, S., SHARLOW, E. R., LAZO, J. S., WANG, Q. J. & WIPF, P. 2011. Design, Synthesis, and Biological Evaluation of PKD Inhibitors. *Pharmaceutics,* 3**,** 186-228.

GIBSON, T. J., SEILER, M. & VEITIA, R. A. 2013. The transience of transient overexpression. *Nat Methods,* 10**,** 715-21.

GILLE, H., KORTENJANN, M., THOMAE, O., MOOMAW, C., SLAUGHTER, C., COBB, M. H. & SHAW, P. E. 1995. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J,* 14**,** 951-62.

GIOIA, M., MONACO, S., FASCIGLIONE, G. F., COLETTI, A., MODESTI, A., MARINI, S. & COLETTA, M. 2007. Characterization of the mechanisms by which gelatinase A, neutrophil collagenase, and membrane-type metalloproteinase MMP-14 recognize collagen I and enzymatically process the two alpha-chains. *J Mol Biol,* 368**,** 1101-13.

GOLDRING, M. B. 2012. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis,* 4**,** 269-85.

GOLDRING, M. B., BIRKHEAD, J., SANDELL, L. J., KIMURA, T. & KRANE, S. M. 1988. Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. *J Clin Invest,* 82**,** 2026-37.

GOLDRING, M. B., BIRKHEAD, J. R., SUEN, L. F., YAMIN, R., MIZUNO, S., GLOWACKI, J., ARBISER, J. L. & APPERLEY, J. F. 1994. Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes. *J Clin Invest,* 94**,** 2307-16.

GOLDRING, M. B. & GOLDRING, S. R. 2007. Osteoarthritis. *J Cell Physiol,* 213**,** 626-34.

GOLDRING, M. B. & MARCU, K. B. 2009. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther,* 11**,** 224.

GOLDRING, M. B. & OTERO, M. 2011. Inflammation in osteoarthritis. *Curr Opin Rheumatol,* 23**,** 471-8.

GOLDRING, M. B., OTERO, M., TSUCHIMOCHI, K., IJIRI, K. & LI, Y. 2008. Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann Rheum Dis,* 67 Suppl 3**,** iii75-82.

GOLDRING, M. B., TSUCHIMOCHI, K. & IJIRI, K. 2006. The control of chondrogenesis. *J Cell Biochem,* 97**,** 33-44.

GOMIS-RUTH, F. X., MASKOS, K., BETZ, M., BERGNER, A., HUBER, R., SUZUKI, K., YOSHIDA, N., NAGASE, H., BREW, K., BOURENKOV, G. P., BARTUNIK, H. & BODE, W. 1997.

Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. *Nature,* 389**,** 77-81.

GOUZE, E., PAWLIUK, R., PILAPIL, C., GOUZE, J. N., FLEET, C., PALMER, G. D., EVANS, C. H., LEBOULCH, P. & GHIVIZZANI, S. C. 2002. In vivo gene delivery to synovium by lentiviral vectors. *Mol Ther,* 5**,** 397-404.

GRINER, E. M. & KAZANIETZ, M. G. 2007. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer,* 7**,** 281-94.

GROSS, J. & LAPIERE, C. M. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A,* 48**,** 1014-22.

GSCHWENDT, M., DIETERICH, S., RENNECKE, J., KITTSTEIN, W., MUELLER, H. J. & JOHANNES, F. J. 1996. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett,* 392**,** 77-80.

HA, C. H., JHUN, B. S., KAO, H. Y. & JIN, Z. G. 2008. VEGF stimulates HDAC7 phosphorylation and cytoplasmic accumulation modulating matrix metalloproteinase expression and angiogenesis. *Arterioscler Thromb Vasc Biol,* 28**,** 1782-8.

HAI, T., WOLFGANG, C. D., MARSEE, D. K., ALLEN, A. E. & SIVAPRASAD, U. 1999. ATF3 and stress responses. *Gene Expr,* 7**,** 321-35.

HAN, J., LUBY-PHELPS, K., DAS, B., SHU, X., XIA, Y., MOSTELLER, R. D., KRISHNA, U. M., FALCK, J. R., WHITE, M. A. & BROEK, D. 1998. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science,* 279**,** 558-60.

HAN, Z., BOYLE, D. L., CHANG, L., BENNETT, B., KARIN, M., YANG, L., MANNING, A. M. & FIRESTEIN, G. S. 2001. c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest,* 108**,** 73-81.

HAO, F., WU, D. D., XU, X. & CUI, M. Z. 2012. Histamine induces activation of protein kinase D that mediates tissue factor expression and activity in human aortic smooth muscle cells. *Am J Physiol Heart Circ Physiol,* 303**,** H1344-52.

HARIKUMAR, K. B., KUNNUMAKKARA, A. B., OCHI, N., TONG, Z., DEORUKHKAR, A., SUNG, B., KELLAND, L., JAMIESON, S., SUTHERLAND, R., RAYNHAM, T., CHARLES, M., BAGHERZADEH, A., FOXTON, C., BOAKES, A., FAROOQ, M., MARU, D., DIAGARADJANE, P., MATSUO, Y., SINNETT-SMITH, J., GELOVANI, J., KRISHNAN, S., AGGARWAL, B. B., ROZENGURT, E., IRESON, C. R. & GUHA, S. 2010. A novel small-molecule inhibitor of protein kinase D blocks pancreatic cancer growth in vitro and in vivo. *Mol Cancer Ther,* 9**,** 1136-46.

HAWKINS, P. T., ANDERSON, K. E., DAVIDSON, K. & STEPHENS, L. R. 2006. Signalling through Class I PI3Ks in mammalian cells. *Biochem Soc Trans,* 34**,** 647-62.

HEALY, A. M., IZMAILOVA, E., FITZGERALD, M., WALKER, R., HATTERSLEY, M., SILVA, M., SIEBERT, E., TERKELSEN, J., PICARELLA, D., PICKARD, M. D., LECLAIR, B., CHANDRA, S. & JAFFEE, B. 2006. PKC-theta-deficient mice are protected from Th1-dependent antigen-induced arthritis. *J Immunol,* 177**,** 1886-93.

HEINRICH, P. C., BEHRMANN, I., HAAN, S., HERMANNS, H. M., MULLER-NEWEN, G. & SCHAPER, F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J,* 374**,** 1-20.

HEMBRY, R. M., BAGGA, M. R., REYNOLDS, J. J. & HAMBLEN, D. L. 1995. Immunolocalisation studies on six matrix metalloproteinases and their inhibitors, TIMP-1 and TIMP-2, in synovia from patients with osteo- and rheumatoid arthritis. *Ann Rheum Dis,* 54**,** 25-32.

HESS, J., ANGEL, P. & SCHORPP-KISTNER, M. 2004. AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci,* 117**,** 5965-73.

HILL, P. A., DOCHERTY, A. J., BOTTOMLEY, K. M., O'CONNELL, J. P., MORPHY, J. R., REYNOLDS, J. J. & MEIKLE, M. C. 1995. Inhibition of bone resorption in vitro by selective inhibitors of gelatinase and collagenase. *Biochem J,* 308 (Pt 1)**,** 167-75.

HINCHLIFFE, K. A. & IRVINE, R. F. 2006. Regulation of type II PIP kinase by PKD phosphorylation. *Cell Signal,* 18**,** 1906-13.

HODGE, C., LIAO, J., STOFEGA, M., GUAN, K., CARTER-SU, C. & SCHWARTZ, J. 1998. Growth hormone stimulates phosphorylation and activation of elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem,* 273**,** 31327-36.

HOF, P., PLUSKEY, S., DHE-PAGANON, S., ECK, M. J. & SHOELSON, S. E. 1998. Crystal structure of the tyrosine phosphatase SHP-2. *Cell,* 92**,** 441-50.

HOSOKAWA, Y., HOSOKAWA, I., OZAKI, K., NAKAE, H. & MATSUO, T. 2010. Oncostatin M synergistically induces CXCL10 and ICAM-1 expression in IL-1beta-stimulated-human gingival fibroblasts. *J Cell Biochem,* 111**,** 40-8.

HUEBER, A. J., ASQUITH, D. L., MILLER, A. M., REILLY, J., KERR, S., LEIPE, J., MELENDEZ, A. J. & MCINNES, I. B. 2010. Mast cells express IL-17A in rheumatoid arthritis synovium. *J Immunol,* 184**,** 3336-40.

HUI, W., BELL, M. & CARROLL, G. 1997. Detection of oncostatin M in synovial fluid from patients with rheumatoid arthritis. *Ann Rheum Dis,* 56**,** 184-7.

HUI, W., CAWSTON, T. & ROWAN, A. D. 2003a. Transforming growth factor beta 1 and insulin-like growth factor 1 block collagen degradation induced by oncostatin M in combination with tumour necrosis factor alpha from bovine cartilage. *Ann Rheum Dis,* 62**,** 172-4.

HUI, W., ROWAN, A. D., RICHARDS, C. D. & CAWSTON, T. E. 2003b. Oncostatin M in combination with tumor necrosis factor alpha induces cartilage damage and matrix metalloproteinase expression in vitro and in vivo. *Arthritis Rheum,* 48**,** 3404-18.

HUNTER, D. J. & FELSON, D. T. 2006. Osteoarthritis. *BMJ,* 332**,** 639-42.

HURD, C., WALDRON, R. T. & ROZENGURT, E. 2002. Protein kinase D complexes with C-Jun N-terminal kinase via activation loop phosphorylation and phosphorylates the C-Jun N-terminus. *Oncogene,* 21**,** 2154-60.

IGLESIAS, T. & ROZENGURT, E. 1998. Protein kinase D activation by mutations within its pleckstrin homology domain. *J Biol Chem,* 273**,** 410-6.

IGLESIAS, T. & ROZENGURT, E. 1999. Protein kinase D activation by deletion of its cysteine-rich motifs. *FEBS Lett,* 454**,** 53-6.

IGLESIAS, T., WALDRON, R. T. & ROZENGURT, E. 1998. Identification of in vivo phosphorylation sites required for protein kinase D activation. *J Biol Chem,* 273**,** 27662-7.

IM, H. J., MUDDASANI, P., NATARAJAN, V., SCHMID, T. M., BLOCK, J. A., DAVIS, F., VAN WIJNEN, A. J. & LOESER, R. F. 2007. Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. *J Biol Chem,* 282**,** 11110-21.

ISAKOV, N. & ALTMAN, A. 2002. Protein kinase C(theta) in T cell activation. *Annu Rev Immunol,* 20**,** 761-94.

ITO, A. & NAGASE, H. 1988. Evidence that human rheumatoid synovial matrix metalloproteinase 3 is an endogenous activator of procollagenase. *Arch Biochem Biophys,* 267**,** 211-6.

ITTNER, A., BLOCK, H., REICHEL, C. A., VARJOSALO, M., GEHART, H., SUMARA, G., GSTAIGER, M., KROMBACH, F., ZARBOCK, A. & RICCI, R. 2012. Regulation of PTEN activity by p38delta-PKD1 signaling in neutrophils confers inflammatory responses in the lung. *J Exp Med,* 209**,** 2229-46.

JAGGI, M., RAO, P. S., SMITH, D. J., HEMSTREET, G. P. & BALAJI, K. C. 2003. Protein kinase C mu is down-regulated in androgen-independent prostate cancer. *Biochem Biophys Res Commun,* 307**,** 254-60.

JAMES, C. G., WOODS, A., UNDERHILL, T. M. & BEIER, F. 2006. The transcription factor ATF3 is upregulated during chondrocyte differentiation and represses cyclin D1 and A gene transcription. *BMC Mol Biol,* 7**,** 30.

JENSEN, E. D., GOPALAKRISHNAN, R. & WESTENDORF, J. J. 2009. Bone morphogenic protein 2 activates protein kinase D to regulate histone deacetylase 7 localization and repression of Runx2. *J Biol Chem,* 284**,** 2225-34.

JOHANNES, F. J., PRESTLE, J., EIS, S., OBERHAGEMANN, P. & PFIZENMAIER, K. 1994. PKCu is a novel, atypical member of the protein kinase C family. *J Biol Chem,* 269**,** 6140-8.

JOHANSSON, N., SAARIALHO-KERE, U., AIROLA, K., HERVA, R., NISSINEN, L., WESTERMARCK, J., VUORIO, E., HEINO, J. & KAHARI, V. M. 1997. Collagenase-3 (MMP-13) is expressed by hypertrophic chondrocytes, periosteal cells, and osteoblasts during human fetal bone development. *Dev Dyn,* 208**,** 387-97.

JUBB, R. W. & FELL, H. B. 1980. The breakdown of collagen by chondrocytes. *J Pathol,* 130**,** 159-162.

KARIN, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem,* 270**,** 16483-6.

KENWORTHY, R., LAMBERT, D., YANG, F., WANG, N., CHEN, Z., ZHU, H., ZHU, F., LIU, C., LI, K. & TANG, H. 2009. Short-hairpin RNAs delivered by lentiviral vector transduction trigger RIG-I-mediated IFN activation. *Nucleic Acids Res,* 37**,** 6587-99.

KEVORKIAN, L., YOUNG, D. A., DARRAH, C., DONELL, S. T., SHEPSTONE, L., PORTER, S., BROCKBANK, S. M., EDWARDS, D. R., PARKER, A. E. & CLARK, I. M. 2004. Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheum,* 50**,** 131-41.

KIM, M., JANG, H. R., KIM, J. H., NOH, S. M., SONG, K. S., CHO, J. S., JEONG, H. Y., NORMAN, J. C., CASWELL, P. T., KANG, G. H., KIM, S. Y., YOO, H. S. & KIM, Y. S. 2008. Epigenetic inactivation of protein kinase D1 in gastric cancer and its role in gastric cancer cell migration and invasion. *Carcinogenesis,* 29**,** 629-37.

KIM, Y. I., PARK, J. E., BRAND, D. D., FITZPATRICK, E. A. & YI, A. K. 2010. Protein kinase D1 is essential for the proinflammatory response induced by hypersensitivity pneumonitis-causing thermophilic actinomycetes Saccharopolyspora rectivirgula. *J Immunol,* 184**,** 3145-56.

KISS-TOTH, E., BAGSTAFF, S. M., SUNG, H. Y., JOZSA, V., DEMPSEY, C., CAUNT, J. C., OXLEY, K. M., WYLLIE, D. H., POLGAR, T., HARTE, M., O'NEILL L, A., QWARNSTROM, E. E. & DOWER, S. K. 2004. Human tribbles, a protein family controlling mitogen-activated protein kinase cascades. *J Biol Chem,* 279**,** 42703-8.

KNAUPER, V., LOPEZ-OTIN, C., SMITH, B., KNIGHT, G. & MURPHY, G. 1996a. Biochemical characterization of human collagenase-3. *J Biol Chem,* 271**,** 1544-50.

KNAUPER, V., MURPHY, G. & TSCHESCHE, H. 1996b. Activation of human neutrophil procollagenase by stromelysin 2. *Eur J Biochem,* 235**,** 187-91.

KNAUPER, V., WILL, H., LOPEZ-OTIN, C., SMITH, B., ATKINSON, S. J., STANTON, H., HEMBRY, R. M. & MURPHY, G. 1996c. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem,* 271**,** 17124-31.

KOK, S. H., HONG, C. Y., KUO, M. Y., WANG, C. C., HOU, K. L., LIN, Y. T., GALSON, D. L. & LIN, S. K. 2009. Oncostatin M-induced CCL2 transcription in osteoblastic cells is mediated by multiple levels of STAT-1 and STAT-3 signaling: an implication for the pathogenesis of arthritis. *Arthritis Rheum,* 60**,** 1451-62.

KOLCH, W. 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J,* 351 Pt 2**,** 289-305.

KONTTINEN, Y. T., CEPONIS, A., TAKAGI, M., AINOLA, M., SORSA, T., SUTINEN, M., SALO, T., MA, J., SANTAVIRTA, S. & SEIKI, M. 1998. New collagenolytic enzymes/cascade

identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above. *Matrix Biol,* 17**,** 585-601.

KORZUS, E., NAGASE, H., RYDELL, R. & TRAVIS, J. 1997. The mitogen-activated protein kinase and JAK-STAT signaling pathways are required for an oncostatin M-responsive element-mediated activation of matrix metalloproteinase 1 gene expression. *J Biol Chem,* 272**,** 1188-96.

KOSHY, P. J., HENDERSON, N., LOGAN, C., LIFE, P. F., CAWSTON, T. E. & ROWAN, A. D. 2002a. Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines. *Ann Rheum Dis,* 61**,** 704-13.

KOSHY, P. J., LUNDY, C. J., ROWAN, A. D., PORTER, S., EDWARDS, D. R., HOGAN, A., CLARK, I. M. & CAWSTON, T. E. 2002b. The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: a time-course study using real-time quantitative reverse transcription-polymerase chain reaction. *Arthritis Rheum,* 46**,** 961-7.

KOVARIK, P., STOIBER, D., EYERS, P. A., MENGHINI, R., NEININGER, A., GAESTEL, M., COHEN, P. & DECKER, T. 1999. Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. *Proc Natl Acad Sci U S A,* 96**,** 13956-61.

KUETTNER, K. E. 1992. Biochemistry of articular cartilage in health and disease. *Clin Biochem,* 25**,** 155-63.

KURZ, B., LEMKE, A. K., FAY, J., PUFE, T., GRODZINSKY, A. J. & SCHUNKE, M. 2005. Pathomechanisms of cartilage destruction by mechanical injury. *Ann Anat,* 187**,** 473-85.

KWOK, S., RITTLING, S. R., PARTRIDGE, N. C., BENSON, C. S., THIYAGARAJ, M., SRINIVASAN, N. & SELVAMURUGAN, N. 2009. Transforming growth factor-beta1 regulation of ATF-3 and identification of ATF-3 target genes in breast cancer cells. *J Cell Biochem,* 108**,** 408-14.

LANGDON, C., KERR, C., HASSEN, M., HARA, T., ARSENAULT, A. L. & RICHARDS, C. D. 2000. Murine oncostatin M stimulates mouse synovial fibroblasts in vitro and induces inflammation and destruction in mouse joints in vivo. *Am J Pathol,* 157**,** 1187-96.

LAUER-FIELDS, J. L., CHALMERS, M. J., BUSBY, S. A., MINOND, D., GRIFFIN, P. R. & FIELDS, G. B. 2009. Identification of specific hemopexin-like domain residues that facilitate matrix metalloproteinase collagenolytic activity. *J Biol Chem,* 284**,** 24017-24.

LEFEBVRE, V., PEETERS-JORIS, C. & VAES, G. 1990. Modulation by interleukin 1 and tumor necrosis factor alpha of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. *Biochim Biophys Acta,* 1052**,** 366-78.

LEHMANN, U., SOMMER, U., SMYCZEK, T., HORTNER, M., FRISCH, W., VOLKMER-ENGERT, R., HEINRICH, P. C., SCHAPER, F. & HAAN, S. 2006. Determinants governing the potency of STAT3 activation via the individual STAT3-recruiting motifs of gp130. *Cell Signal,* 18**,** 40-9.

LEIPE, J., GRUNKE, M., DECHANT, C., REINDL, C., KERZENDORF, U., SCHULZE-KOOPS, H. & SKAPENKO, A. 2010. Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum,* 62**,** 2876-85.

LEMONNIER, J., GHAYOR, C., GUICHEUX, J. & CAVERZASIO, J. 2004. Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. *J Biol Chem,* 279**,** 259-64.

LEPPA, S., SAFFRICH, R., ANSORGE, W. & BOHMANN, D. 1998. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *EMBO J,* 17**,** 4404-13.

LI, C., LI, J., CAI, X., SUN, H., JIAO, J., BAI, T., ZHOU, X. W., CHEN, X., GILL, D. L. & TANG, X. D. 2011. Protein kinase D3 is a pivotal activator of pathological cardiac hypertrophy by

selectively increasing the expression of hypertrophic transcription factors. *J Biol Chem,* 286**,** 40782-91.

LI, W. Q., DEHNADE, F. & ZAFARULLAH, M. 2001. Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway. *J Immunol,* 166**,** 3491-8.

LI, Y., TEW, S. R., RUSSELL, A. M., GONZALEZ, K. R., HARDINGHAM, T. E. & HAWKINS, R. E. 2004. Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9. *Tissue Eng,* 10**,** 575-84.

LIACINI, A., SYLVESTER, J., LI, W. Q. & ZAFARULLAH, M. 2002. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-kappa B) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. *Matrix Biol,* 21**,** 251-62.

LIM, C. P. & CAO, X. 1999. Serine phosphorylation and negative regulation of Stat3 by JNK. *J Biol Chem,* 274**,** 31055-61.

LIM, H. & KIM, H. P. 2011. Matrix metalloproteinase-13 expression in IL-1beta-treated chondrocytes by activation of the p38 MAPK/c-Fos/AP-1 and JAK/STAT pathways. *Arch Pharm Res,* 34**,** 109-17.

LINT, J. V., RYKX, A., VANTUS, T. & VANDENHEEDE, J. R. 2002. Getting to know protein kinase D. *Int J Biochem Cell Biol,* 34**,** 577-81.

LITHERLAND, G. J., DIXON, C., LAKEY, R. L., ROBSON, T., JONES, D., YOUNG, D. A., CAWSTON, T. E. & ROWAN, A. D. 2008. Synergistic collagenase expression and cartilage collagenolysis are phosphatidylinositol 3-kinase/Akt signaling-dependent. *J Biol Chem,* 283**,** 14221-9.

LITHERLAND, G. J., ELIAS, M. S., HUI, W., MACDONALD, C. D., CATTERALL, J. B., BARTER, M. J., FARREN, M. J., JEFFERSON, M. & ROWAN, A. D. 2010. Protein kinase C isoforms zeta and iota mediate collagenase expression and cartilage destruction via STAT3- and ERK-dependent c-fos induction. *J Biol Chem,* 285**,** 22414-25.

LITHERLAND, G. J., HUI, W., ELIAS, M. S., WILKINSON, D. J., WATSON, S., HUESA, C., YOUNG, D. A. & ROWAN, A. D. 2014. Glycogen synthase kinase-3 INHIBITION STIMULATES human CARTILAGE DESTRUCTION and exacerbates murine osteoarthritis. *Arthritis Rheumatol*.

LIU, J., XIONG, W., BACA-REGEN, L., NAGASE, H. & BAXTER, B. T. 2003. Mechanism of inhibition of matrix metalloproteinase-2 expression by doxycycline in human aortic smooth muscle cells. *J Vasc Surg,* 38**,** 1376-83.

LOTZ, M. & GUERNE, P. A. 1991. Interleukin-6 induces the synthesis of tissue inhibitor of metalloproteinases-1/erythroid potentiating activity (TIMP-1/EPA). *J Biol Chem,* 266**,** 2017-20.

LU, D., CHEN, J. & HAI, T. 2007. The regulation of ATF3 gene expression by mitogen-activated protein kinases. *Biochem J,* 401**,** 559-67.

LUBBERTS, E., JOOSTEN, L. A., CHABAUD, M., VAN DEN BERSSELAAR, L., OPPERS, B., COENEN-DE ROO, C. J., RICHARDS, C. D., MIOSSEC, P. & VAN DEN BERG, W. B. 2000. IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. *J Clin Invest,* 105**,** 1697-710.

LUBBERTS, E., KOENDERS, M. I. & VAN DEN BERG, W. B. 2005. The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. *Arthritis Res Ther,* 7**,** 29-37.

LUCAS, B. 2004. Nursing management issues in hip and knee replacement surgery. *Br J Nurs,* 13**,** 782-7.

LUTTICKEN, C., WEGENKA, U. M., YUAN, J., BUSCHMANN, J., SCHINDLER, C., ZIEMIECKI, A., HARPUR, A. G., WILKS, A. F., YASUKAWA, K., TAGA, T. & ET AL. 1994. Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science,* 263**,** 89-92.

LV, D., MENG, D., ZOU, F. F., FAN, L., ZHANG, P., YU, Y. & FANG, J. 2011. Activating transcription factor 3 regulates survivability and migration of vascular smooth muscle cells. *IUBMB Life,* 63**,** 62-9.

LYNCH, C. M., LEANDRY, L. A. & MATHENY, R. W., JR. 2013. Lysophosphatidic acid-stimulated phosphorylation of PKD2 is mediated by PI3K p110beta and PKCdelta in myoblasts. *J Recept Signal Transduct Res,* 33**,** 41-8.

MACDONALD, C. 2013. *Synergistic transcriptional regulation of collagenase gene expression in chondrocytes.* Newcastle University.

MACNAUL, K. L., CHARTRAIN, N., LARK, M., TOCCI, M. J. & HUTCHINSON, N. I. 1990. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts. Synergistic effects of interleukin-1 and tumor necrosis factor-alpha on stromelysin expression. *J Biol Chem,* 265**,** 17238-45.

MARCU, K. B., OTERO, M., OLIVOTTO, E., BORZI, R. M. & GOLDRING, M. B. 2010. NF-kappaB signaling: multiple angles to target OA. *Curr Drug Targets,* 11**,** 599-613.

MARTEL-PELLETIER, J., BOILEAU, C., PELLETIER, J. P. & ROUGHLEY, P. J. 2008. Cartilage in normal and osteoarthritis conditions. *Best Pract Res Clin Rheumatol,* 22**,** 351-84.

MARTEL-PELLETIER, J., MCCOLLUM, R., DIBATTISTA, J., FAURE, M. P., CHIN, J. A., FOURNIER, S., SARFATI, M. & PELLETIER, J. P. 1992. The interleukin-1 receptor in normal and osteoarthritic human articular chondrocytes. Identification as the type I receptor and analysis of binding kinetics and biologic function. *Arthritis Rheum,* 35**,** 530-40.

MARTIGNETTI, J. A., AQEEL, A. A., SEWAIRI, W. A., BOUMAH, C. E., KAMBOURIS, M., MAYOUF, S. A., SHETH, K. V., EID, W. A., DOWLING, O., HARRIS, J., GLUCKSMAN, M. J., BAHABRI, S., MEYER, B. F. & DESNICK, R. J. 2001. Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nat Genet,* 28**,** 261-5.

MARTINY-BARON, G., KAZANIETZ, M. G., MISCHAK, H., BLUMBERG, P. M., KOCHS, G., HUG, H., MARME, D. & SCHACHTELE, C. 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem,* 268**,** 9194-7.

MATRISIAN, L. M. 1992. The matrix-degrading metalloproteinases. *Bioessays,* 14**,** 455-63.

MATTHEWS, S., IGLESIAS, T., CANTRELL, D. & ROZENGURT, E. 1999. Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. *FEBS Lett,* 457**,** 515-21.

MATTHEWS, S. A., NAVARRO, M. N., SINCLAIR, L. V., EMSLIE, E., FEIJOO-CARNERO, C. & CANTRELL, D. A. 2010. Unique functions for protein kinase D1 and protein kinase D2 in mammalian cells. *Biochem J,* 432**,** 153-63.

MCCAWLEY, L. J. & MATRISIAN, L. M. 2001. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol,* 13**,** 534-40.

MCENEANEY, V., DOOLEY, R., HARVEY, B. J. & THOMAS, W. 2010. Protein kinase D stabilizes aldosterone-induced ERK1/2 MAP kinase activation in M1 renal cortical collecting duct cells to promote cell proliferation. *J Steroid Biochem Mol Biol,* 118**,** 18-28.

MCINNES, I. B. & SCHETT, G. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol,* 7**,** 429-42.

MENGSHOL, J. A., VINCENTI, M. P. & BRINCKERHOFF, C. E. 2001. IL-1 induces collagenase-3 (MMP-13) promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation by p38 MAPK and JNK pathways. *Nucleic Acids Res,* 29**,** 4361-72.

MENGSHOL, J. A., VINCENTI, M. P., COON, C. I., BARCHOWSKY, A. & BRINCKERHOFF, C. E. 2000. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum,* 43**,** 801-11.

MIHAILOVIC, T., MARX, M., AUER, A., VAN LINT, J., SCHMID, M., WEBER, C. & SEUFFERLEIN, T. 2004. Protein kinase D2 mediates activation of nuclear factor kappaB by Bcr-Abl in Bcr-Abl+ human myeloid leukemia cells. *Cancer Res,* 64**,** 8939-44.

MILNER, J. M., ELLIOTT, S. F. & CAWSTON, T. E. 2001. Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways. *Arthritis Rheum,* 44**,** 2084-96.

MILNER, J. M., PATEL, A., DAVIDSON, R. K., SWINGLER, T. E., DESILETS, A., YOUNG, D. A., KELSO, E. B., DONELL, S. T., CAWSTON, T. E., CLARK, I. M., FERRELL, W. R., PLEVIN, R., LOCKHART, J. C., LEDUC, R. & ROWAN, A. D. 2010. Matriptase is a novel initiator of cartilage matrix degradation in osteoarthritis. *Arthritis Rheum,* 62**,** 1955-66.

MIOT, S., GIANNI-BARRERA, R., PELTTARI, K., ACHARYA, C., MAINIL-VARLET, P., JUELKE, H., JAQUIERY, C., CANDRIAN, C., BARBERO, A. & MARTIN, I. 2010. In vitro and in vivo validation of human and goat chondrocyte labeling by green fluorescent protein lentivirus transduction. *Tissue Eng Part C Methods,* 16**,** 11-21.

MORGAN, E. E., CHANDLER, M. P., YOUNG, M. E., MCELFRESH, T. A., KUNG, T. A., RENNISON, J. H., TSERNG, K. Y., HOIT, B. D. & STANLEY, W. C. 2006. Dissociation between gene and protein expression of metabolic enzymes in a rodent model of heart failure. *Eur J Heart Fail,* 8**,** 687-93.

MOSIG, R. A., DOWLING, O., DIFEO, A., RAMIREZ, M. C., PARKER, I. C., ABE, E., DIOURI, J., AQEEL, A. A., WYLIE, J. D., OBLANDER, S. A., MADRI, J., BIANCO, P., APTE, S. S., ZAIDI, M., DOTY, S. B., MAJESKA, R. J., SCHAFFLER, M. B. & MARTIGNETTI, J. A. 2007. Loss of MMP-2 disrupts skeletal and craniofacial development and results in decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast growth. *Hum Mol Genet,* 16**,** 1113-23.

MOSLEY, B., DE IMUS, C., FRIEND, D., BOIANI, N., THOMA, B., PARK, L. S. & COSMAN, D. 1996. Dual oncostatin M (OSM) receptors. Cloning and characterization of an alternative signaling subunit conferring OSM-specific receptor activation. *J Biol Chem,* 271**,** 32635-43.

MUIR, H. 1995. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. *Bioessays,* 17**,** 1039-48.

MURPHY, G., HEMBRY, R. M. & REYNOLDS, J. J. 1986. Characterization of a specific antiserum to rabbit stromelysin and demonstration of the synthesis of collagenase and stromelysin by stimulated rabbit articular chondrocytes. *Coll Relat Res,* 6**,** 351-63.

MURPHY, G., HOUBRECHTS, A., COCKETT, M. I., WILLIAMSON, R. A., O'SHEA, M. & DOCHERTY, A. J. 1991. The N-terminal domain of tissue inhibitor of metalloproteinases retains metalloproteinase inhibitory activity. *Biochemistry,* 30**,** 8097-102.

MURPHY, G., KNAUPER, V., COWELL, S., HEMBRY, R., STANTON, H., BUTLER, G., FREIJE, J., PENDAS, A. M. & LOPEZ-OTIN, C. 1999. Evaluation of some newer matrix metalloproteinases. *Ann N Y Acad Sci,* 878**,** 25-39.

MURPHY, G. & NAGASE, H. 2008. Progress in matrix metalloproteinase research. *Mol Aspects Med,* 29**,** 290-308.

NAGASE, H., ENGHILD, J. J., SUZUKI, K. & SALVESEN, G. 1990. Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. *Biochemistry,* 29**,** 5783-9.

NAGASE, H., VISSE, R. & MURPHY, G. 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res,* 69**,** 562-73.

NAKAE, S., NAMBU, A., SUDO, K. & IWAKURA, Y. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol,* 171**,** 6173-7.

NEMOTO, O., YAMADA, H., MUKAIDA, M. & SHIMMEI, M. 1996. Stimulation of TIMP-1 production by oncostatin M in human articular cartilage. *Arthritis Rheum,* 39**,** 560-6.

NI, Y., SINNETT-SMITH, J., YOUNG, S. H. & ROZENGURT, E. 2013. PKD1 mediates negative feedback of PI3K/Akt activation in response to G protein-coupled receptors. *PLoS One,* 8**,** e73149.

NINOMIYA-TSUJI, J., KISHIMOTO, K., HIYAMA, A., INOUE, J., CAO, Z. & MATSUMOTO, K. 1999. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature,* 398**,** 252-6.

NRAS, N. R. A. S. 2010. The Economic Burden of Rheumatoid Arthritis.

NRAS, N. R. A. S. 2013. Breaking Down Barriers. 5.

O'DOHERTY, U., SWIGGARD, W. J. & MALIM, M. H. 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J Virol,* 74**,** 10074-80.

O'NEILL, L. A. & GREENE, C. 1998. Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J Leukoc Biol,* 63**,** 650-7.

OGATA, Y., ENGHILD, J. J. & NAGASE, H. 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem,* 267**,** 3581-4.

OHUCHI, E., IMAI, K., FUJII, Y., SATO, H., SEIKI, M. & OKADA, Y. 1997. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J Biol Chem,* 272**,** 2446-51.

OKAMOTO, A., IWAMOTO, Y. & MARU, Y. 2006. Oxidative stress-responsive transcription factor ATF3 potentially mediates diabetic angiopathy. *Mol Cell Biol,* 26**,** 1087-97.

ONISHI, Y., KAWAMOTO, T., KISHIMOTO, K., HARA, H., FUKASE, N., TODA, M., HARADA, R., KUROSAKA, M. & AKISUE, T. 2012. PKD1 negatively regulates cell invasion, migration and proliferation ability of human osteosarcoma. *Int J Oncol,* 40**,** 1839-48.

OSTER, H., ABRAHAM, D. & LEITGES, M. 2006. Expression of the protein kinase D (PKD) family during mouse embryogenesis. *Gene Expr Patterns,* 6**,** 400-8.

OTERO, M. & GOLDRING, M. B. 2007. Cells of the synovium in rheumatoid arthritis. Chondrocytes. *Arthritis Res Ther,* 9**,** 220.

PANAYI, G. S. 2006. Even though T-cell-directed trials have been of limited success, is there reason for optimism? *Nat Clin Pract Rheumatol,* 2**,** 58-9.

PARK, E. J., KWON, H. K., CHOI, Y. M., SHIN, H. J. & CHOI, S. 2012. Doxorubicin induces cytotoxicity through upregulation of pERK-dependent ATF3. *PLoS One,* 7**,** e44990.

PARK, J. E., KIM, Y. I. & YI, A. K. 2009. Protein kinase D1 is essential for MyD88-dependent TLR signaling pathway. *J Immunol,* 182**,** 6316-27.

PEARLE, A. D., WARREN, R. F. & RODEO, S. A. 2005. Basic science of articular cartilage and osteoarthritis. *Clin Sports Med,* 24**,** 1-12.

PELLETIER, J. P., MARTEL-PELLETIER, J. & ABRAMSON, S. B. 2001. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum,* 44**,** 1237-47.

PESSLER, F., DAI, L., CRON, R. Q. & SCHUMACHER, H. R. 2006. NFAT transcription factors--new players in the pathogenesis of inflammatory arthropathies? *Autoimmun Rev,* 5**,** 106-10.

PESSLER, F., DAI, L., DIAZ-TORNE, C., GOMEZ-VAQUERO, C., PAESSLER, M. E., ZHENG, D. H., EINHORN, E., RANGE, U., SCANZELLO, C. & SCHUMACHER, H. R. 2008. The synovitis of "non-inflammatory" orthopaedic arthropathies: a quantitative histological and immunohistochemical analysis. *Ann Rheum Dis,* 67**,** 1184-7.

PETTIPHER, E. R., HIGGS, G. A. & HENDERSON, B. 1986. Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. *Proc Natl Acad Sci U S A,* 83**,** 8749-53.

POSTLETHWAITE, A. E., LACHMAN, L. B., MAINARDI, C. L. & KANG, A. H. 1983. Interleukin 1 stimulation of collagenase production by cultured fibroblasts. *J Exp Med,* 157**,** 801-6.

PRICE, J. S., WATERS, J. G., DARRAH, C., PENNINGTON, C., EDWARDS, D. R., DONELL, S. T. & CLARK, I. M. 2002. The role of chondrocyte senescence in osteoarthritis. *Aging Cell,* 1**,** 57-65.

PULITI, M., MOMI, S., FALCINELLI, E., GRESELE, P., BISTONI, F. & TISSI, L. 2012. Contribution of matrix metalloproteinase 2 to joint destruction in group B Streptococcus-induced murine arthritis. *Arthritis Rheum,* 64**,** 1089-97.

PULVERER, B. J., KYRIAKIS, J. M., AVRUCH, J., NIKOLAKAKI, E. & WOODGETT, J. R. 1991. Phosphorylation of c-jun mediated by MAP kinases. *Nature,* 353**,** 670-4.

PUSHPARAJ, P. N. & MELENDEZ, A. J. 2006. Short interfering RNA (siRNA) as a novel therapeutic. *Clin Exp Pharmacol Physiol,* 33**,** 504-10.

RADTKE, S., WULLER, S., YANG, X. P., LIPPOK, B. E., MUTZE, B., MAIS, C., DE LEUR, H. S., BODE, J. G., GAESTEL, M., HEINRICH, P. C., BEHRMANN, I., SCHAPER, F. & HERMANNS, H. M. 2010. Cross-regulation of cytokine signalling: pro-inflammatory cytokines restrict IL-6 signalling through receptor internalisation and degradation. *J Cell Sci,* 123**,** 947-59.

RADWAN, M., GAVRIILIDIS, C., ROBINSON, J. H., DAVIDSON, R., CLARK, I. M., ROWAN, A. D. & YOUNG, D. A. 2013. Matrix metalloproteinase 13 expression in response to double-stranded RNA in human chondrocytes. *Arthritis Rheum,* 65**,** 1290-301.

RAKIEH, C. & CONAGHAN, P. G. 2013. Tofacitinib for treatment of rheumatoid arthritis. *Adv Ther,* 30**,** 713-26.

RAO, D. D., VORHIES, J. S., SENZER, N. & NEMUNAITIS, J. 2009. siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev,* 61**,** 746-59.

RAVANTI, L. & KAHARI, V. M. 2000. Matrix metalloproteinases in wound repair (review). *Int J Mol Med,* 6**,** 391-407.

RAYMOND, L., ECK, S., MOLLMARK, J., HAYS, E., TOMEK, I., KANTOR, S., ELLIOTT, S. & VINCENTI, M. 2006. Interleukin-1 beta induction of matrix metalloproteinase-1 transcription in chondrocytes requires ERK-dependent activation of CCAAT enhancer-binding protein-beta. *J Cell Physiol,* 207**,** 683-8.

REGISTRY, N. J. 2011. 8th Annual Report.

RENGEL, Y., OSPELT, C. & GAY, S. 2007. Proteinases in the joint: clinical relevance of proteinases in joint destruction. *Arthritis Res Ther,* 9**,** 221.

REUBEN, P. M., SUN, Y. & CHEUNG, H. S. 2004. Basic calcium phosphate crystals activate p44/42 MAPK signal transduction pathway via protein kinase Cmicro in human fibroblasts. *J Biol Chem,* 279**,** 35719-25.

REUNANEN, N., LI, S. P., AHONEN, M., FOSCHI, M., HAN, J. & KAHARI, V. M. 2002. Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. *J Biol Chem,* 277**,** 32360-8.

REY, O., PAPAZYAN, R., WALDRON, R. T., YOUNG, S. H., LIPPINCOTT-SCHWARTZ, J., JACAMO, R. & ROZENGURT, E. 2006. The nuclear import of protein kinase D3 requires its catalytic activity. *J Biol Chem,* 281**,** 5149-57.

REY, O., YOUNG, S. H., CANTRELL, D. & ROZENGURT, E. 2001. Rapid protein kinase D translocation in response to G protein-coupled receptor activation. Dependence on protein kinase C. *J Biol Chem,* 276**,** 32616-26.

REYNOLDS, A., LEAKE, D., BOESE, Q., SCARINGE, S., MARSHALL, W. S. & KHVOROVA, A. 2004. Rational siRNA design for RNA interference. *Nat Biotechnol,* 22**,** 326-30.

RICHARDS, C. D., CATTERALL, J., CAWSTON, T. E. & ROWAN, A. D. 2001. Synergistic regulation of chondrocytes by IL-1 and oncostatin M (OSM) involves interaction of JAK/STAT, MAP kinase and c-jun. *Arthritis and Rheumatism,* 44**,** S112-S112.

RIDLEY, S. H., SARSFIELD, S. J., LEE, J. C., BIGG, H. F., CAWSTON, T. E., TAYLOR, D. J., DEWITT, D. L. & SAKLATVALA, J. 1997. Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J Immunol,* 158**,** 3165-73.

ROCKEL, J. S., BERNIER, S. M. & LEASK, A. 2009. Egr-1 inhibits the expression of extracellular matrix genes in chondrocytes by TNFalpha-induced MEK/ERK signalling. *Arthritis Res Ther,* 11**,** R8.

RODRIGUEZ-VICIANA, P., SABATIER, C. & MCCORMICK, F. 2004. Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Mol Cell Biol,* 24**,** 4943-54.

ROFFEY, J., ROSSE, C., LINCH, M., HIBBERT, A., MCDONALD, N. Q. & PARKER, P. J. 2009. Protein kinase C intervention: the state of play. *Curr Opin Cell Biol,* 21**,** 268-79.

ROGERS, E. M., HSIUNG, F., RODRIGUES, A. B. & MOSES, K. 2005. Slingshot cofilin phosphatase localization is regulated by receptor tyrosine kinases and regulates cytoskeletal structure in the developing Drosophila eye. *Mech Dev,* 122**,** 1194-205.

ROWAN, A. D., HUI, W., CAWSTON, T. E. & RICHARDS, C. D. 2003. Adenoviral gene transfer of interleukin-1 in combination with oncostatin M induces significant joint damage in a murine model. *Am J Pathol,* 162**,** 1975-84.

ROWAN, A. D., KOSHY, P. J., SHINGLETON, W. D., DEGNAN, B. A., HEATH, J. K., VERNALLIS, A. B., SPAULL, J. R., LIFE, P. F., HUDSON, K. & CAWSTON, T. E. 2001. Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. *Arthritis Rheum,* 44**,** 1620-32.

ROWAN, A. D. & YOUNG, D. A. 2007. Collagenase gene regulation by pro-inflammatory cytokines in cartilage. *Front Biosci,* 12**,** 536-50.

ROZENGURT, E. 2011. Protein kinase D signaling: multiple biological functions in health and disease. *Physiology (Bethesda),* 26**,** 23-33.

RYBIN, V. O., GUO, J. & STEINBERG, S. F. 2009. Protein kinase D1 autophosphorylation via distinct mechanisms at Ser744/Ser748 and Ser916. *J Biol Chem,* 284**,** 2332-43.

SAKUMA, T., BARRY, M. A. & IKEDA, Y. 2012. Lentiviral vectors: basic to translational. *Biochem J,* 443**,** 603-18.

SANDY, J. D., ADAMS, M. E., BILLINGHAM, M. E., PLAAS, A. & MUIR, H. 1984. In vivo and in vitro stimulation of chondrocyte biosynthetic activity in early experimental osteoarthritis. *Arthritis Rheum,* 27**,** 388-97.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-101.

SCHEIDEREIT, C. 2006. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene,* 25**,** 6685-705.

SCHELLEKENS, G. A., DE JONG, B. A., VAN DEN HOOGEN, F. H., VAN DE PUTTE, L. B. & VAN VENROOIJ, W. J. 1998. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest,* 101**,** 273-81.

SCHWANHAUSSER, B., BUSSE, D., LI, N., DITTMAR, G., SCHUCHHARDT, J., WOLF, J., CHEN, W. & SELBACH, M. 2011. Global quantification of mammalian gene expression control. *Nature,* 473**,** 337-42.

SEKHON, B. S. 2010. Matrix metalloproteinase- an overview. *Research and Reports in Biology,* 2010:1**,** 1-20.

SEMIZAROV, D., FROST, L., SARTHY, A., KROEGER, P., HALBERT, D. N. & FESIK, S. W. 2003. Specificity of short interfering RNA determined through gene expression signatures. *Proc Natl Acad Sci U S A,* 100**,** 6347-52.

SHARLOW, E. R., GIRIDHAR, K. V., LAVALLE, C. R., CHEN, J., LEIMGRUBER, S., BARRETT, R., BRAVO-ALTAMIRANO, K., WIPF, P., LAZO, J. S. & WANG, Q. J. 2008. Potent and selective disruption of protein kinase D functionality by a benzoxoloazepinolone. *J Biol Chem,* 283**,** 33516-26.

SHLOPOV, B. V., STUART, J. M., GUMANOVSKAYA, M. L. & HASTY, K. A. 2001. Regulation of cartilage collagenase by doxycycline. *J Rheumatol,* 28**,** 835-42.

SHOUDA, T., YOSHIDA, T., HANADA, T., WAKIOKA, T., OISHI, M., MIYOSHI, K., KOMIYA, S., KOSAI, K., HANAKAWA, Y., HASHIMOTO, K., NAGATA, K. & YOSHIMURA, A. 2001.

Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. *J Clin Invest,* 108**,** 1781-8.

SHOULDERS, M. D. & RAINES, R. T. 2009. Collagen structure and stability. *Annu Rev Biochem,* 78**,** 929-58.

SILVA, J. M., LI, M. Z., CHANG, K., GE, W., GOLDING, M. C., RICKLES, R. J., SIOLAS, D., HU, G., PADDISON, P. J., SCHLABACH, M. R., SHETH, N., BRADSHAW, J., BURCHARD, J., KULKARNI, A., CAVET, G., SACHIDANANDAM, R., MCCOMBIE, W. R., CLEARY, M. A., ELLEDGE, S. J. & HANNON, G. J. 2005. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet,* 37**,** 1281-8.

SINNETT-SMITH, J., JACAMO, R., KUI, R., WANG, Y. M., YOUNG, S. H., REY, O., WALDRON, R. T. & ROZENGURT, E. 2009. Protein kinase D mediates mitogenic signaling by Gq-coupled receptors through protein kinase C-independent regulation of activation loop Ser744 and Ser748 phosphorylation. *J Biol Chem,* 284**,** 13434-45.

SINNETT-SMITH, J., ROZENGURT, N., KUI, R., HUANG, C. & ROZENGURT, E. 2011. Protein kinase D1 mediates stimulation of DNA synthesis and proliferation in intestinal epithelial IEC-18 cells and in mouse intestinal crypts. *J Biol Chem,* 286**,** 511-20.

SINNETT-SMITH, J., ZHUKOVA, E., HSIEH, N., JIANG, X. & ROZENGURT, E. 2004. Protein kinase D potentiates DNA synthesis induced by Gq-coupled receptors by increasing the duration of ERK signaling in swiss 3T3 cells. *J Biol Chem,* 279**,** 16883-93.

SINNETT-SMITH, J., ZHUKOVA, E., REY, O. & ROZENGURT, E. 2007a. Protein kinase D2 potentiates MEK/ERK/RSK signaling, c-Fos accumulation and DNA synthesis induced by bombesin in Swiss 3T3 cells. *J Cell Physiol,* 211**,** 781-90.

SINNETT-SMITH, J., ZHUKOVA, E., REY, O. & ROZENGURT, E. 2007b. Protein kinase D2 potentiates MEK/ERK/RSK signaling, c-Fos accumulation and DNA synthesis induced by Bombesin in Swiss 3T3 cells. *Journal of Cellular Physiology,* 211**,** 781-790.

SMEETS, T. J., BARG, E. C., KRAAN, M. C., SMITH, M. D., BREEDVELD, F. C. & TAK, P. P. 2003. Analysis of the cell infiltrate and expression of proinflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis. *Ann Rheum Dis,* 62**,** 635-8.

SMITH, M. D., TRIANTAFILLOU, S., PARKER, A., YOUSSEF, P. P. & COLEMAN, M. 1997. Synovial membrane inflammation and cytokine production in patients with early osteoarthritis. *J Rheumatol,* 24**,** 365-71.

SONG, J., LI, J., QIAO, J., JAIN, S., MARK EVERS, B. & CHUNG, D. H. 2009. PKD prevents H2O2-induced apoptosis via NF-kappaB and p38 MAPK in RIE-1 cells. *Biochem Biophys Res Commun,* 378**,** 610-4.

SONG, M. J., WANG, Y. Q. & WU, G. C. 2007. Lipopolysaccharide-induced protein kinase D activation mediated by interleukin-1beta and protein kinase C. *Brain Res,* 1145**,** 19-27.

STARR, R. & HILTON, D. J. 1999. Negative regulation of the JAK/STAT pathway. *Bioessays,* 21**,** 47-52.

STAUNTON, D., HUDSON, K. R. & HEATH, J. K. 1998. The interactions of the cytokine-binding homology region and immunoglobulin-like domains of gp130 with oncostatin M: implications for receptor complex formation. *Protein Eng,* 11**,** 1093-102.

STEARNS, M. E., KIM, G., GARCIA, F. & WANG, M. 2004. Interleukin-10 induced activating transcription factor 3 transcriptional suppression of matrix metalloproteinase-2 gene expression in human prostate CPTX-1532 Cells. *Mol Cancer Res,* 2**,** 403-16.

STEIN, G. S., LIAN, J. B., VAN WIJNEN, A. J., STEIN, J. L., MONTECINO, M., JAVED, A., ZAIDI, S. K., YOUNG, D. W., CHOI, J. Y. & POCKWINSE, S. M. 2004. Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene,* 23**,** 4315-29.

STEINBERG, S. F. 2008. Structural basis of protein kinase C isoform function. *Physiol Rev,* 88**,** 1341-78.

STORZ, P., DOPPLER, H., JOHANNES, F. J. & TOKER, A. 2003. Tyrosine phosphorylation of protein kinase D in the pleckstrin homology domain leads to activation. *J Biol Chem,* 278**,** 17969-76.

STORZ, P. & TOKER, A. 2003. Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. *EMBO J,* 22**,** 109-20.

STRANG, B. L., TAKEUCHI, Y., RELANDER, T., RICHTER, J., BAILEY, R., SANDERS, D. A., COLLINS, M. K. & IKEDA, Y. 2005. Human immunodeficiency virus type 1 vectors with alphavirus envelope glycoproteins produced from stable packaging cells. *J Virol,* 79**,** 1765-71.

STREMME, S., DUERR, S., BAU, B., SCHMID, E. & AIGNER, T. 2003. MMP-8 is only a minor gene product of human adult articular chondrocytes of the knee. *Clin Exp Rheumatol,* 21**,** 205-9.

SUMARA, G., FORMENTINI, I., COLLINS, S., SUMARA, I., WINDAK, R., BODENMILLER, B., RAMRACHEYA, R., CAILLE, D., JIANG, H., PLATT, K. A., MEDA, P., AEBERSOLD, R., RORSMAN, P. & RICCI, R. 2009. Regulation of PKD by the MAPK p38delta in insulin secretion and glucose homeostasis. *Cell,* 136**,** 235-48.

SWEENEY, S. E. & FIRESTEIN, G. S. 2004. Rheumatoid arthritis: regulation of synovial inflammation. *Int J Biochem Cell Biol,* 36**,** 372-8.

TAKAESU, G., KISHIDA, S., HIYAMA, A., YAMAGUCHI, K., SHIBUYA, H., IRIE, K., NINOMIYA-TSUJI, J. & MATSUMOTO, K. 2000. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell,* 5**,** 649-58.

TARDIF, G., PELLETIER, J. P., DUPUIS, M., GENG, C., CLOUTIER, J. M. & MARTEL-PELLETIER, J. 1999. Collagenase 3 production by human osteoarthritic chondrocytes in response to growth factors and cytokines is a function of the physiologic state of the cells. *Arthritis Rheum,* 42**,** 1147-58.

TEMENOFF, J. S. & MIKOS, A. G. 2000. Review: tissue engineering for regeneration of articular cartilage. *Biomaterials,* 21**,** 431-40.

TOWLE, C. A., HUNG, H. H., BONASSAR, L. J., TREADWELL, B. V. & MANGHAM, D. C. 1997. Detection of interleukin-1 in the cartilage of patients with osteoarthritis: a possible autocrine/paracrine role in pathogenesis. *Osteoarthritis Cartilage,* 5**,** 293-300.

TRABANDT, A., AICHER, W. K., GAY, R. E., SUKHATME, V. P., FASSBENDER, H. G. & GAY, S. 1992. Spontaneous expression of immediately-early response genes c-fos and egr-1 in collagenase-producing rheumatoid synovial fibroblasts. *Rheumatol Int,* 12**,** 53-9.

TSUJI, M., HIRAKAWA, K., KATO, A. & FUJII, K. 2000. The possible role of c-fos expression in rheumatoid cartilage destruction. *J Rheumatol,* 27**,** 1606-21.

TYLER, J. A. & BENTON, H. P. 1988. Synthesis of type II collagen is decreased in cartilage cultured with interleukin 1 while the rate of intracellular degradation remains unchanged. *Coll Relat Res,* 8**,** 393-405.

VALVERDE, A. M., SINNETT-SMITH, J., VAN LINT, J. & ROZENGURT, E. 1994. Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc Natl Acad Sci U S A,* 91**,** 8572-6.

VAN DEN BERG, W. B. 1999. The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. *Z Rheumatol,* 58**,** 136-41.

VAN DEN BERG, W. B. & VAN RIEL, P. L. 2005. Uncoupling of inflammation and destruction in rheumatoid arthritis: myth or reality? *Arthritis Rheum,* 52**,** 995-9.

VAN DER KRAAN, P. M. & VAN DEN BERG, W. B. 2012. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartilage,* 20**,** 223-32.

VAN LINT, J., RYKX, A., MAEDA, Y., VANTUS, T., STURANY, S., MALHOTRA, V., VANDENHEEDE, J. R. & SEUFFERLEIN, T. 2002. Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol,* 12**,** 193-200.

VAN LINT, J. V., SINNETT-SMITH, J. & ROZENGURT, E. 1995. Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. *J Biol Chem,* 270**,** 1455-61.

VANTUS, T., VERTOMMEN, D., SAELENS, X., RYKX, A., DE KIMPE, L., VANCAUWENBERGH, S., MIKHALAP, S., WAELKENS, E., KERI, G., SEUFFERLEIN, T., VANDENABEELE, P., RIDER, M. H., VANDENHEEDE, J. R. & VAN LINT, J. 2004. Doxorubicin-induced activation of protein kinase D1 through caspase-mediated proteolytic cleavage: identification of two cleavage sites by microsequencing. *Cell Signal,* 16**,** 703-9.

VERTOMMEN, D., RIDER, M., NI, Y., WAELKENS, E., MERLEVEDE, W., VANDENHEEDE, J. R. & VAN LINT, J. 2000. Regulation of protein kinase D by multisite phosphorylation. Identification of phosphorylation sites by mass spectrometry and characterization by site-directed mutagenesis. *J Biol Chem,* 275**,** 19567-76.

VERZIJL, N., DEGROOT, J., THORPE, S. R., BANK, R. A., SHAW, J. N., LYONS, T. J., BIJLSMA, J. W., LAFEBER, F. P., BAYNES, J. W. & TEKOPPELE, J. M. 2000. Effect of collagen turnover on the accumulation of advanced glycation end products. *J Biol Chem,* 275**,** 39027-31.

VINCENTI, M. P. & BRINCKERHOFF, C. E. 2002. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. *Arthritis Res,* 4**,** 157-64.

VINCENTI, M. P., COON, C. I. & BRINCKERHOFF, C. E. 1998. Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts. *Arthritis Rheum,* 41**,** 1987-94.

VISSE, R. & NAGASE, H. 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res,* 92**,** 827-39.

WALDRON, R. T., IGLESIAS, T. & ROZENGURT, E. 1999. The pleckstrin homology domain of protein kinase D interacts preferentially with the eta isoform of protein kinase C. *J Biol Chem,* 274**,** 9224-30.

WALDRON, R. T., REY, O., ZHUKOVA, E. & ROZENGURT, E. 2004. Oxidative stress induces protein kinase C-mediated activation loop phosphorylation and nuclear redistribution of protein kinase D. *J Biol Chem,* 279**,** 27482-93.

WALDRON, R. T., WHITELEGGE, J. P., FAULL, K. F. & ROZENGURT, E. 2007. Identification of a novel phosphorylation site in c-jun directly targeted in vitro by protein kinase D. *Biochem Biophys Res Commun,* 356**,** 361-7.

WANG, Q. J. 2006. PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol Sci,* 27**,** 317-23.

WANG, Y. & FULLER, G. M. 1994. Phosphorylation and internalization of gp130 occur after IL-6 activation of Jak2 kinase in hepatocytes. *Mol Biol Cell,* 5**,** 819-28.

WANG, Y., SCHATTENBERG, J. M., RIGOLI, R. M., STORZ, P. & CZAJA, M. J. 2004. Hepatocyte resistance to oxidative stress is dependent on protein kinase C-mediated down-regulation of c-Jun/AP-1. *J Biol Chem,* 279**,** 31089-97.

WANG, Y., WALDRON, R. T., DHAKA, A., PATEL, A., RILEY, M. M., ROZENGURT, E. & COLICELLI, J. 2002. The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. *Mol Cell Biol,* 22**,** 916-26.

WESCHE, H., KORHERR, C., KRACHT, M., FALK, W., RESCH, K. & MARTIN, M. U. 1997. The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J Biol Chem,* 272**,** 7727-31.

WESTERMARCK, J., LI, S. P., KALLUNKI, T., HAN, J. & KAHARI, V. M. 2001. p38 mitogen-activated protein kinase-dependent activation of protein phosphatases 1 and 2A inhibits MEK1 and MEK2 activity and collagenase 1 (MMP-1) gene expression. *Mol Cell Biol,* 21**,** 2373-83.

WHITMARSH, A. J., YANG, S. H., SU, M. S., SHARROCKS, A. D. & DAVIS, R. J. 1997. Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol Cell Biol,* 17**,** 2360-71.

WICKREMASINGHE, M. I., THOMAS, L. H. & FRIEDLAND, J. S. 1999. Pulmonary epithelial cells are a source of IL-8 in the response to Mycobacterium tuberculosis: essential role of IL-1 from infected monocytes in a NF-kappa B-dependent network. *J Immunol,* 163**,** 3936-47.

WILL, H., ATKINSON, S. J., BUTLER, G. S., SMITH, B. & MURPHY, G. 1996. The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. *J Biol Chem,* 271**,** 17119-23.

WILLE, C., KOHLER, C., ARMACKI, M., JAMALI, A., GOSSELE, U., PFIZENMAIER, K., SEUFFERLEIN, T. & EISELER, T. 2013. Protein Kinase D2 induces invasion of pancreatic cancer cells by regulating matrix metallo-proteinases. *Mol Biol Cell*.

WORONICZ, J. D., GAO, X., CAO, Z., ROTHE, M. & GOEDDEL, D. V. 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science,* 278**,** 866-9.

YAMAMOTO, H., OUE, N., SATO, A., HASEGAWA, Y., MATSUBARA, A., YASUI, W. & KIKUCHI, A. 2010. Wnt5a signaling is involved in the aggressiveness of prostate cancer and expression of metalloproteinase. *Oncogene,* 29**,** 2036-46.

YAMASHITA, K., GON, Y., SHIMOKAWA, T., NUNOMURA, S., ENDO, D., MIYATA, N., HASHIMOTO, S., VAN LINT, J. & RA, C. 2010. High affinity receptor for IgE stimulation activates protein kinase D augmenting activator protein-1 activity for cytokine producing in mast cells. *Int Immunopharmacol,* 10**,** 277-83.

YAN, C. & BOYD, D. D. 2007. Regulation of matrix metalloproteinase gene expression. *J Cell Physiol,* 211**,** 19-26.

YAN, C., WANG, H. & BOYD, D. D. 2002. ATF3 represses 72-kDa type IV collagenase (MMP-2) expression by antagonizing p53-dependent trans-activation of the collagenase promoter. *J Biol Chem,* 277**,** 10804-12.

YASUDA, T. 2006. Cartilage destruction by matrix degradation products. *Mod Rheumatol,* 16**,** 197-205.

YOO, S. A., PARK, B. H., YOON, H. J., LEE, J. Y., SONG, J. H., KIM, H. A., CHO, C. S. & KIM, W. U. 2007. Calcineurin modulates the catabolic and anabolic activity of chondrocytes and participates in the progression of experimental osteoarthritis. *Arthritis Rheum,* 56**,** 2299-311.

YOON, Y. M., KIM, S. J., OH, C. D., JU, J. W., SONG, W. K., YOO, Y. J., HUH, T. L. & CHUN, J. S. 2002. Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem,* 277**,** 8412-20.

YOSHIHARA, Y., NAKAMURA, H., OBATA, K., YAMADA, H., HAYAKAWA, T., FUJIKAWA, K. & OKADA, Y. 2000. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann Rheum Dis,* 59**,** 455-61.

YUAN, J., BAE, D., CANTRELL, D., NEL, A. E. & ROZENGURT, E. 2002. Protein kinase D is a downstream target of protein kinase Ctheta. *Biochem Biophys Res Commun,* 291**,** 444-52.

ZHANG, W., ZHENG, S., STORZ, P. & MIN, W. 2005. Protein kinase D specifically mediates apoptosis signal-regulating kinase 1-JNK signaling induced by H2O2 but not tumor necrosis factor. *J Biol Chem,* 280**,** 19036-44.

ZIEGLER, S., EISELER, T., SCHOLZ, R. P., BECK, A., LINK, G. & HAUSSER, A. 2011. A novel protein kinase D phosphorylation site in the tumor suppressor Rab interactor 1 is critical for coordination of cell migration. *Mol Biol Cell,* 22**,** 570-80.

ZOU, Z., ZENG, F., XU, W., WANG, C., KE, Z., WANG, Q. J. & DENG, F. 2012. PKD2 and PKD3 promote prostate cancer cell invasion by modulating NF-kappaB- and HDAC1-mediated expression and activation of uPA. *J Cell Sci,* 125**,** 4800-11.

ZUGAZA, J. L., SINNETT-SMITH, J., VAN LINT, J. & ROZENGURT, E. 1996. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J,* 15**,** 6220-30.