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Synergistic transcriptional regulation of collagenase gene expression in chondrocytes

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Abstract

The proteolytic degradation of articular cartilage in load-bearing joints is a key pathological step in the progression of arthritis, a process mediated by enzymes called collagenases (specifically MMP-1 and MMP-13). My research has focused on the transcriptional regulation of these enzymes in cartilage cells (chondrocytes) in response to a pro-catabolic stimulus which mimics the complex milieu of elevated cytokines found within the arthritic joint.

Activating Protein (AP)-1 transcription factors, specifically the c-Fos/c-Jun heterodimer, have previously been shown to be crucial in collagenase gene regulation. c-Fos/c-Jun gene expression, protein production and collagenase promoter enrichment studies identified a temporal deficit between transient c-Fos/c-Jun peak following 1 hour stimulation and the initiation of collagenase gene transcription following 6 hours stimulation. Protein synthesis inhibitor studies indicated that although c-Fos/c-Jun are indeed important, they are not the sole regulators of collagenase gene expression.

DNA microarray studies highlighted a number of genes that contributed to transcriptional regulation early within this temporal deficit. Collagenase gene expression was assessed following the siRNA-mediated silencing of these factors. This confirmed that new factors, not previously associated with collagenase gene regulation, were demonstrated to have a significant role in initiating their transcription. This included factors such as activating transcription factor (ATF)3 and early growth response (EGR)2 which demonstrated differential regulation of the collagenase genes, with their silencing affecting *MMP13* expression alone.

Having identified a number of contributing factors, I then assessed their temporal gene expression and protein production. Comparisons to c-Fos/c-Jun induction confirmed that a number of these factors were transient, similar to AP-1, yet they peaked following longer durations of stimulation. Subsequent siRNA gene silencing of c-Fos and c-Jun led to decreased expression of some of these factors. This demonstrated that these factors may be regulating collagenase expression indirectly by controlling the expression of other transcription factors that, themselves contribute to the regulation of collagenase gene.

The present study improves our understanding of how collagenases are regulated in chondrocytes in response to pro-catabolic stimuli. With an improved knowledge of regulation it may be possible to specifically abrogate aberrant collagenases expression in disease. Moreover, by exploiting the differential regulation of collagenases exhibited by some of these factors, there is the potential to mitigate the side effects associated with broad-spectrum collagenase inhibition, thereby removing the barrier to successful treatment.

Acknowledgements

Declaration

This thesis is based on research performed in the Musculoskeletal Research Group (Rheumatology), Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK. Except for commonly held concepts, and where specific reference is made to other work, the content of the thesis is original. No part of this thesis has been submitted for the award of any other degree.

Table of Contents

List of Figures

List of Tables

List of abbreviations

3C	Chromatin conformation capture
Ac	Acetylated
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AK	Adenylate kinase
AMP	Adenosine monophosphate
AP-1	Activating Protein-1
APS	Ammonium peroxodisulphate
ATF	Activating transcription factor
AXUD1	Axin 1 upregulated
BMP2	Bone morphogenetic protein 2
bp	Base Pairs
BSA	Bovine serum albumin
BTG2	B-cell translocation gene 2
bZIP	Basic leucine zipper
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin-dependent Kinase
CEB	Cytoplasmic extraction buffer
CIA	Collagen-induced arthritis
CIF	Ciliary neurotrophic factor
CpG	Cytosine phosphodiester Guanine
CRE	cAMP responsive element
CREB	cAMP response element binding
CT-1	Cardiotrophin-1
CTD	Carboxy terminal domain
DAG	Diacylglycerol
DAPA	DNA affinity pulldown assay
dI-dC	Poly deoxyinosinic-deoxycytidylic
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyl transferase
dNTP	Deoxyribonucleotide
DPBS	Dulbecco's Phosphate Buffered Saline
DSIF	DRB-sensitivity-inducing factor
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
Fra-1	Fos-related antigen 1
GAG	Glycosaminoglycan
Gp 130	Glycoprotein 130

GTF	General transcription factors
HAC	Human articular chondrocytes
HAT	Histone acetyl-transferase
HBSS	Hank's-Buffered Saline Solution
HCl	Hydrochloric acid
HDAC	Histone de-acetylase
HRP	Horseradish peroxidase
IEG	Immediate early gene
IER	Immediate early response
IEX-1	Immediate early response gene X-1
IFNγ	Interferon γ
IL	Interleukin
IL-1AcP	IL-1 receptor accessory protein
IL-1ra	IL-1 receptor antagonist
IP3	Inositol trisphosphate
IRAK	IL-1 receptor-associated kinase
IκB	Inhibitor of NF-κB
Jak	Januse kinase
JNK	Jun N-terminal kinase
JUN	Jun proto-oncogene
K	Lysine
kbp	Kilobase pair
LIF	Leukaemia inhibitory factor
M/C	Micro-centrifuge
MAPK	Mitogen-activated protein kinase
Me	Methylated
MEB	Membrane extraction buffer
MMLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MT	Membrane-type
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrocarbonate
NEB	Nuclear extraction buffer
NELF	Negative elongation factor
NFATc1	Nuclear factor of activated T-cells cytoplasmic 1
NF-κB	Nuclear factor κB
NIK	NF-κB-inducing kinase
NO	Nitric oxide
OA	Osteoarthritis
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrphoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
$Pd(N)_6$	Random hexamers
PDK1	3-phosphoinositide dependent protein kinase-1
PEB	pellet extraction buffer

PI	Protease inhibitors
PI3K	Phosphatidylinositol 3-kinases
PIC	Pre-initiation Complex
PIP	Phosphatidylinositol-3-Phosphate
PIP2	Phosphatidylinositol-3,4-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
Pol II	RNA polymerase II
P-TEFb	Positive transcription elongation factor b
PTGS2	Prostaglandin-endoperoxide synthase 2
PVDF	Polyvinylidene fluoride
RA	Rheumatoid Arthritis
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
Runx-2	Runt-related transcription factor-2
SDS	Sodium dodecyl sulfate
Ser	Serine
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TBP	TATA binding protien
TBS-T	Tris-buffered saline-Tween
TEMED	N,N,N'N'-tetramethylenediamine
TF	Transcription factors
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-α
TORC2	Transducer of regulated CREB protein 2
TRAF	TNF receptor associated factor
TRE	TPA responsive element
TSA	Trichostatin A
TSS	Transcription start site
Zn	Zinc
β-ME	β-mercaptoethanol

1 Chapter 1: Introduction

The cleavage of type II collagen in articular cartilage represents a key pathological process that, once initiated, leads to a cascade of catabolic events culminating in the degradation of a tissue which is crucial for normal load bearing and articulation of the synovial joints within the body. This loss of function increases disability and is the root cause of pain experienced in patients suffering from arthritis. This process is mediated by the aberrant expression of matrix metalloproteinase (MMP) enzymes called collagenases by the sole cartilage cell type, the chondrocyte. Previous broad-spectrum metalloproteinase inhibition demonstrated no benefit to arthritis patients due to a lack of specificity [1] and so targeting of this pathological process remains ineffective. By studying collagenase gene regulation I aim to elucidate specific transcriptional events that could be targeted in order to reduce the MMP-dependent cartilage destruction.

1.1 Articular Cartilage

1.1.1 Structure and Function

The synovial joint is a highly articular joint in mammalian bodies. This articulation is, in-part provided by the presence of a lubricant called synovial fluid which is compartmentalised between the two ends of the bones within the joint. Articular cartilage also plays a key role in the functionality of the synovial joint. This tissue surrounds the epiphysis of the bones in the joint allowing load-bearing through a range of functional motions minimizing the stress on subchondral bone. This is achieved by creating a compressive, friction-less surface upon which the bones can articulate [2].

Unlike the majority of other tissues, articular cartilage is aneuronal, alymphatic and avascular. It is made up of 4 zones that layer upon the subchondral bone. The superficial zone is the articular surface exposed to shear stress generated by movement and makes up 10-20% of cartilage thickness. Collagen fibres are aligned parallel to the joint surface providing protection to deeper zones which are crucial for resisting the forces applied to the joint. The next layer and largest zone of articular cartilage is the middle zone which makes up 40-60% of the volume. Collagen fibres are

thicker than in the superficial zone and so provide greater resistance to compressive force. The deep zone which sits between the middle zone and calcified cartilage anchors cartilage to bone and provides the most resistance to compressive force. Fibres are thickest within this region as they are bolstered by high proteoglycan content. They are also aligned perpendicularly to the joint surface and hence to the direction of force. These properties make the deep zone the most important for physiological cartilage function [3].

1.1.2 Chondrocytes

Despite the resulting low oxygen environment, the tissue is populated (be it only sparsely) by a sole cell type, the chondrocyte. As a consequence of the cartilage environment the majority of chondrocytic energy production is achieved through anaerobic glycolysis [4]. Chondrocytes have different morphology depending on the zone of cartilage in which they reside: Chondrocytes within the superficial zone are small, flattened and relatively densely populated, whilst in the middle zone they are more disperse. In the deep zone they align into columns parallel to the collagen fibres surrounding them. Chondrocytes are crucial in the development and maintenance of cartilage and bone. In the context of bone, cells differentiate from chondroprogenitor cells into hypertrophic, then mature chondrocytes capable of mineralizing the cartilaginous growth plate into bone bringing about endochondral ossification. Once bone has been laid down these chondrocytes are then capable of undergoing apoptosis. Conversely, articular chondrocytes (those cells that exist within articular cartilage) do not fully mature and remain arrested before terminal hypertrophic differentiation. These cells retain the ability produce all the components of the extracellular matrix (ECM) that surrounds the chondrocyte and constitutes the articular cartilage. They are also capable of remodelling cartilage through the production of enzymes that can degrade the components of the ECM. What was once regarded as inert tissue is actually constantly remodelling in response to external stimuli (e.g. compression and cytokines) to improve tissue functionality in a chondrocyte-dependent manner [5].

1.1.3 Extracellular Matrix

Cartilage is a complex structure that is made up of ECM generated by the chondrocytes which populate the cartilage. The functional aim of this meshwork of proteins

produced by chondrocytes is to trap water in the tissue thereby creating compressive resistance to force. This is achieved through the production of only two main protein types: collagen and aggrecan. These proteins make up the vast majority of the tissue with only 0.4-2% of the mass of cartilage accounted for by chondrocytes [6].

1.1.3.1 Collagen

The main structural component of articular cartilage is type II collagen making up 90-98% of total collagens within the tissue. It is composed of 3 identical polypeptide chains (α1) which intertwine forming a triple helical structure, a property imparted by the presence of a glycine at every third amino acid in the protein sequence. These trimers then congregate into collagen fibrils formed through bonding of the telopeptide regions of one collagen molecule to the trimeric region of another. This imparts a great deal of tensile strength to the fibrils and with time, as molecular cross-linkage continues, type II collagen becomes very resistant to proteolytic degradation. Type II collagen is not the sole collagen type within articular cartilage. Type XI and IX cartilage specific collagens, as well as type X and IV, are also present however, they comprise a negligible proportion of the cartilage at less than 5% of the total volume of collagen [6].

The collagen within cartilage forms a complex structure which is essential to function as it provides a framework of proteins that enmesh other crucial cartilage components within. The turnover of collagen is therefore very slow indeed with collagen laid down during development will endure for an entire lifetime [7, 8]. As a result of this the loss of type II collagen has been suggested to be a rate-limiting step in cartilage breakdown in diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) [9-12].

1.1.3.2 Aggrecan

The other major structural component of cartilage produced by the chondrocyte is the proteoglycan, aggrecan. These molecules form proteoglycan aggregates consisting of up to 100 aggrecan molecules attached through link proteins to a single hyaluronan filament. These in turn are encased within a type II collagen framework. Aggrecan itself is made up of 3 core globular domains (G1-3) which are intersected by a glycosaminoglycan (GAG)-rich region comprised of a keratan sulphate region directly adjacent the G2 domain followed by 2 chondroitin sulphate regions (Figure 1). These

regions impart the functional qualities of aggrecan. These sulphated side chains are highly negatively charged which creates a hydrophilic environment resulting in hydration of aggrecan. This allows it to resist compression from loading and prevents chondrocytic stress that can lead to aberrant protease production and cartilage destruction [6, 13, 14].

These aggregates exhibit a relatively high amount of plasticity in comparison to collagen. As the cartilage ages cleavage events re-model the aggrecan molecules bound to the hyaluronan leading to altered proteoglycan composition. However, with time sulphated regions are lost and the tissue is less able to resist compression, a suggested pathological process occurring in OA and RA.
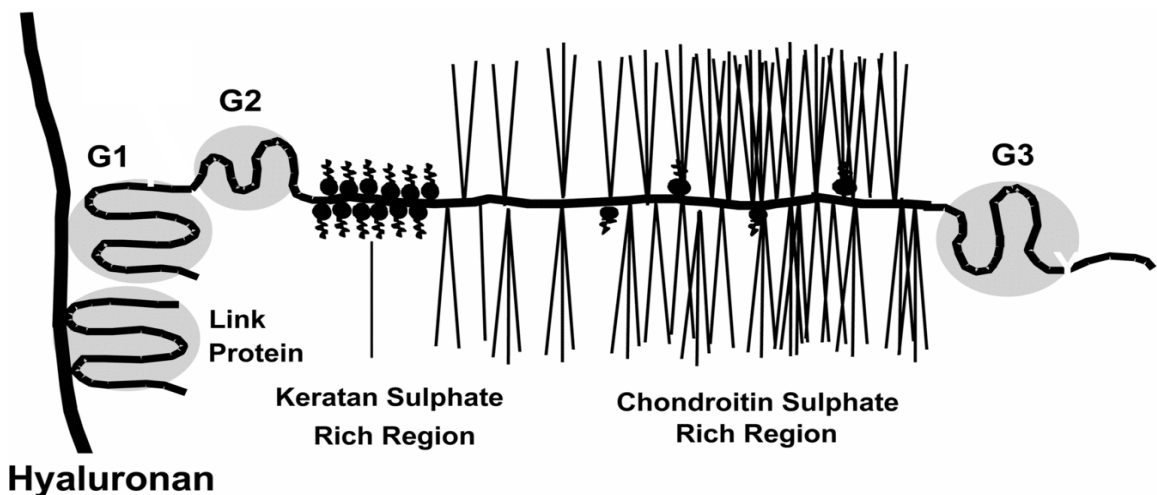
Figure 1: Protein structure of aggrecan showing three globular domains encompassing glycosaminoglycan region, attached to hyaluronan via link protein. Taken from Porter et al [15]

1.2 Arthritis

As previously mentioned, the destruction of key components of cartilage, namely aggrecan and type II collagen lead to the progression of cartilage destruction that characterise the disease endpoints of both RA and OA. This loss of cartilage leads to pain and narrowing in the range of movement, increasing disability and decreasing the patients' quality of life. The process of articular cartilage degradation is due to an altered balance of anabolic and catabolic factors produced and orchestrated by a number of cells within the joint including the chondrocyte. Under normal physiological conditions chondrocytes subtly re-model their ECM in response to pressure exerted on the cartilage due to loading of the joint. Cartilage degradation occurs when this balance is disturbed and the catabolic factors produced by the chondrocyte surpass

anabolic factor production. The reasons behind this imbalance are dependent on specific arthritic disease and its pathogenesis.

1.2.1 Rheumatoid arthritis

RA is a chronic autoimmune inflammatory disorder which affects the synovial joints (including hip and knee joints) of the body. It affects between 0.5-1% of population in developed countries, with incidence increasing with age and is three times more frequent in women than men [16]. In an ever aging population RA is a significant economic burden with the total average medical cost amounting to approximately £3600 per person and the loss of 27 to 30 working days/year/patient [17].
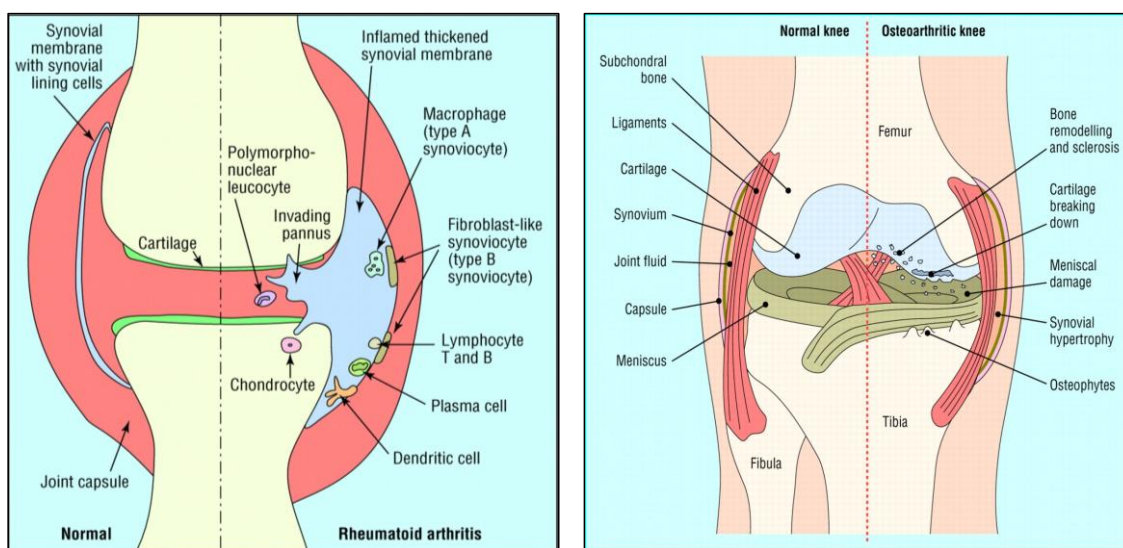
Figure 2: Pathological changes in a knee joint due to rheumatoid arthritis and osteoarthritis. Taken from Buckley [18] and Hunter et al. [19]

In the initial stages of pathology, CD4+ T helper cells orchestrate an adaptive immune response upon recognition of auto-antigens presented by B cells. In the context of RA, this consists of activation and subsequent infiltration of T and B cells, macrophages, dendritic cells and other inflammatory infiltrates (Figure 2) into the synovial lining of the joint cavity. Here, they exert their pathological effects by releasing pro-inflammatory cytokines into joint tissues [20]. Tumour necrosis factor (TNF)-α has been demonstrated to be one of the key cytokines that drive the disease. This is demonstrated by the striking efficacy of anti-TNF treatment in some RA patients. However, there are still sub-populations of disease-sufferers that do not respond to anti-TNF highlighting the important role of other pro-inflammatory cytokines

5

(reviewed in Feldmann, 2002 [21]). Interleukin (IL)-1, -6 and -17 have also been shown to have important roles in the pathogenesis of RA [20].

In response to this complex cytokine milieu within the joint, many cell types are phenotypically altered. Synoviocytes (macrophage-like and fibroblast-like), under the influence of these pro-inflammatory cytokines, perpetuate inflammation and bring about synovial vascularisation and angiogenesis within the synovial lining. This forms pannus, a synovial infiltrate that begins to cover the surface of the articular cartilage [16]. Chondrocytes within the cartilage shift the balance between anabolic and catabolic factor production, as mentioned previously in response to pro-inflammatory cytokines. This leads to degradation of cartilage and the subchondral bone.

1.2.2 Osteoarthritis

Osteoarthritis (OA) is the most prevalent arthritic disease, affecting the majority of over 65 year olds. The symptoms include joint pain, stiffness, swelling and loss of mobility. Hence, OA is the leading cause of mobility disability in this age group [19]. Although associated with age, OA can lead to about 6% of 30 year olds exhibiting disease symptoms [22]. The process by which synovial joint function fails is due to mechanical factors although other factors such as increasing age and weight, an individuals' genetic profile and gender can affect susceptibility to OA. Local mechanical disturbances like abnormal joint loading, joint misalignment and muscle weakness lead to loss of control of homeostasis within cartilage [7, 19, 23]. Though it has been demonstrated that OA has an inflammatory component [24], it is not the driving mechanism behind cartilage degradation as it is in RA. Rather it is secondary to it, with abnormal mechanical force causing chondrocytes (as well as synoviocytes) to not only release catabolic factors but cease producing anti-anabolic factors. Pro-inflammatory cytokines are also released which perpetuate cartilage degradation and exacerbate the aforementioned inflammation [7, 9, 23]. This eventually leads to advanced cartilage loss and damage to the tissues within the joint (Figure 2) leading to attenuated mobility.

1.3 Enzymes and cartilage degradation

With cartilage loss a common feature in the pathologies of both RA and OA, the process by which this occurs is crucial to our understanding of these diseases. As mentioned previously, the imbalance in favour of increased production of catabolic factors leads to increased cartilage degradation. These catabolic factors include various enzymes within the proteinase family that degrade articular collagen. Their actions lead to irreversible cartilage damage with chondrocytes unable to lay down enough type II collagen to recapitulate the normal cartilage architecture laid down during development. This is due to a very low collagen turnover rate that has an estimated half-life over 100 years [8]. In contrast, aggrecan can be replenished by chondrocytes following proteinase-mediated cartilage resorption with a more modest half-life of 3-24 years [25, 26].
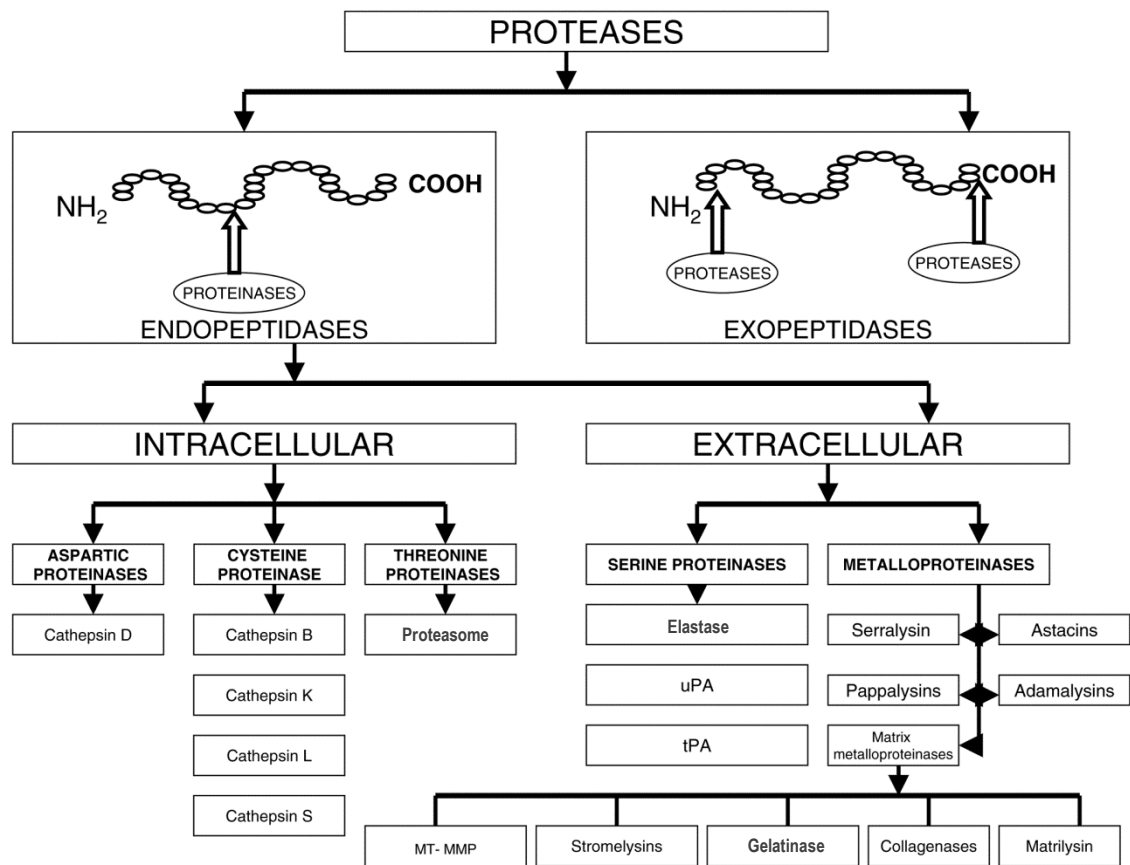
Figure 3: Schematic of the sub-divisions of protease family. Taken from Rengel et al. [27]

The physiological role of proteinases is to cleave peptide bonds that hold amino acids together in the polypeptide chains that make up proteins. This can then allow for the catabolism of proteins for degradation or formation of new, active proteins. There are

5 subclasses of proteinase based on their catalytic activity. These are: aspartate, cysteine, threonine, serine and metallo catalytic types. In the context of collagen degradation serine and metalloproteinases are the only extracellular members and so are able to directly affect collagen breakdown. For a detailed overview of proteinases and its sub-classifications see Figure 3.

Serine proteinases have indirect effects on collagen breakdown. For example Milner et al. [28] demonstrated that inhibition of various serine proteinases led to significant reduction of collagen release from human articular cartilage explants following pro-catabolic stimulus. However, rather than actively cleaving collagen fragments in the cartilage, serine proteinases were shown to cleave pro-forms of members of the MMP (a sub-division of metalloproteinases) into active enzymes capable of a direct proteolytic effect on collagen within the cartilage [29, 30].

1.4   Matrix Metalloproteinases
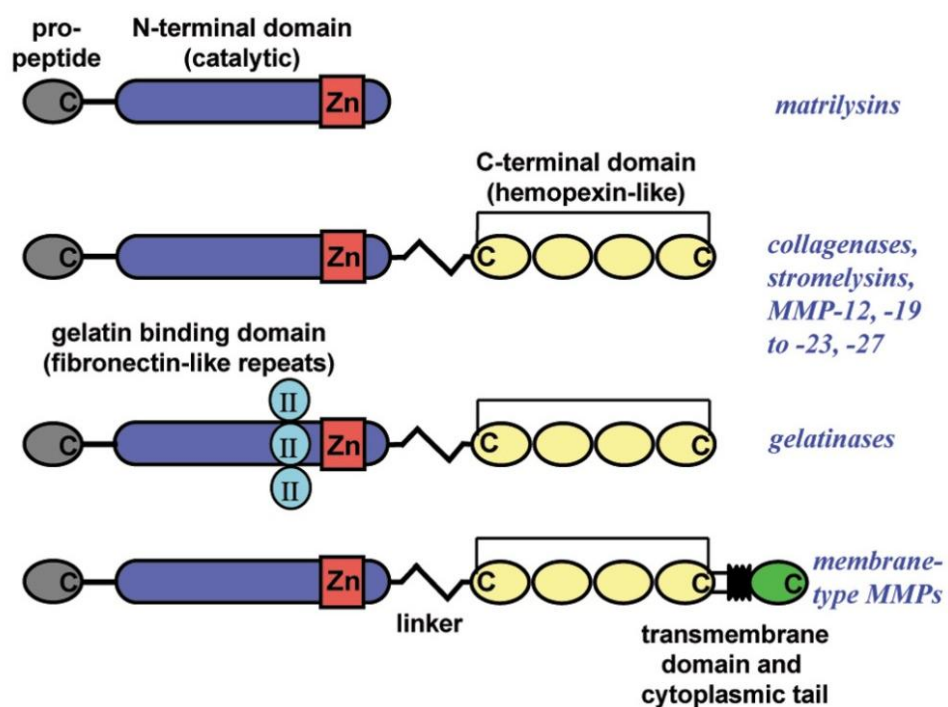
Figure 4: Schematic of the domains that make up various MMPs. Adapted from Murphy et al [31]

The human MMP family of endopeptidases consists of 24 zinc-dependent enzymes. These are categorised into groups by their structure (Figure 4). All contain a pro-peptide domain which retains the enzyme in a latent form. This pro-peptide is

positioned close to the active site and prevents water binding in the cleft (thus preventing peptide hydrolysis) through interaction of a conserved cysteine residue with the zinc ion in the catalytic domain [32]. This domain is also common to all MMPs. As well as the catalytic zinc ion which is necessary for substrate hydrolysis, a secondary 'structural zinc' and additional calcium ions confer function to the domain [31]. The C-terminal hemopoxin domain is another domain shared by the vast majority of MMPs (excluding matrilysins). Small structural variations within this domain have been suggested to give MMPs their respective specificity to certain substrates and proteolytic function [33]. Other more specific structural features of MMPs include a furin recognition motif (MMP-11, -14 to -17, -21 to -25 and -28) allowing activation by furin-like proteinases; fibronectin-like (gelatinases) and vibronectin-like (MMP-21) domains that provide greater substrate specificity as well as transmembrane domains that anchor membrane-type (MT)- MMPs at the cell surface [34].

1.5 Collagenases

Collagenase	Alternate names	Substrate Specificity (collagen type)	Expressed by
MMP-1	Interstitial collagenase; Fibroblast collagenase; Collagenase-1	III > I > II [35]	Macrophages, fibroblasts, chondrocytes, epithial cells, endothelial cells [36, 37]
MMP-8	Neutophil collagenase; Collagenase II	I > II > III [35]	Chondrocytes, neutrophils, fibroblasts, endothelial cells [38]
MMP-13	Collagenase III	II > I and III [39]	Chondrocytes, macrophages, fibroblasts [36, 37]

Table 1: Collagenase activities and expression

In the context of pathological cartilage degradation the most pertinent of the sub-classes of MMP are the collagenases (MMP-1, -8 and -13). As the name suggests, this is because they are the most crucial MMPs in the cleavage of fibrillar collagen within articular cartilage. They are the only proteolytic enzymes that are capable of cleaving the three α1 chains of type I, II, and III collagen. Other MMPs can target and cleave collagen (MMP-2 and -14) but at an incomparable efficiency to the collagenases [40]. For more detailed information on collagenases see Table 1. Cleavage of collagen fibrils occurs at a glycine-isoleucine bond resulting in ¾ and ¼ fragments which in turn unwind at physiological temperatures, making them susceptible to further degradation by other less specific proteinases [9, 31]. Collagens provide high tensile stiffness and strength to the cartilage as well as heavily cross-linking with, and sequestering hydrated proteoglycans which allows cartilage to resist compression. As such, this is seen as the rate-limiting step in cartilage degradation as this cleavage leads to a cascade of proteolytic events which ends with the structural and functional loss of cartilage.

1.5.1 Collagenases in arthritis

All the collagenases have been demonstrated to be present in cartilage and are significantly elevated in both OA and RA articular cartilage [37, 41]. This is also true of the synovial fluid however levels are considerably higher in RA samples compared to OA [42]. This is in line with the differential pathologies, with RA cartilage degradation driven by *MMP* expression and secretion by the inflammatory infiltrate within the synovial lining. The difference between these two arthritic diseases is not only evident in their respective pathologies but also by the differences in their MMP profile. This is because *MMP13* is preferentially expressed by chondrocytes and *MMP1* by synovial cells [9]. As such, current dogma implies that RA is mainly associated with MMP-1-driven cartilage degradation whereas MMP-13 has a more important role in OA. Corroborating cartilage localisation studies demonstrate that MMP-13 is found in areas of cartilage degradation occurring at deep layers within the articular cartilage suggesting that proteolysis is chondrocyte-driven. Conversely, MMP-1 has been localised to cartilage degradation in the superficial layer adjacent to the invading pannus leading to cartilage degradation driven by synovial cells or chondrocytes in

response to pro-inflammatory cytokines released from the synovium, at the site of contact between synovial pannus and/or fluid and articular cartilage [37, 43-46].

Although this paradigm is widely accepted, there is evidence to suggest a less specific association. Studies have shown that both MMP-1 and MMP-13 are co-localised in areas of type II collagen cleavage in samples of OA cartilage. The localisation of these collagenases was peri-cellular to chondrocytes demonstrating that it is indeed the chondrocyte that mediates collagen proteolysis but that this process is driven by not only MMP-13 but equally MMP-1 [47]. Expression studies also highlight that the deficiency between MMP-1 and MMP-13 ability to cleave type II collagen is negated by the fact that chondrocytes express around 10 times more *MMP1* than *MMP13* [45]. The assumption that cartilage degradation in RA is dependent on *MMP1* expressed by synovial cells has also been called into question. Studies have shown that both collagen and proteoglycan loss is present throughout the cartilage of RA patient samples irrespective of age [48]. It has also been shown that destruction of cartilage at the pannus boundary is orchestrated by chondrocytes in response to pro-inflammatory cytokines released by the synovial cells [49] and even that MMP-1 is derived from the chondrocyte and not the pannus [50].

These conflicting studies only serve to highlight that no definite rule can be applied to the orchestration of cartilage breakdown in either OA or RA and, whilst key to cartilage destruction, the disease-specific collagenase profile is not certain.

The fact that MMP-1 and MMP-13 are not solely responsible for the proteolytic cleavage of collagen in either OA or RA is somewhat overlooked. A third collagenase, MMP-8 has been demonstrated to be up-regulated in cartilage samples taken from OA knees indicating that MMP-8 may well be important in the pathogenesis of OA [51, 52]. However, previous study has shown that *MMP8* is not expressed in HAC [53]. As such, it may well be other cell types, such as fibroblasts or infiltrating neutrophils [38], within the synovial joint that are implicated in the increased production of MMP-8 in disease.

1.5.1.1 Other MMPs in Arthritis

Though not seen to be as pivotal in cartilage breakdown, the other members of the MMP family have an association with arthritis. The gelatinases (MMP-2 and MMP-9)

are produced by the chondrocyte and are increased in OA articular cartilage [54]. Their substrate specificities differ from collagenases as they cleave different forms of collagen as well as denatured collagens generated through the actions of collagenases. It is suggested that gelatinases have a more crucial role in activation of pro-MMPs, like pro-MMP-13, rather than in collagenolysis which is modest [55].

The matrilysins (MMP-7 and MMP-26) have also been shown to be elevated in OA cartilage, however their substrate specificity favours cleavage of proteoglycan rather than the structurally integral collagens within articular cartilage [54].

The stromelysins (MMP-3 and MMP-10) have been demonstrated to be crucial in collaganolysis whilst only demonstrating substrate specificity to proteoglycans within ECM [56]. Found in arthritic joints, they are essential in activating latent pro-MMPs (including MMP-1, -7, -8 and -13) and are therefore key regulators of cartilage breakdown [28, 57]. Specifically, MMP-3 is a marker of disease activity in RA [58] and OA [59].

Membrane-type (MT) MMP (specifically MMP-14 and -16) have both been shown to play a role in RA and OA pathogenesis. MMP-14 (MT1-MMP) is found in the superficial and middle cartilage zones of OA patient samples [60]. Synovial cells from RA patients as well as chondrocytes have also been demonstrated to express MT1-MMP [61, 62]. MMP-16 (MT3-MMP) shares a similar expression profile in RA tissues [63], however is not expressed by chondrocytes leading more to RA pathology than to OA [61]. They also have a common ability to cleave latent pro-MMP, for example MMP-13 [55], into its active form and whilst they have some collagenolytic capacity [64], MMP activation is suggested to be the more crucial function in disease pathology.

1.6   Pro-Inflammatory Cytokines

Although RA and OA are different in their pathogenesis the disease outcome is none-the-less the same; irreversible cartilage degradation leading to pain and loss of joint function in the patient. Furthermore, this is driven, in-part, by a common pathway whereby chondrocytes produce pro-catabolic factors in response to a complex milieu of elevated pro-inflammatory cytokines produced in the joint locality.

As mentioned previously, TNFα was demonstrated to be important in inflammatory pathways in RA hence its therapeutic targeting. However, other pro-inflammatory cytokines have been shown to not only be elevated in both rheumatoid and osteoarthritic joints but also lead to chondrocytes releasing catabolic factors which degrade articular cartilage.

1.6.1 Interleukin-1

One of the first cytokines to be demonstrated to be within the synovial fluid of a RA joint was IL-1β [65]. The IL-1 family is composed of the peptides; IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra). IL-1ra has no agonistic effect however it binds to both IL-1 receptors in a similar configuration to IL-1α/β thus achieving its antagonism. Many cell types produce IL-1α/β including monocytes, osteoblasts, synovial cells and chondrocytes [37]. All the genes for the IL-1 cytokines and receptors are found on the long arm of chromosome 2. IL-1α/β initially exists as atypical 31kD pro-peptides which are cleaved to biologically active 17kD, mature forms. However, the pro-peptides have some activity, with pro-IL-1α exhibiting DNA binding capabilities and a capacity to act as an autocrine factor in some cell types. IL-1β is much less active as cleavage occurs extracellularly, unlike IL-1α which is cleaved intracellularly and released in an active form [66].

There are two IL-1 receptors. Type I receptors are synthesised as an 80kD protein and type II as a smaller, 67kD protein. Both are monomeric transmembrane receptors, however, the shorter type II receptor has a small cytosolic component and as such has been termed a 'decoy' receptor as it has capacity to bind IL-1α/β yet lacks the structure to transduce an intracellular response [66]. When IL-1α/β binds, type I receptors recruit proteins called IL-1 receptor accessory proteins (IL-1AcP). These proteins facilitate the transduction of IL-1 signalling. Further recruitment of IL-1 receptor-associated kinases (IRAK) and TNF receptor associated factor (TRAF) accessory proteins follows allowing initiation of several signalling pathways [67].

Within the context of disease, IL-1 has been demonstrated to be one of the key inflammatory mediators in RA and OA. Studies have shown that IL-1 co-localises with collagenases and other pro-inflammatory cytokines in areas where ECM depletion has been observed [7, 37]. In RA, IL-1 is also found to be significantly increased in the

synovial fluid and inflamed synovium compared to healthy joint [7, 68, 69]). Chondrocytes have also been shown to produce IL-1, suggesting a pathological autocrine function [37, 47, 70, 71]. The importance of IL-1 in the destruction of articular cartilage is shown in IL-1 stimulations of primary human articular chondrocytes (HAC) isolated from OA cartilage resulting in a significant increase in collagenase expression as well as marked cartilage degradation in mouse models and both bovine and human nasal cartilage [72-74].

1.6.2 Pro-Inflammatory Cytokine Synergy

IL-17, -32 and -6 are among the other examples of elevated pro-inflammatory cytokine associated with arthritic disease. A common characteristic of a great number of these key mediators is their ability to synergise with one another to bring about a chondrocytic response greater than the sum of their individual effects. Many of these cytokines, including IL-1, TNFα and IL-17 have been shown to synergise amongst themselves and with Oncostatin M (OSM), a member of the IL-6-type cytokine family [75-77].

1.6.2.1 Oncostatin M

OSM, so named due to its discovery whereby it repressed the growth of cancerous cells, is a member of the IL-6-type cytokine family. Other members of this family, include leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CIF), cardiotrophin-1 (CT-1) and IL-11 as well as IL-6 itself. They share common structure and functional properties as well as an association with the glycoprotein (gp)130 transmembrane receptor. OSM, like IL-1α/β is secreted as a pro-peptide which is cleaved resulting in the active cytokine [78]. This synthesis and secretion is observed in many different induced cells but is primarily produced in T cells, monocytes and macrophages [79]. Upon release into the extracellular space, IL-6-type cytokines generally bind to a α-chain receptor (IL-6, IL-11 and CNTF) which associate with a corresponding signal transduction subunit, which then results in heterodimerisation with a β-chain receptor (gp130, LIFR and OSMR). gp130 β-chains also have the capacity to form homodimers [80].

LIF and OSM exhibit a great deal of homology in both structure and biological actions. This is demonstrated in their neighbouring gene locations (27% sequence homology) and the ability of OSM to signal via binding of both OSM receptor/gp130 and LIF receptor/gp130 heterodimer receptors [80]. However, along with IL-6, only OSM has the ability to cause proteolytic breakdown of ECM. Furthermore, the distribution of these two heterodimers throughout cell types may offer an explanation as to why OSM brings about proteolytic ECM degradation and LIF does not. Studies of chondrocyte surface receptor profile using flow cytometry have shown the presence of gp130 and OSMR and the absence of LIFR [75]. Further study, showing that OSM but not LIF can signal through OSMR/gp130 heterodimer [81] highlights the potential biological actions of OSM in relation to chondrocytes [82].

In relation to disease OSM levels are elevated in both RA and OA synovial fluid [83, 84]. However, its pathological role is somewhat unclear. Contradictory evidence suggests that OSM has both pro- and anti-catabolic function. Research has shown that stimulation of HAC and chondrocytic cell lines with OSM leads to an increase in production of anti-catabolic factors called tissue inhibitors of metalloproteinases (TIMPs) [85]. These studies suggest that OSM is a "chondroprotective" cytokine. Conversely, other studies have shown pro-inflammatory and pro-catabolic function of OSM in murine models [84, 86, 87] and others have demonstrated that whilst there is an increase in TIMP there is also a corresponding increase in proteolytic enzymes maintaining the anabolic-catabolic balance within the cartilage [88]. However, the capacity for OSM to induce TIMP production by chondrocytes remains well established which only serves to highlight that the context of other cytokines is an important determinant as to whether a pro- or anti-inflammatory affect is likely.

1.6.2.2 Interleukin-1 in combination with Oncostatin M

Cawston et al [76] demonstrated that stimulation with IL-1α in combination with OSM of immortalized chondrocyte cell lines, bovine nasal cartilage and primary HAC led to a dose dependent increase in collagenase production and collagen fragment release from cartilage explants. A corresponding decrease in TIMP-1 release compared to control and IL-I and OSM independently was also shown. Other IL-6-type cytokines, other than IL-6+soluble receptor, do not share this synergistic ability [75]. These findings show that a combination of arthritis associated cytokines leads to a marked

increase in collagenase expression and release, a finding which is supported by many other studies [72, 73, 77, 82]. This may represent a significant contributing factor in the breakdown of type II collagen associated with cartilage degradation of the arthritic joint.

It must be said that IL-1+OSM is by no means the only synergistic cytokine combination that has a marked increase on *MMP* expression in chondrocytes. Many cytokines other than IL-1 have been shown to synergise with OSM and IL-6+soluble receptor [75-77]. The reason for its use in the present study is because of the large body of work that has been built up around this specific synergy. With this work has come a great deal of understanding into the cellular events that occur in a synergistically stimulated chondrocyte. Perhaps most importantly, however, is that the pathology of the disease strongly suggests that it is a combination of the many significantly elevated cytokines rather than a single stimulus that brings about cartilage degradation in arthritis. Therefore, the IL-1+OSM cytokine combination offers a well-studied candidate for complex, pathological synergy.

1.7 IL-1- and OSM-mediated cell signalling pathways

1.7.1 MAPK

In order for these previously mentioned pro-inflammatory cytokines to illicit a pathophysiological response by the chondrocyte (e.g. to produce cartilage degrading enzymes) there needs to be an intracellular transduction of this signal in order to induce transcription of collagenases. This has been demonstrated to occur through a number of cell signalling pathways within chondrocytes. The mitogen-activated protein kinase (MAPK) cascade is one such pathway. It consists of serine and threonine protein kinases that function to transduce signal (cytokine) from binding to a receptor residing on the cell membrane to a series of phosphorylation cascades that culminate in the activation of effector molecules such as transcription factors that induce transcription of certain genes. Three distinct pathways are encompassed within the MAPK, with their differentiation from one-another dependent on the downstream end point kinase that they activate; extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) or p38 kinase. These three pathways have been specifically linked to transduction of pro-inflammatory cytokine signals which elicit collagenase induction

and/or cartilage destruction. JNK and p38 kinase pathways are associated with IL-1 and are implicated in *MMP1 and MMP13* regulation in HAC [89, 90], observations which are supported by studies using p38 kinase inhibitors in which IL-1-mediated cartilage degradation was blocked by these compounds [91]. JNK inhibitors are less established but do show amelioration of adjuvant-induced arthritis in rats [92]. Mengshol *et al* [93] further demonstrated the importance of these MAPK pathways in IL-1-mediated *MMP* gene regulation in the chondrosarcoma cell line, SW1353 and in HAC. Inhibition of p38 kinase led to the complete block of *MMP13* expression in both cell populations with similar effects on *MMP1* in SW1353. JNK inhibition led to a 60% decrease in IL-1 induced *MMP13* expression in SW1353 with no effect on *MMP1* in chondrocytes. Complete suppression of IL-1-induced JNK-dependent collagenase expression was also demonstrated in synoviocytes treated with a novel JNK inhibitor. This inhibition translated to total abrogation of radiographic evidence of arthritis in treated mice [92]. The same study showed only modest effects with ERK inhibition. However, other studies implicate the ERK pathway in pro-inflammatory cytokine induced *MMP* expression in chondrocytes [94, 95]. Specifically, ERK inhibition and gene silencing with siRNA led to decreases in cartilage explant resorption and *MMP13* expression, respectively [96, 97].

1.7.2 Jak/STAT

As previously discussed OSM exerts its effects on chondrocytes through binding to OSMR/gp130 heterodimer. This binding leads to recruitment of a Januse kinase (Jak) to the cytoplasmic tail of the receptor complex. Auto-phosphorylation of Jak and gp130 follows and latent cytoplasmic signal transducer and activator of transcription (STAT) proteins are recruited to the receptor/Jak complex. Further tyrosine phosphorylation events on STAT lead to dimerisation allowing their translocation into the nucleus where they directly regulate transcription of genes [98]. Evidence for the involvement of this pathway in collagenase gene transcription is illustrated in studies where STAT inhibition led to decreased activation of a *MMP1* promoter-luciferase construct [82]. Litherland *et al* [97] also demonstrated that *STAT3* silencing with small interfering (si)RNA led to a significant decrease in *MMP1* and *MMP13* mRNA levels in IL-1+OSM stimulated HAC. Other studies have also demonstrated reduced STAT-1

phosphorylation and collagenase gene expression as a result of Jak inhibition in OSM-stimulated chondrocytes [94].

1.7.3 Phosphatidylinositol 3-kinase (PI3K)/Akt pathway

As well as Jak/STAT pathway, PI3K/Akt pathway is also involved in chondrocytic OSM-dependent collagenase production. This pathway consists of lipid kinases that have been associated with large number of cellular functions that include the ability to activate Akt (Protein Kinase B). This kinase is a hub for a vast array of downstream events corresponding to cell function. This activation is brought about through a complex cascade of intracellular signalling events. Firstly the binding of a substrate to a receptor leads to the localisation of PI3K. The receptor being bound to dictates the class of PI3K which localises to the specific receptor: auto phosphorylation of tyrosine-kinase receptors bring about the binding of class IA (catalytic subunit: p110α, β, and δ and regulatory subunit: p85 and p50-55), whilst uncoupling of G-protein receptors allows class IB binding (catalytic subunit: p110γ and regulatory subunit p84 or p101) [99]. These kinases then phosphorylate membrane-bound phosphatidylinositides. This generates phosphatidylinositides-3,4-biphoshate (PIP2) and PI-3,4,5-triphosphate (PIP3) allowing the binding and co-localising of Akt and 3-phosphoinositide dependent protein kinase-1 (PDK1) which results in the partial activation of Akt. Full activation is achieved through the actions of the mTOR (mammalian target of rapamycin) protein kinase and TORC2 (transducer of regulated CREB protein 2) [100], a co-activator of cyclic adenosine monophosphate (AMP) response element binding (CREB) transcription factor, a key *MMP13* regulator [101, 102]. An insight into the importance PI3K/Akt pathway in the context of disease was demonstrated in mouse models. PI3K inhibition resulted in significant reduction in joint damage in RA models [103]. Studies into cell signalling events demonstrated that OSM-dependent *MMP13* induction was down-regulated upon PI3K and Akt inhibition [96]. Further study silencing specific Akt and PDK isotypes in HAC highlight the potential for the differential regulation of distinct collagenases by these different isotypes following OSM stimulation and the complex signalling inter-play with other pro-inflammatory cytokines [104].

1.7.4 PI3K/Protein Kinase C pathway

The phosphorylation of PI bound to the membrane can also take a different cell signalling route and is not confined to Akt activation. PIP2 can act as a molecular

anchor for other signalling molecules. Phospholipase C (PLC) hydrolyses PIP2 into diacylglycerol (DAG) and inositol trisphosphate (IP3), which facilitate the opening of intracellular calcium channels and release of calcium from intracellular stores increase cellular calcium concentrations. The second messenger, calcium, then binds to the serine/threonine kinase, protein kinase C (PKC) allowing its activation by DAG. PKC can then activate RAF through dis-inhibition. RAF functions as a MAP3K initiating a phosphorylation cascade that activates ERK. As mentioned previously, the ERK MAPK pathway is important in signal induction culminating in *collagenase* expression in the chondrocyte. Study on isoforms of PKC showed that silencing of this kinase leads to diminished IL-1+OSM-dependent ERK phosphorylation and collagenase production in HAC [96, 97].

1.7.5 NF-κB

The Nuclear Factor κB (NF-κB) pathway is another intracellular signalling pathway suggested to be involved in pro-inflammatory cytokine-dependent *MMP* regulation in chondrocytes. The NF-κB family of proteins share a Rel-homology domain within the N-terminal region and a transactivation domain within the C-terminal region. The canonical pathway is activated by a number of stimuli including IL-1. This pro-inflammatory stimulus leads to the activation of inhibitor of κB (IκB) kinase (IKK) complex leading to the subsequent phosphorylation of IκB which is bound to NF-κB dimers in the un-stimulated cell. This targets IκB for poly-ubiquitination and proteasomal degradation leaving free NF-κB. Dis-inhibition allows NF-κB dimer to translocate to the nucleus where it binds to DNA thereby regulating transcription of genes [67]. The blocking of components of this pathway has demonstrated that NF-κB does indeed have transcriptional control over collagenase expression post IL-1 stimulation in synovial fibroblasts and both human and bovine chondrocytes [105-108]. Furthermore, treatment of chondrocytes with NFκB inhibitors leads to the reduced production of MMP-1 and MMP-13 [109]. Conversely, other studies have demonstrated an increase in *MMP1* gene expression with NFκB silencing in chondrocytes [110]. Contradicting studies of the NF-κB pathway and its regulation of collagenase genes suggest a regulatory role for NF-κB but as to whether or not this is positive or negative is yet to be determined.

1.8 Cis-regulatory elements and trans-activators

The aforementioned cellular signalling pathways culminate in the activation of transcription factors (trans-activators) that work in concert, in order to regulate gene transcription. This is achieved through the binding to specific regions of DNA called cis-binding elements which are conventionally just upstream of the gene being regulated. However the sites can also be found several kb upstream, within the 3'UTR or within an intron of the gene. The process of binding to these elements then allows the recruitment of transcriptional machinery containing numerous factors that initiate and perpetuate the transcription of a gene.

The characterisation of collagenase cis-binding elements has given insight into transcription factors that potentially regulate their transcription. *MMP1* and *MMP13* along with many other MMPs contain a TATA box approximately 30 base pairs (bp) upstream of the transcription start site (TSS) and a binding element for the family of transcription factors called Activating Protein (AP)-1 at approximately 70bp upstream of the TSS [111]. Other cis-element have been identified within the promoter regions of various MMP, for example Ets [111], NFκB [112], STAT [95], RUNX-2 [113] and Sp-1 [114] DNA binding sites (Figure 5).
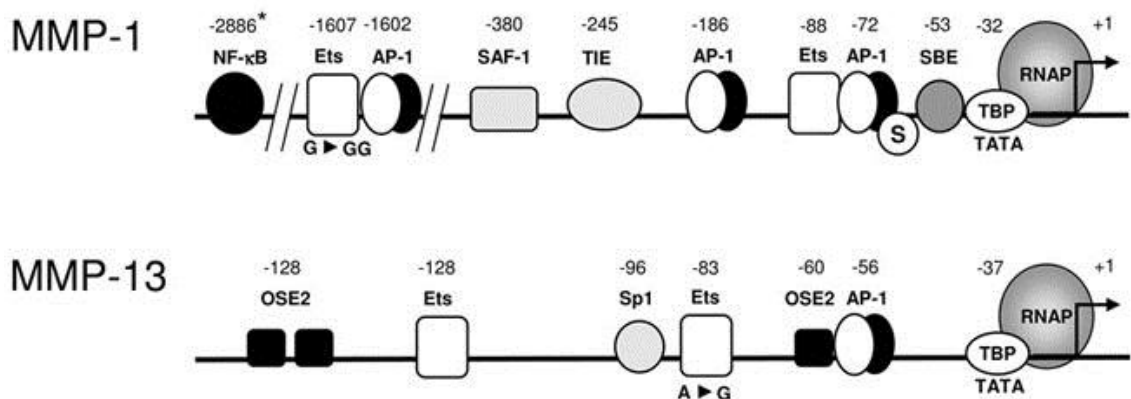
Figure 5: Schematic of MMP-1 and -13 promoter regions. Consensus transcription factor binding sites and the positions relative to transcription start site; SBE, STAT binding element; TIE, TGF-β inhibitory factor; S, Smad binding site; SAF-1, serum amyloid A-activating factor; OSE2, osteoblast-specific element-2; TBP, TATA binding protein; RNAP, RNA polymerase II complex. Taken from Rowan and Young [2]

The profile of cis-binding elements within promoter regions is somewhat subjective to the individual MMP, providing an explanation for differential regulation of the members of this endopeptidase family. However, the majority of the proteinases contain at least one, if not several, AP-1 binding sites within the promoter region of

their genes. These AP-1 binding elements can be found within very close proximity to the TSS emphasising its potential in orchestrating the recruitment of co-factors necessary for transcription. Other AP-1 binding elements can also be found anywhere from within close proximity to the TTS to approximately 20kb upstream of the TSS [115]. Complemented by the fact that many of the aforementioned cell signalling pathways culminate in the activation/expression of many AP-1 members, these factors, therefore, play a crucial role in the regulation of collagenase expression following IL-1+OSM stimulation in chondrocytes.

1.8.1 Immediate early genes

Immediate early genes (IEG) encode numerous proteins involved in various cellular functions such as cell proliferation and differentiation. One of the most well characterised IEG is *FOS* (FBJ murine osteosarcoma viral oncogene homolog), which encodes c-Fos, a key binding partner in many AP-1 heterodimers. *FOS*, as well as many other IEG encode transcription factors and play a key role in controlling gene expression and the regulation of cellular function. All IEG are rapidly expressed in response to stimulation, a characteristic which is imparted by a number of regulatory controls; IEG are very short primary transcipts with very few, if any, exons and their promoter regions contain a great number of transcription factor binding sites and high affinity TATA boxes [116]; The structure of the chromatin associated with these genes is also open and therefore highly permissive to gene activation by transcription factors [117]; Moreover, transcriptionally active RNA polymerase II (pol II) is found in a 'paused' state at the promoters of IEG ready to initiate transcription [118]; Finally, IEG require no *de novo* synthesis of proteins in order to bring about their expression [119] as such transcription factors that regulate their expression are constitutively present and therefore IEG can be expressed very rapidly following stimulation.

However, because of the prominent role that these IEG have in regulating other genes that control cell cycle progression and apoptosis, strict cellular controls exist to make their rapid induction countered by a similarly rapid reduction. This serves to mitigate the response to the robust induction of IEG thereby preventing unregulated functioning synonymous with cancer development in which high, sustained levels of IEG correlate with cancer progression, angiogenesis and metastasis [117, 120]. The attenuation of IEG is controlled at a number of levels including mRNA and protein. The

mRNA of IEG is very unstable which is conferred by active targeted degradation by RNA-binding proteins [121] but also by microRNA and RNA interference [122]. Protein products are also rapidly targeted for protesomal degradation [123]. Moreover, the transcription factors encoded by IEG also control their own expression via auto-repression [124-126].

There are other groups of genes that do not require the *de novo* synthesis of proteins in order to allow their expression to take place. Delayed primary response genes [116] or 'slow' IEG [127] differ from IEG as they do not have many of the gene characteristics associated with IEG that confer rapid induction and therefore take longer to be expressed. As such it is proposed that IEG function as mediators of signalling in which their expression leads to the control of secondary response genes, which require *de novo* synthesis resulting from transcription factors encoded by IEG. Delayed primary response/'slow' IEG may contribute to controlling the expression of secondary response genes or are themselves the effectors at the end of signalling.

1.8.2 Activator Protein-1

AP-1 transcription factor is an umbrella term used for a dimer that is predominantly made up from the Jun (proto-oncogene) (c-Jun, Jun B and Jun D) and Fos (c-Fos, Fos B, Fos-related antigen (Fra)-1 and Fra-2) protein families. Activating Transcription Factor (ATF) and Musculoaponeurotic Fibrosarcoma (MAF) members are also capable of heterodimerising with Jun and Fos members [128, 129]. These proteins display similar structural and functional properties which are conferred by the presence of an evolutionarily conserved basic leucine zipper (bZIP) domain [130]. This allows these proteins to dimerise with one another through the leucine zipper motif thereby forming a bipartite DNA-binding domain [131, 132]. AP-1 dimers control the transcription of many genes that contain an AP-1 consensus binding site, also known as TPA responsive element (TRE) (5'-TGAG/CTCA-3') within their promoter and/or enhancer elements [133]. Other consensus sequences also exist that also provide a target for DNA binding by AP-1 dimers; the cAMP (cyclic adenosine monophosphate) responsive element (CRE) and the MAF-recognition elements (MAREs) [134]. These sequences are preferentially bound by different AP-1 heterodimers. For instance, Jun-Fos heterodimers and Jun-Jun homodimers bind with greater affinity to TRE than CRE whereas ATF-Jun or ATF-ATF bind more readily to CRE [135]. The individual

components of these dimers also have varying trans-activating capacity with members such as Jun, Fos and Fos B considered strong activators whilst other members such as Jun B, Jun D, Fra-1 and Fra-2 displaying modest transactivation [136]. Moreover, the constituents of these dimers also govern their capacity to trans-activate gene promoters. Fos members are incapable of forming homodimers capable of binding to DNA [137] and therefore require heterodimerisation with Jun members to bind to DNA [137, 138]. However, Jun homodimers can bind to DNA yet lack the affinity with which Fos/Jun heterodimers bind [138]. As such the ability of these transcription factors to form any number of possible homo- or hetero-dimers, the preferential binding of those dimers and their ability to actually activate gene expression confers varying regulatory function in relation to different genes and therefore different cellular processes (Figure 6).
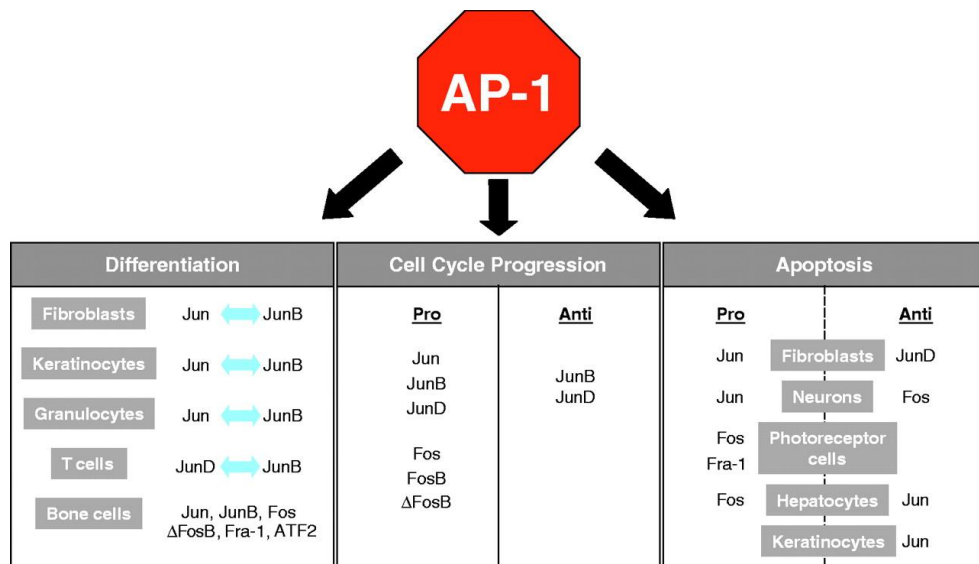
Figure 6: Functions of AP-1 subunits in various cellular processes. Adapted from Hess et al [136].

Their main functional role within the body is that of control over genes involved in cell proliferation and differentiation. These genes include many of the key regulators of the cell cycle progression which include cyclins and cyclin-dependent kinases (CDK) [139]. The capacity for varying AP-1 dimer combinations and the level of regulation that this lends is characterised by the variety of cell types and the dimer-specific functions. For example, Jun family members are crucial for regulating the proliferation of keratinocytes and hepatocytes [136]. Likewise, Fos members have been demonstrated to play a key role in osteoblast/osteoclastogenesis [140, 141] and chondrogenesis [142] over that of Jun members. Contrasting roles within the protein families are also

evident. JunB inhibits proliferation in the context of lymphoid cells [143] but, in contrast, positively regulates growth in mouse fibroblasts [144] and the production of cytokines which drive differentiation in T Helper cells [145]. AP-1 also regulates apoptosis with Jun and Fos members often having contrasting apoptotic regulation within a number of different cell types.

1.8.3 AP-1 and collagenase gene regulation

AP-1 members have been demonstrated to be involved in normal cellular function such as chondrogenesis and act to balance catabolic and anabolic factors that remodel cartilage throughout the body. In the context of pathological cartilage degradation, a great deal of study has implicated AP-1 in the aberrant regulation and increased expression of collagenases in disease. This is demonstrated in immunohistochemical studies of patient synovium samples, that show correlation between c-Fos and active RA [146].

General consensus strongly suggests that IL-1 stimulation of cells leads to the AP-1-dependent trans-activation of many *MMPs* including collagenases through the MAPK pathways, JNK, p38 and ERK. Study has shown that increased *MMP1/13* expression is due to ERK activation following IL-1 stimulation [93, 147, 148]. Cell lines that exhibit constitutively elevated *MMP1* levels have been shown to do so via ERK1/2-dependent phosphorylation, activation and subsequent promoter binding of AP-1 [149]. Inhibition of PKC isoforms upstream of MEK in the ERK pathway lead to decreased collagenase expression and cartilage breakdown. Furthermore, AP-1 monomer, *FOS* mRNA and protein levels were shown to significantly decrease with ERK and PKC silencing. Decreased IL-1+OSM-dependent *MMP1 and MMP13* mRNA expression was also demonstrated with *FOS* silencing [97]. The JNK pathway also leads to the activation of key AP-1 trans-activators such as c-Jun and ATF2 [150] and has also been clearly demonstrated to be important in IL-1-dependent *MMP13* expression [93]. Cis-element mutations via transfected reporter studies have shown that p38, as well as JNK, are important in AP-1-dependent collagenase transcription following IL-1 stimulation in chondrocytes [113]. p38 has also been shown to increase the levels of c-Fos and c-Jun thereby directly contributing to transcriptional regulation of collagenases [151, 152].

Many studies have demonstrated how the direct binding of AP-1 elements to *MMP* genes is crucial for transactivation and expression [153-158]. Han *et al* [92] showed that inhibition of IL-1-dependent JNK signalling pathways in synoviocytes resulted in decreased *JUN* expression, phosphorylation, accumulation and collagenase promoter binding. Martens *et al* [158] demonstrated that both c-Fos and c-Jun are transiently and rapidly enriched on *MMP1* proximal promoter in chondrocytes and are vital components in the assembly of the pre-initiation complex that transcribes the gene. A study by Aikawa *et al* [159] provides probably the most robust demonstration of the importance of AP-1 in the trans-activation of *MMP* within the context of arthritic cartilage degradation. In this study the authors synthesised an inhibitor of c-Fos/AP-1 binding by 3D pharmacophore modelling through previous study which elucidated the X-ray crystal structure of AP-1-DNA complex [130]. Use of this inhibitor in both synoviocytes and chondrocytes stimulated with IL-1 led to significant reduction in *MMP13*. Administration of this inhibitor to mice with collagen-induced arthritis (CIA) resulted in >91% reduction in arthritis (blinded scoring of paws) with significant reduction in *MMP13* expression in hind paws.

Recent study has also highlighted an important role for enhancer regions in the regulation of collagenase. Enhancer elements are regions of DNA distinct from the proximal promoter regions that have binding sites for transcription factors that contribute to regulation. These elements are usually many kb away from the proximal promoter/TSS but dynamic structural changes in the chromatin allow folding, bringing a distal enhancer into close proximity to other transcription factors bound to the proximal promoter to effect full activation. Schmucker *et al* [115] demonstrated that a -20kb distal enhancer region is crucial for IL-1-dependent *MMP13* induction in chondrocytes. ChIP revealed binding of not only c-Fos and c-Jun to this site but also markers of active transcription, H3K4me2 and AcH4 modifications and pol II. Chromatin conformation capture (3C) highlighted an association between this enhancer element and the proximal promoter. Reporter studies then showed a recapitulation of endogenous IL-1-dependent *MMP13* expression when the enhancer region was combined with the proximal promoter region of *MMP13*.

1.8.4 Other tran-activators and collagenase gene regulation

In the case of Jak/STAT and NFκB pathways the transduction of signal through cascading phosphorylation events culminates with direct activation of pathway-specific transcription factors. A STAT binding element is found near the proximal AP-1 binding element in the *MMP1* promoter [160]. STAT-1 has been demonstrated to be involved in collagenase regulation as Jak inhibition in chondrocytes leads to decreased STAT-1 phosphorylation and binding to *MMP1 and MMP13* [94]. Inhibition of STAT-3 as well as direct *STAT3* gene silencing leads to significant reduction in collagenase expression in HAC [82, 97]. However, direct binding to the *MMP1* STAT element in chondrocytes was unable to be shown. Interestingly though, STAT-3 activation was still required for transcriptional activation despite lacking the capacity to bind to the promoter of *MMP1* [82]. It is suggested that it is the indirect effect of STAT-3 on *FOS* expression that plays a role in collagenase transcription.

NF-κB has also been shown to have a role in IL-1-dependent collagenase transcription, as discussed above. As with STATs, NFκB has a cis-element in the *MMP1* promoter but at nearly 3kb upstream of the TSS this is most likely in an enhancer region which is looped into proximity with trans-activation. Although studies have demonstrated NFκB binding to this site following IL-1 stimulation [112], binding in chondrocytes has yet to be demonstrated.

The tissue-specific transcription factor, Runx-2 (runt-related transcription factor-2) is crucial in osteoblast and chondrocyte differentiation and skeletal morphogenesis [161], co-ordinating the resorption of cartilage into bone during development. Study has also demonstrated the pathological role of Runx-2 in *MMP13* transcription following IL-1 stimulation of the chondrocyte. It demonstrates that *MMP13* induction by IL-1 required Runx-2 and that binding of Runx-2 to a conserved binding site -138bp from the TSS in combination with JNK-dependent AP-1 binding acted synergistically to induce *MMP13* expression [113].

1.9 Epigenetic regulation

The culmination of cell signalling events to the binding of the *trans*-activators to *cis*-elements is an important process yet sits amongst many other regulatory steps: Chromatin remodelling, assembly of the machinery necessary for transcription, mRNA

splicing and post-transcriptional modifications all exist to exercise control over the production of proteins that ultimately control cellular function.

1.9.1 Chromatin remodelling

The transcription of genes is regulated at a number of different levels. Regulation at points post transcription factor activation is limited by the accessibility of the gene. Chromatin is the structure formed by association of DNA with histone octamers (H2A, H2B, H3 and H4 in duplicate) at regular intervals of approximately 150bp wound around the histone core to form what is known as a nucleosome. Packaging of the DNA into nucleosomes increases the stability and prevents damage of the DNA at the same time compressing the entire genome within the confines of the cell nucleus. What regulates the access of transcriptional machinery to genes within the DNA is governed by the state of the chromatin, a role which represents an important regulatory step in transcription. Modifications of these histones around which DNA is wound allows this otherwise very stable complex to dynamically remodel in order to allow access to genes within the DNA. Euchromatin, the open-unwound form of chromatin is brought about through histone modifications of specific amino acid residues on histone tails (e.g. lysine, serine, and threonine) that include methylation, acetylation, ubiquitination and phosphorylation. An ATP-dependent chromatin remodelling complex, SWI/SNF can also facilitate transcription factor access [162]. These modifications act to alter the charge of the histones thus repelling the negatively charged DNA and opening the chromatin. Conversely, other modifications such as methylation events on histone (H) 3 lead to the re-coiling of chromatin into heterochromatin which masks genes. These modifications are targeted events thus allowing distinct regions of chromatin i.e. around specific genes to open, allowing access to the gene. These modifications have also been proposed to act as targets for proteins that play a role in transcriptional regulation [163].

Once this euchromatin state is achieved, transcription factors can bind to specific sequences within the promoter regions of these revealed genes. These binding sites are often in a region slightly upstream of the transcription start site of the gene where there is a low level of nucleosome occupancy [164]. This binding brings about the recruitment of co-activator complexes that both further remodel the chromatin to

allow the access of factors necessary for transcription to the proximal promoter and allow greater binding of other co-activators to initiate transcription [165].

1.9.2 The Transcriptional Cycle
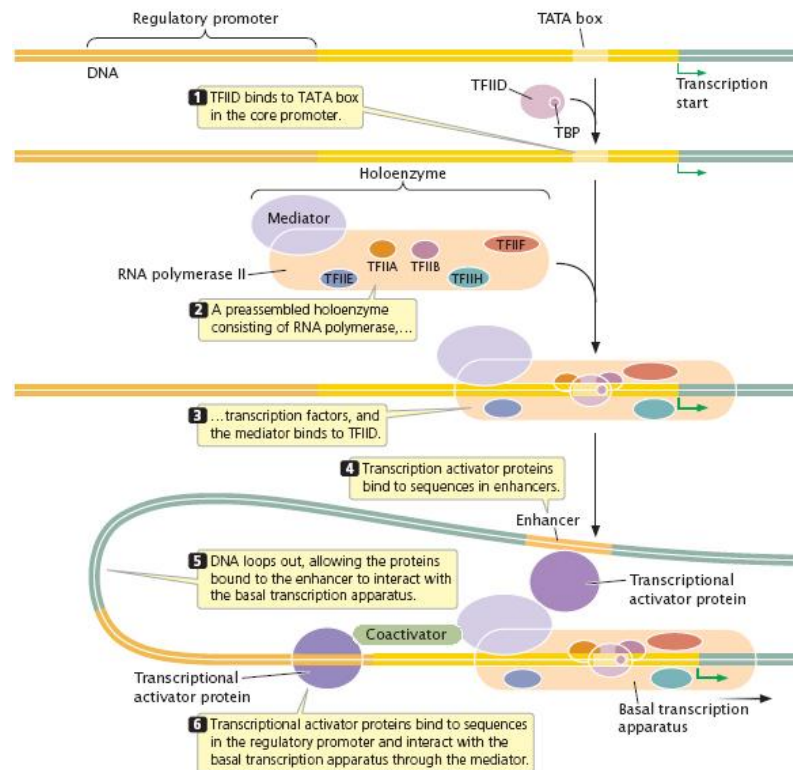
Figure 7: Recruitment of transcriptional machinery to gene promoter. Adapted from [166]

1.9.2.1 Initiation

Transcription initiation is a complex process of factor recruitment in a carefully orchestrated manner in order to bring together all the transcriptional machinery necessary for transcription (Figure 7). Firstly, a pre-initiation complex (PIC) is formed. This consists of the enzyme pol II along with various general transcription factors (GTF) and the Mediator complex. Core promoter binding is governed by TFIID and TATA binding protein (TBP). Whilst the PIC remains promoter bound it is prone to abortive initiation in which only small nascent RNA transcripts (2-3 nucleotides) are produced. In order to allow promoter escape the C-terminal domain (CTD) of pol II must undergo ser 5 phosphorylation which in turn recruits RNA capping machinery to modify the 5' end of the nascent RNA transcript [167]. This stabilises the short transcript allowing rearrangements of the GTFs within the PIC. Increasing transcript length and the association of RNA to DNA infers greater complex stability [168, 169]. This stabilised complex can now facilitate the transition into the next phase of transcription.

1.9.2.2 Elongation

Elongation is initiated by the ser 2 phosphorylation on the CTD of pol II by Positive Transcription Elongation Factor b (P-TEFb) [170]. This leads to an alteration in the profile of GTF that constitute the PIC and removes the inhibition of negatively regulating factors that normally keep the pol II in a paused state. Highly stable productive elongation then ensues, concerting RNA production with transcript processing events that splice intron-containing pre-mRNA to mature mRNA which is then translated to protein. Once the elongating pol II complex reaches the end of the gene transcription is terminated through nascent transcript cleavage and 3' polyadenylation and enzyme release from template DNA [171].

1.9.3 Epigenetic regulation of collagenase genes

Transcriptional regulation of collagenases is by no means solely governed by the binding of transcription factors to DNA. Study has demonstrated that acetylation of histones is an important step in allowing collagenase gene induction to occur. IL-1 stimulated HAC were treated with the Histone de-acetylase (HDAC) inhibitor, Trichostatin A (TSA) resulting in genome-wide increase in histone acetylation. This allows for more permissive DNA which was reflected in an increase in *MMP1* and *MMP1*3 gene expression, collagenolysis and subsequent explant degradation [172, 173]. With regards to disease, the expression of HDACs in normal and OA cartilage is somewhat unclear with studies showing opposing expression [174-176].

DNA methylation is another epigenetic modification that acts to regulate gene transcription. The addition of a methyl group by DNA methyl transferases (DNMT) to the cytosine within cytosine phosphodiester guanine (CpG) dinucleotides leads to repression of gene expression. DNA methylation is associated with distinct regions within the promoters of genes, called CpG islands. These islands, that vary in size from as little as 200 bp to several 1000 bp in length, have a GC percentage of >50% and are present in approximately 40% of all mammalian gene promoters [177, 178]. The exact mechanism by which methylation represses transcription is not fully understood: Methyl groups may act to prevent the binding of transcription factors to the DNA. Alternatively, these modifications may provide new binding sites for proteins that orchestrate co-repressors and HDACs in bringing about chromatin condensation [179]. In disease, DNA methylation has been suggested to play a role in the pathologies of

both RA [180] and OA [181, 182]. With regards to collagenase expression, Roach *et al* [183] showed that the *MMP13* promoter in OA chondrocytes had a significant decrease in methylation. Further study demonstrated that CpG islands in the *MMP13* proximal promoter of HAC have decreased methylation which affects its transcriptional activity leading to an increase in *MMP13* gene expression [102, 184].

1.10 Summary and aims of the study

The cleavage of type II collagen and subsequent cartilage degradation represents a threshold in the pathological progression of arthritic disease. Type II collagen is the main structural component of articular cartilage which imparts tensile strength to the tissue. It also acts as a scaffold in which proteins called aggrecan are held, providing resistance against compression following joint loading. These two molecules are therefore crucial to the healthy functioning of cartilage in the articulating joint. Pain and disability in patients which follow irreparable damage to cartilage architecture comprise a series of events that stem from aggrecan loss and type II collagen destruction.

The collagenases, MMP-1 and MMP-13 are responsible for this process. In cartilage degradation associated with arthritic disease, the physiological role of MMP-1 and MMP-13 in subtly remodelling cartilage in response to joint loading is replaced by aberrant proteolytic events. Taken together with the lack of opposing anabolism and the slow recapitulation of cartilage architecture, the cleavage of type II collagen by MMP-1 and MMP-13 is a threshold event leading to irreversible disease progression.

The chondrocyte, which resides within the articular cartilage, orchestrates not only normal remodelling but also pathological cartilage destruction. The latter, is a direct result of collagenase production by the chondrocyte in response to elevated levels of pro-inflammatory cytokines such as IL-1 and OSM in arthritic patients.

The transduction of pro-inflammatory cytokine signals into excessive collagenase production by the chondrocyte is achieved by many cell signalling pathways, which is, in-part, due to the cytokine milieu found in disease. Therefore, when trying to attenuate collagenase expression by the chondrocyte the targeting of any one

pathway can be ineffective. Moreover, broad spectrum inhibition of MMP is equally ineffective due to the lack of specificity.

The inflammatory response mediated by cytokines and tissue remodelling by MMP are homeostatic processes that serve to maintain normal physiological function in the body. As such, interventions that are intended to reduce cartilage degradation by the reduction of *MMP* expression need to be as specific as possible. I postulate that specificity may be gained at the level of gene regulation. All genes contain a specific binding element profile in their promoter region and so only certain transcription factors can bring about the transcription of certain genes. A greater understanding of these factors, crucial to gene regulation of the MMPs that degrade cartilage, could therefore help to develop more targeted intervention.

AP-1 transcription factor family members are ubiquitous proteins that regulate many genes throughout the body. Previous study has demonstrated the necessity of AP-1 heterodimer c-Fos/c-Jun for efficient collagenase transcription. However, targeting this ubiquitous regulating factor would lack specificity, as with direct MMP inhibition.

In the following study I intend to better understand how c-Fos and c-Jun are contributing to the regulation of the collagenase genes, *MMP1* and *MMP13* following pro-inflammatory cytokine stimulus. With an improved knowledge I hope to be able to elucidate the mechanisms by which different *MMP* are regulated and therefore how the expression of specific *MMP* can be reduced in arthritic disease.

2 Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Antibodies

Antibody (Dilution for WB)	Source	Product code
Acetyl-Histone H3	Millipore	06-599
ATF3 (1/500)	Santa Cruz Tech (SC)	sc-188
AXUD1 (1/500)	SC	sc-81191
Caveolin (1/2000)	Cell signalling (CS)	3238
c-Fos (1/500)	SC	sc-7202
c-Jun (1/500)	SC	sc-1694
c-Jun-phospho ser63 (1/500)	SC	sc-7908-R
EGR2 (1/1000)	Epitomics	3172-1
GAPDH (1/40000)	CS	2118
Histone H3 (1/2000)	CS	9715
Lamin A/C (1/10000)	CS	4777
NFATc1 (1/500)	SC	Sc-7294
RNApolII-phospho CTD ser5	Abcam	ab5131
β-tubulin (1/2000)	CS	4967

Table 2: Detailed list of antibodies used in study

Antibody isotype controls, rabbit and mouse IgG from sera were purchased from Sigma-Aldrich (Poole, UK) and mouse monoclonal IgG was a kind gift from Dr Jelena Mann.

2.1.2 Cytokines

IL-1α was a gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, UK) and was stored at -20 °C until use. Recombinant OSM was made in-house (method described in Staunton et al [185]) 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. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich. Immediately prior to

use IL-1α, OSM and PMA were diluted to appropriate working concentrations in culture medium.

2.1.3 Cell and Tissue Culture Reagents

Dulbecco's modified Eagle's medium (DMEM), DMEM-F12, foetal bovine serum (FBS) and nystatin were obtained from Gibco BRL (Paisley, UK). L-Glutamine, penicillin-streptomycin and trypsin (type III, from bovine pancreas) were obtained from Sigma-Aldrich. Plates (96-well and 6-well) and vented cell culture flasks (75 cm^2 and 162 cm^2) were obtained from Corning/Costar UK Ltd. (High Wycombe, UK). Sterile, vented 100 mm cell culture dishes and universals (20 ml and 5 ml) were obtained from Greiner Bio-One (Gloucestershire, UK). Centrifuge tubes, 50 ml and 15 ml were obtained from Corning/Costar UK Ltd. 50 ml syringes were obtained from Scientific Laboratory Supplies Ltd. (Nottingham, UK) and syringe driven 0.22 μm filters were obtained from Millipore. DPBS was obtained from Lonza (Wokingham, UK). Hyaluronidase (from bovine testes; 439 U/mg), trypsin (from porcine pancreas; 1020 U/mg) and collagenase (from Clostridium histolyticum type I; 125 U/mg) were obtained from Sigma-Aldrich. Cell Strainers (100 μm) were obtained from Scientific Laboratory Supplies (Nottingham, UK).

2.1.4 Commercially available kits

RNeasy Mini Kit, QIAShredder and QIAQuick PCR Purification Kit were purchased from Qiagen (Crawley, UK). EZ-ChIP Kit: Chromatin Immunoprecipitation Kit was purchased from Millipore. Subcellular Protein Fractionation Kit and NE-PER Nuclear and Cytoplasmic Extraction Reagents were obtained from ThermoScientific (Loughborough, UK). ToxiLight BioAssay Kit: Non-Destructive Cytotoxicity Assay was purchased from Lonza. Taqman gene expression assays were purchased from Applied Biosystems (CA, USA). Universal ProbeLibrary System was obtained from Roche Diagnostics (Burgess Hill, UK).

2.1.5 Immunoblotting Reagents

BSA, ammonium peroxodisulphate (APS), β-mercaptoethanol (β-ME), 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). PageRuler

prestained protein ladder was purchased from ThermoScientific. Enhanced chemiluminescence (ECL) western blot detection reagents were obtained from Amersham Biosciences (Little Chalfont, UK). Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate was purchased from Millipore. Reagents for the iBlot gel Transfer System were purchased from Invitrogen (Paisley, UK). Bradford ULTRA was purchased from Novexin (Invitrogen).

2.1.6 Cell Lines

SW1353 chondrosarcoma cell lines were purchased from the American type culture collection (ATCC). They are derived from a primary grade II chondrosarcoma of the right humerus obtained from a 72 year old female Caucasian. SW1353 cells were cultured in DMEM-F12 containing 10 % (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin, 200 μg/ml streptomycin and 40 IU/ml Nystatin.

2.1.7 Biochemical Reagents

Glycine, sodium chloride, sodium dodecyl sulfate (SDS) and tris-hydrochloric acid were purchased from Sigma-Aldrich. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA) was purchased from BDH.

2.1.8 Molecular Biology Reagents

SideStep Lysis and Stabilisation Buffer was purchased from Agilent Technologies (CA, USA). Cells-to-cDNA II was purchased from Ambion (Invitrogen). Real-time polymerase chain reaction (PCR) primers and probes were purchased from Sigma-Aldrich. SuperScript III Reverse Transcriptase, Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and RNase OUT were purchased from Life Tech (Invitrogen). RQ1 RNase-free DNase was purchased from Promega (Southampton, UK). SYBR green PCR Master Mix and Titanium Taq polymerase were purchased from Clontech (Paris, FR). Taqman Fast Universal PCR Master Mix (2x) and Taqman Universal Master Mix II were purchased from Applied Biosystems. Agarose (electophoretic grade) were purchased from Invitrogen. RNase- and DNase-free water was purchased from Sigma-Aldrich. Protein synthesis inhibitor, emetine (dihydrochloride) and transcription inhibitor, actinomycin D were purchased from CalbioChem (Merck Chemicals Ltd, Nottingham, UK). Protein A and Protein G agarose beads were purchased from Santa Cruz

Biotechnology Inc. (Heidelberg, Germany). Dharmacon ON-TARGET plus SMARTpools siRNAs, Dharmacon ON-TARGETplus Non-targeting Pool control siRNA and DharmaFECT 1 Transfection reagent were purchased from ThermoFisher Scientific. FlexiTube siRNA GeneSolution and FlexiPlate siRNA were purchased from Qiagen. Complete protease inhibitor Mini tablet was purchased from Roche Diagnostics.

2.2 Methods

2.2.1 Cartilage Sample Collection

Human articular cartilage samples were obtained from patients with osteoarthritis undergoing total hip and total knee replacement surgery at the Freeman Hospital, Newcastle Upon Tyne. Appropriate informed consent and ethical approval was obtained prior to surgery. Samples were stored at 4° C in Hank's Balanced Salt Solution (HBSS) (Lonza), supplemented with 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin and were assessed for tissue viability within 24 hours post-surgery.

2.2.2 Chondrocyte Extraction and Culture

Human cartilage samples from patients undergoing knee and hip replacement surgery were used as a source of chondrocytes for subsequent *in vivo* and *in vitro* experiments. Sequential enzymatic digestion of ECM using hyaluronidase (for hyaluronan), trypsin (for aggrecan) and collagenase (for collagen) released articular chondrocytes allowing them to be cultured in monolayer.

Reagents:

- Phosphate buffered saline (PBS) containing 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin
- DMEM culture medium containing 10% (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin
- Hyaluronidase (1 mg/mL in PBS, 5 ml/g cartilage)
- Trypsin (2.5 mg/mL in PBS, 5 ml/g cartilage)
- Collagenase (2.5 mg/mL in DMEM containing 10% FBS, 3 ml/g cartilage)

Macroscopically normal cartilage tissue was removed from the sub-chondral bone and washed with PBS containing antibiotics. Cartilage was then cut into small pieces with a

scalpel thereby allowing a greater surface area for complete enzymatic digestion and placed in a 50 ml Falcon. The weighed cartilage was then washed three times with excess PBS. Cartilage was then incubated with hyaluronidase at 37 °C for 15 minutes with rotation. The hyaluronidase was then removed and cartilage was washed, three times with PBS. Cartilage was then incubated with trypsin at 37 °C for 30 minutes with rotation. The trypsin was removed and the cartilage was washed three times with excess DMEM+FBS. Cartilage was then incubated with collagenase at 35.5 °C overnight. The next morning the product of the enzymatic digestion was passed through a 100 µm Cell Strainer (BD Falcon) and centrifuged at 400 g for 5 minutes. The collagenase-containing DMEM was then aspirated from the chondrocyte pellet which was then resuspended in an excess of PBS to remove any residual collagenase and centrifuged as before. The PBS was then aspirated and the pelleted chondrocytes resuspended in DMEM+FBS.

2.2.3 Cell Culture

Following extraction from ECM, chondrocytes were required to be cultured in growth medium in order to allow the cells to adhere to the tissue culture surface they were placed on. Primary cells where then cultured until in growth phase and approximately 70-80 % confluent. The chondrocytic cell line, SW1353, was mainly used for ChIP optimisation.

Growth media:

- HAC: DMEM culture medium containing 10% (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin

- SW1353: DMEM-F12 culture medium containing 10% (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin

Method: Isolated human articular chondrocytes and SW1353 cell lines were seeded at a density of 40,000 cells/cm² in 96-well plates, 6-well plates, 10cm cell culture dishes, T75 cm² or T25 cm² culture flasks. Cells were then cultured until 80-90% confluent at 37°C in 5% (v/v) CO_2/humidified air. Prior to cytokine stimulation, culture medium was removed and cells washed of any remaining serum with PBS. Cells were then cultured overnight in serum-free DMEM.

2.2.4 Real-time PCR

2.2.4.1 Taqman® Probe-Based Real-Time RT-PCR

This method of measuring mRNA expression of cells in culture relies on a gene specific fluorescent probe that lends this technique a greater degree of specificity and proportionality. The fluorescent probe binds to the region between the gene specific primers. The probe consists of a fluorophore at the 5' end and a quencher molecule at the 3' end. As the Taq polymerase extends the primer, the 5' to 3' exonuclease activity of the enzyme cleaves the 5' fluorophore. This spatially dissociates the fluorophore from the quencher allowing fluorescence. Fluorescence is therefore directly proportional to the amount of product produced in the PCR reaction, thereby allowing quantification without interference from non-specific products or primer-dimer. To normalise for different amounts of total RNA present in each sample, 18S ribosomal (r)RNA was used as an endogenous control. Oligonucleotide primers and probes were designed using Primer Express 1.0 (Applied Biosystems) and probes from the Universal Probe Library (UPL) purchased from Roche. See Table 3 and Table 5 for details on gene-specific primers and UPL probes. All reactions were performed using the ABI 7900HT Fast Real-Time PCR system.

Reagents:

- TaqMan Fast Universal PCR Master Mix (2x)

Method: cDNA (4 µl/well) was placed into a Fast Optical 96-well reaction plate. To this, 5 µl of TaqMan Fast Universal PCR Master Mix (2x) was added along with 0.2 µl of forward and reverse primer (at a final concentration of 300 nM each) and 0.1µl of probe (at a final concentration of 150 nM) making a 10 µl reaction volume. This was then subject to an initial denaturation stage (95 °C for 20 seconds) followed by 40 cycles of 95 °C for 1 seconds and 60 °C for 20 seconds.

2.2.4.2 SYBR Green Real-Time PCR

In this PCR method SYBR green II dye intercalates into all double-stranded DNA. In this state dye has increased fluorescence compared to the unbound dye. As the amplification reaction occurs the dye is incorporated into all new double-stranded DNA. As such the level of fluorescence is directly proportional to amount of double-

stranded DNA present, which in an efficient and stringent reaction should be almost all amplimer, see Table 4 for details on gene-specific primers. All reactions were performed using the ABI 7900HT Fast Real-Time PCR system.

Reagents:

- SYBR Green PCR Master Mix (2x)

Method: cDNA or gDNA, (1µl/well), depending on the upstream technique used, was placed into a Fast Optical 96-well reaction plate. To this, 5 µl of SYBR Green PCR Master Mix (2x) was added along with 0.1 µl of forward and reverse primer (at a final concentration of 10 nM) and 3 µl of RNase- and DNase-free water making a 10 µl reaction volume. This was then subject to an initial denaturation stage (95 °C for 30 seconds) followed by 40 cycles of 95 °C for 5 sec and 60 °C for 30 seconds. All SYBR Green Real-Time PCR reaction contained an end dissociation stage (95 °C for 15 seconds, 60 °C for 15 seconds and 95 °C for 15 seconds) to assess amplification product for primer-dimer or secondary structures, as shown in Figure 8.
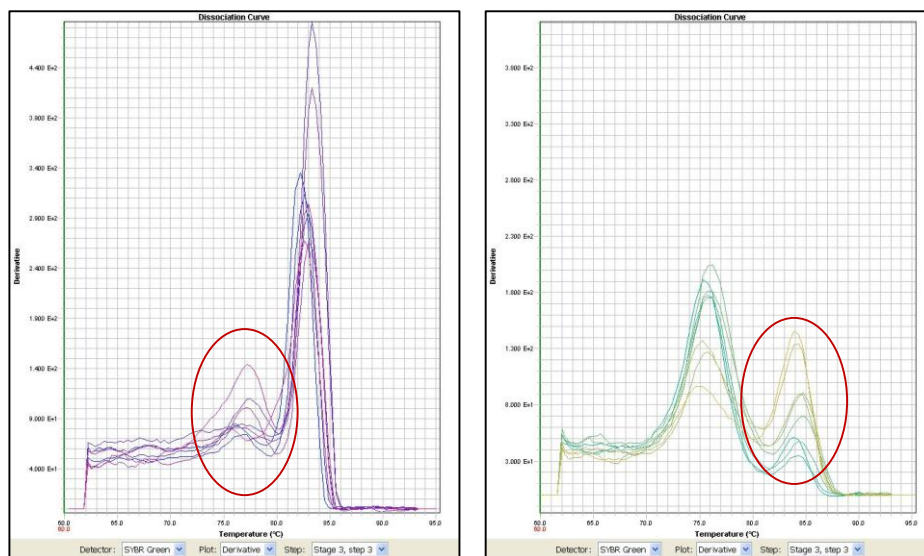
Figure 8: Screen capture image of dissociation stage following SYBR green real-time PCR. Examples of primer-dimer (left) and secondary structures (right) highlighted in red.

Gene	Sequence (5'-3')	Probe
18s	For: CGAATGGCTCATTAAATCAGTTATGG Rev: TATTAGCTCTAGAATTACCACAGTTA TCC	FAM- CAGAGAGTACAACTTACATCGTGTTGC GGCTC-TAMRA
MMP-1	For: AAGATGAAAGGTGGACCAACAATT Rev: CCAAGAGAATGGCCGAGTTC	FAM- CAGAGAGTACAACTTACATCGTGTTGC GGCTC-TAMRA
MMP-13	For: AAATTATGGAGGAGATGCCCATT Rev: TCCTTGGAGTGGTCAAGACCTAA	FAM- CTACAACTTGTTTCTTGTTGCTGCGCAT GA-TAMRA

Table 3: TaqMan Real-time PCR primer and probe sequences.

Gene	Region	Sequence (Distance from TSS)
MMP-1	Proximal promoter/transcription start site	For: TCAGTACAGGTGCCGAACAG (-431) Rev: CAAGATGTGTGCGAAGGAGA (-207)
	3' untranslated region (UTR)	For: ACTGAATGGGCAAAAACTGG (+8042) Rev: TGCAAACAGGGACAATTTGA (+8139)
MMP-13	Proximal promoter/transcription start site	For: GAAAAAGTCGCCACGTAAGC (-116) Rev: CGACAATGAGTCCAGCTCAA (+62)
	3' untranslated region (UTR)	For: TCGGCACAAAATACAGGTCA (+15750) Rev: GCCTCCCCTTTTTAGACCAC (+15932)

Table 4: SYBR Real-time PCR primer sequences.

Gene	Sequence (5'-3')	Universal Probe Library Probe number
ATF3	For: TTTGCCATCCAGAACAAGC	53
	Rev: CATCTTCTTCAGGGGCTACCT	
AXUD1	For: CCTGCCTGACCGTGACTT	57
	Rev: AGCCCGCTTCAGGATAGAC	
BMP2	For: GACTGCGGTCTCCTAAAGGTC	49
	Rev: GGAAGCAGCAACGCTAGAAG	
BTG2	For: GCGAGCAGAGGCTTAAGGT	1
	Rev: GGGAAACCAAGTGGTGTTTGTA	
EGR1	For: AGCCCTACGAGCACCTGAC	22
	Rev: GGTTTGGCTGGGGTAACTG	
EGR2	For: TGGTTTCTAGGTGCAGAGACG	3
	Rev: TGGTTTCTAGGTGCAGAGAGACG	
EGR3	For: CAATCTGTCACCCCGAGGAGA	74
	Rev: CAGACCGATGTCCATTACATTC	
FOSB	For: TCTGTCTTCGGTGGACTCCT	20
	Rev: GTTGCACAAGCCACTGGA	
FOSL1	For: AACCGGAGGAAGGAACTGAC	4
	Rev: CTGCAGCCCAGATTTCTCAT	
HIF1A	For: CAGCTATTTGCGTGTGAGGA	89
	Rev: TTCATCTGTGCTTTCATGTCATC	
IER2	For: TGTCAGTGGAAAGGCATAAGG	54
	Rev: AGATTGGCCCAGTTTCACC	
IER3	For: GGGTCGTAAGTTTAGGAGGTGA	88
	Rev: CGCCGAAGTCTCACACAGTA	
IER5	For: GTCGGGGACGTTTTTCAG	1
	Rev: GCTCAGAAGGCCACGATG	
JUN	For: CCAAAGGATAGTGCGATGTTT	19
	Rev: CTGTCCCTCTCCACTGCAAC	
JUNB	For: CACAAGATGAACCACGTGACA	10
	Rev: GGAGTAGCTGCTGAGGTTGG	
NFATC1	For: CCAAGGTCATTTTCGTGGAG	45
	Rev: GGTCAGTTTTCGCTTCCATC	

Table 5: TaqMan Real-time PCR primer sequences and corresponding UPL probe numbers.

2.2.5 RNA extraction and reverse transcription

Prior to PCR, mRNA/RNA was extracted from cultured cells and converted to cDNA. This allows Taq Polymerase to synthesize amplicons of a specific region allowing quantification. RNA was extracted from HAC using SideStep^{TM} Kit for cells cultured in 96-well plates and RNeasy® Mini Kit (Qiagen) for cells cultured in 6-well plates and using Cells-to-cDNA for SW1353.

Reagents:

- SideStep Lysis and Stabilisation buffer

- RNeasy® Mini Kit

- RNaseOUT™ Recombinant Ribonuclease Inhibitor

- SuperScript® III Reverse Transcriptase

- MMLV Reverse Transcriptase

2.2.5.1 RNA extracted using SideStep Kit

Method: Following appropriate culture, serum-starvation and cytokine stimulation, HAC cultured in a 96-well plate were placed on ice. Cytokine-containing DMEM was aspirated from all the wells. Each well was then washed twice with an excess of ice-cold PBS aspirating PBS after each wash. SideStep Lysis Buffer (20 µl/well) was added, the plate sealed with polyolefin film plate sealers and then agitated on a plate vortex (low setting) for 2 minutes to ensure complete cell lysis. At this point the cell lysate can be stored at -80 °C until later use. The lysate was then diluted 4-fold with RNase and DNase-free water (Sigma) and 4 µl of this dilution transferred to a 96-well PCR plate. To each well of the 96-well plate 1 µl of random hexamers (Pd(N)$_6$) (0.2 µg/ml), 3 µl deoxyribonucleotide (dNTP)s (2.5 mM) and 4 µl of DNase- and RNase-free water was added and the plate heated to 70°C for 5 minutes in order to denature the DNA and allow binding of the hexamers. The plate was immediately placed on ice in order to prevent enzyme denaturation, where 4 µl of 5x First Strand Synthesis Buffer, 2 µl dithiothreitol (DTT) (0.1M), 0.125 µl RNaseOUT (40 U/µl), 0.5 µl MMLV (200U/µl) and 1.375 µl of DNase- and RNase-free water was added to each well. The plate was incubated at 37 °C for 50 minutes, followed by 70 °C for 15 minutes. Human cDNA was

diluted 1:20 for house-keeping gene quantification. All cDNA was stored at -20 °C until required.

2.2.5.2 RNA extracted using Cells-to-cDNA II kit

Method: As above until addition of RNA extraction buffer. Ice-cold Cells-to-cDNA buffer was added (30 µl/well) and cells lysed by tip transferring the full volume to a PCR plate. The plate was then sealed with a polyolefin film plate sealer and lysates heated to 75°C for 10 minutes. Plates were then placed on ice and add 1 unit of RQ1 RNase-free DNase added to each well. The PCR plate was then incubated to 37 °C for 15 minutes and then DNase deactivated the with a 5 minute incubation at 75 °C. At this point the cell lysate could be stored at -80 °C until later use. Cell lysate (8 µl) was then transferred to new PCR plate and 1 µl of random hexamers (Pd(N)$_6$) (0.2 µg/ml) and 3 µl dNTPs (2.5 mM) were added to each well, the plate sealed and the incubated at 70 °C for 5 minutes. The plate was then immediately placed on ice and 4 µl of 5x First Strand Synthesis Buffer, 2 µl DTT (0.1 M), 0.125 µl RNaseOUT (40 U/µl), 0.5 µl MMLV (200U/µl) and 1.375 µl of DNase- and RNase-free water was added to each well. The plate was then incubated at 37 °C for 50 minutes, followed by 70 °C for 15 minutes.

2.2.5.3 RNA extracted using RNeasy Mini Kit

Method: Cytokine-containing DMEM was aspirated from all the wells of a 6-well plate following appropriate culture, serum-starvation and cytokine stimulation of cultured HAC. Each well was then washed twice with an excess of PBS, aspirating PBS after each wash. RLT Buffer (350 µl)+1% β-ME was added to each well. The HAC were then lifted from the tissue culture plastic with a cell scraper and the buffer containing the cells transferred to a 1.5 ml microcentrifuge (M/C) tube. These were then stored at -80 °C. All the frozen samples were allowed to warm up to room temperature. The entire sample volume was placed in a QIAshredder spin column and centrifuged at full speed (approximately 12,000 g) for 2 minutes. This homogenises the sample preventing clogging of the spin columns at later stages. Ethanol (350 µl) at 70 % was added to each homogenised sample and mixed with a pipette. The entire volume was transferred to an RNeasy spin column within a 2 ml collection tube and centrifuge at ≥8000 g for 15 seconds. Flow through was discarded and 700 µl of RW1 Buffer was

added to the same spin column and centrifuge at ≥8000 *g* for 15 seconds. Flow through was discarded and 500 µl of RPE Buffer was added to the same column and centrifuge at ≥8000 *g* for 2 minutes. The spin column was then placed in a new collection tube and used collection tube and flow through within it discarded. Spin column was then centrifuged at full speed (approximately ≥8000 *g*) for 1 minute to eliminate any possible carryover of RPE Buffer into final buffer. The spin column was then placed in a new collection tube, 40 µl of RNase-free water added to the column and centrifuged at ≥8000 *g* for 1 minute to elute the RNA.

RNA was quantified by spectrophotometry (Nanodrop ND 1000 spectrophotometer). RNA (200 ng) from each sample was then placed in a nuclease-free 0.2 ml PCR tube. To this 1 µl of random hexamers (Pd(N)$_6$) (0.2 µg/ml) and 1 µl dNTPs (10 mM) were added as well as enough DNase- and RNase-free water to make a final volume of 13 µl. The tubes were then heated to 65 °C for 5 minutes and then immediately placed on ice and 4 µl of 5x First Strand Synthesis Buffer, 2 µl DTT (0.1 M), 0.25 µl RNaseOUT (40 U/µl), 0.25 µl SuperScript III (200 U/µl) and 0.5 µl of DNase- and RNase-free water was added to each well. The tubes were then incubated at 25 °C for 5 minutes, then at 50 °C for 1 hour followed by 70 °C for 15 minutes. cDNA was then diluted 2.5-fold. A further 20-fold dilution was carried out for house-keeping gene quantification. All cDNA was stored at -20 °C until required.

2.2.6 Chromatin Immunoprecipitation (ChIP)

This is a very powerful technique which provides a "snap-shot" of protein-DNA interactions occurring within cells at any given point following stimulation. This allows us to assess transcription factor binding to specific regions in the genome and offers insight into the transcriptional regulation of genes of interest. Briefly, the protocol consists of 5 stages:

- **Cross-linkage:** Formaldehyde is added to the medium of cytokine-stimulated HAC leading to the cross-linkage of DNA and proteins.

- **Cell Lysis and Chromatin Shearing:** These cells are then lysed in a SDS lysis buffer and subject to carefully optimised sonication which, shears the chromatin into approximately 500bp fragments. This is done in order to

increase the specificity of the procedure allowing us to determine the presence of a protein within a short region of the genome, e.g. the gene promoter.

- **Immunoprecipitation:** Antibodies for specific proteins are then used to immune-select these proteins which when pulled-down with agarose beads co-precipitate with genome fragments that were bound at the time of cross-linkage.

- **DNA purification:** Co-precipitated DNA is then eluted from beads, washed, detached from proteins and purified using spin columns.

- **Detection:** The immune-selected co-precipitated DNA is then used in a real-time PCR reaction with a primer set specific for a region of the genome you want to assess for transcription factor association.

Reagents:

- EZ-ChIP Kit: Chromatin Immunoprecipitation Kit (Millipore)

- Formaldehyde solution-for molecular biology, 36.5-38 % in H$_2$O (Sigma)

Method: HAC were cultured until 80-90 % confluent in 10 cm culture dishes. The cells were then serum-starved overnight and then stimulated the following morning. After appropriate stimulation duration, formaldehyde solution 37 % (Sigma) was added to medium to a 1 % (w/v) final percentage. The plates were then incubated at room temperature for 5 minutes with frequent agitation. To quench the formaldehyde and stop cross-linkage, ice-cold glycine was added to the medium-formaldehyde solution with a final concentration of 0.125 M. Glycine has a free amine group and so competes for the formaldehyde-dependent amino/imino group cross linkage. The plates were then incubated at room temperature for 5 minutes with frequent agitation. The quenched solution was then removed and the cells washed twice with ice-cold PBS. PBS (1 ml) was supplemented with protease inhibitors (PI) and added to each 10 cm culture dish. The cells were then removed from the surface of the plate using a cell lifter (CoStar) and PBS containing cells transferred to 1.5 ml M/C tube. The cells were then centrifuged for 5 minutes at 700 *g*. Pellets were then snap frozen and stored at -80 °C for further use.

Cell pellets were thawed on ice and then lysed with 200 µl of SDS Lysis Buffer supplemented with PI on ice for 15 minutes. The cell lysates were then placed in a Bioruptor (Diagenode). This high energy sonicating water bath yields reliable and uniform chromatin shearing whilst keeping sample temperature low. The samples were subject to three rounds of 5x 30 second pulses with each pulse followed by a 30 second rest. In between rounds of sonication, a portion of water was removed and replaced with ice in order to reduce the temperature in the water bath in order to prevent protein denaturation. The concentration of chromatin was quantified by spectrophotometry (Nanodrop ND 1000 spectrophotometer) and 100 µg added to silconised 1.5 ml M/C tubes. The cell lysate was then diluted to 1 ml using ChIP dilution buffer supplemented with PI and pre-cleared with the addition of 60 µl of protein A/G agarose beads (Santa Cruz) incubated for 1 hour with rotation at 4 °C. Samples were then centrifuged at 4000 g for 1 minute. The supernatant was then removed and transferred to a new silconised 1.5 ml M/C tube. Immunoprecipitating antibody (5 µg) was then added to each sample and incubated overnight with rotation at 4 °C.

The following morning 60 µl of protein A/G agarose beads was added to the sample and incubated for 1 hour with rotation at 4 °C in order to capture antibody/protein/chromatin complex. Samples were centrifuged at 4000 g for 1 minute and supernatant removed. The antibody/protein/chromatin complex was than washed by resuspending the pellet with cold buffers in the order listed below and incubate samples for 4 minutes at 4 °C with agitation. Samples were than centrifuged at 4000 g for 1 minute and the supernatant removed.

a) Low Salt Immune Complex Wash buffer, 1 wash

b) High Salt Immune Complex Wash buffer, 1 wash

c) LiCl Salt Immune Complex Wash buffer, 1 wash

d) Tris-EDTA Buffer, 2 washes

Following the removal of the final supernatant, 100 µl of Elution Buffer (5 µl 20 % SDS, 10 µl 1 M NaHCO$_3$ and 85 µl of sterile water) was added to each pellet, gently resuspended with pipette and then incubated at room temperature for 15 minutes. Agarose beads were then pelleted by centrifuging at 4000 g for 1 minute, collecting

the supernatant into new silconised 1.5 ml M/C tube. Another 100 µl of Elution Buffer was added to the agarose pellets, gently mixed and incubate as before, combining the eluates.

Sodium chloride (NaCl) (8 µl at 5 M) was added to the samples and incubated at 65 °C with agitation for 4-5 hours to reverse the cross-links. RNase A (1 µl/1 U) was added and incubated at 37 °C with agitation for 30 minutes. This was followed by the addition of 4 µl 0.5 M EDTA, 8 µl 1 M Tris-hydrochloric acid (HCl) and 1 µl of Proteinase K (1 U) to each sample and incubation at 45 °C for 1-2 hours with agitation.

Bind Reagent A (5 volumes) was added to each sample and gently mixed with a pipette. Half the volume was then transferred to a spin filter, placed in a collection tube and centrifuged at 13,000 g for 30 seconds. Liquid in the collection tube was then discarded, the second half of the sample added to the same spin filter and centrifuged as before. Liquid in the collection tube was then discarded and 500 µl of Wash Reagent B added to the spin filter and centrifuged as before. Liquid in the collection tube was then discarded. Spin filter was then placed back into the collection tube and centrifuged as before. The spin filter was then placed in a new collection tube and the used collection tube, along with any liquid was discarded. Elution Buffer C (50 µl) was added to the spin filter in the new collection tube and centrifuged as before. The spin filter was then removed and discarded. Purified gDNA was stored at -20 °C.

Immunoselected, pull-down gDNA was then used in real-time PCR to assess fold enrichment of chosen proteins to specific regions in the genome. This was performed as described in the SYBR Green Real-Time PCR methodology section above.

2.2.6.1 ChIP primer design

A crucial element of a reliable ChIP protocol is stringent primer design. When assessing the fold enrichment of a protein to a specific region of the genome between different durations of cytokine stimulation with real-time PCR you have to be confident that your primers for that specific region reflect a doubling of template in the Ct read by the PCR machine. The gold standard of primer design is that a primer set leads to an increase of a single Ct following a doubling of the template DNA in the PCR reaction. As such any analysis of the data then reflects actual fold changes in enrichment and small discrepancies are not amplified during PCR.

Method: A region of approximately 4000 bp upstream and 1000 bp downstream of the transcription start site of a gene of interest was placed into a transcription factor binding site prediction software (PROMO 3). Based on predicted sites a region of 1000 bp which sits over the proximal promoter and transcription start site was then placed into primer design software (Primer 3.0). A number of primer sets were then chosen based on their comparable melting temperature and the absence of predicted secondary structure, self-annealing or primer dimer. These primer sets were then tested with real-time PCR (as described in TaqMan Real-Time PCR methodology section above) at various concentrations using doubling dilutions of pooled-gDNA. The PCR products were checked for the absence of primer-dimer or secondary products by electrophoresing them on a 2 % agarose gel (as described in agarose gel electrophoresis methodology section, below).

2.2.7 Semi-Quantitative PCR

This method allows us to compare immune-selected gDNA from ChIP and their controls in order to assess whether or not successful ChIP has occurred. This is achieved by amplifying the gDNA pulled down in all samples equally thereby boosting the signal so it can be visualised by electrophoresing the PCR product on an agarose gel (see below).

Reagents:

- Titanium Taq Polymerase (Clontech)

Method: In a single well of a 96-well PCR plate, PCR reaction was carried out in 15 µl containing 0.5 µl of gDNA, 0.075 µl of each primer at a concentration of 10 µM, 1.5 µl of 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 0.8 µl, 1.2 µl or 1.8 µl of 25 mM $MgCl_2$ to a concentration of 1 mM, 2 mM or 3 mM respectively depending on the required $MgCl_2$ concentration, 0.375 µl dNTPs (10nM), 0.08 µl of Titanium Taq Polymerase (1 U/µl) and made up to 15 µl with DNase- and RNase-free water. Thermocycling conditions were an initial denaturation at 95 °C for 14 minutes, followed by 35 cycles of 95 °C for 30 seconds annealing at temperatures between 55 °C and 65 °C depending on the specific conditions for the primers used, for 30 seconds, 72 °C for 30 seconds and a final extension at 72 °C for 5 minutes.

2.2.7.1 *Agarose Gel Electrophoresis*

This technique separates DNA fragments based on their size and was employed to visualise amplified ChIP-gDNA in order to compare abundance. It was also used to check for primer-dimer and secondary products following real-time PCR ChIP primer optimisation and also to confirm appropriate chromatin shearing following sonication of HAC.

Reagents:

- 4x Loading Buffer: 0.125 M Tris (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol and 0.001 % (w/v) bromophenol blue

- Tris-acetate-ethylenediamine tetraacetic acid (TAE) buffer: 0.04 M Tris (pH 8), 5.7 % (v/v) glacial acetic acid and 0.001 M EDTA

Method: Agarose 3 % (w/v) gels were prepared by dissolving agarose TAE buffer by boiling. Ethidium Bromide (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide) was then added to the cooled agarose-TAE solution at a final concentration of 0.2 µg/ml. This was poured into a gel form and allowed to set. Once set the gel was placed into an electrophoresis tank (BioRad Wide mini-gel Sub cell GT) and submerged in TAE buffer. Loading buffer was then added to samples to be electrophoresed and then samples, as well as DNA ladder, were loaded into the wells within the gel. A 100 V electric field was then applied across the gel attracting the negatively charged DNA fragments to the positive terminus. The DNA fragments were then visualised using ChemiGenius II BioImager (Syngene, Cambridge, UK).

2.2.8 Subcellular Protein Fractionation

This method allows the stepwise separation of cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extracts from HAC. The first buffer allows the permeabilisation of cell membrane, releasing soluble cytoplasmic contents. The second buffer selectively dissolves plasma, endoplasmic reticulum/golgi and mitochondrial membranes without de-stabilising the nuclear membrane. The third buffer releases nuclear content. The same buffer is then supplemented with micrococcal nuclease which cleaves chromatin into 146 bp fragments allowing the

extraction of chromatin-bound proteins. A final buffer was added to the remaining insoluble pellet isolating cytoskeletal proteins. These extracts were then resolved by SDS-PAGE and analysed by western blotting to assess the abundance and distribution within the cellular compartments of specific proteins following various cytokine stimulations and durations.

Reagents:

- Subcellular Protein Fractionation Kit

Method: HAC were cultured until 80-90 % confluent in 6-well plates. The cells were then subject to serum-starvation overnight and stimulated the following morning. After appropriate stimulation duration cytokine-containing medium was then aspirated off the wells and washed twice with ice-cold PBS. A cell scraper was then used to remove the cells from the tissue culture surface into 150 µl of ice-cold PBS. This was then transferred to a 1.5 ml M/C tube and centrifuged at 500 g for 3 minutes. The supernatant was then removed and discarded leaving a semi-dry pellet. A rough pellet volume was then estimated using known volume water comparisons. Pellet volume then governed the volumes of the buffers used throughout the procedure. For example, for every 10 µl packed cell volume, a 100 µl of cytoplasmic extraction buffer (CEB) and membrane extraction buffer (MEB) and 50 µl of nuclear extraction buffer (NEB) and pellet extraction buffer (PEB) are used in each stage.

Ice-cold CEB containing protease inhibitors was then added to the pellet and incubated at 4 °C for 10 minutes with gentle agitation. Samples were then centrifuged at 500 g for 5 minutes and supernatant (Cytoplasmic extract) transferred to a clean, pre-chilled 1.5 ml M/C tube. Ice-cold MEB containing protease inhibitors was then added to the pellet and vortexed for 5 seconds on the highest setting followed by incubation at 4 °C for 10 minutes with gentle agitation. Samples were then centrifuged at 3000 g for 5 minutes and supernatant (Membrane extract) transferred to a clean, pre-chilled 1.5 ml M/C tubes. Ice-cold NEB containing protease inhibitors was then added to the pellet and vortexed for 15 seconds on the highest setting followed by incubation at 4 °C for 30 minutes with gentle agitation. Samples were then centrifuged at 5000 g for 5 minutes. During this time, chromatin-bound extraction buffer was prepared by combining 5 µl of 100 mM $CaCl_2$ and 3 µl of micrococcal nuclease (300 units) per 100 µl

of room temperature NEB. The supernatant (soluble nuclear extract) was then transferred to a clean, pre-chilled 1.5 ml M/C tube. Room temperature NEB containing protease inhibitors, CaCl$_2$ and micrococcal nuclease was added to the pellet and vortexed on highest setting for 15 seconds. Samples were then incubated in a water bath at a temperature of 37 °C for 5 minutes. The samples were then vortexed on the highest setting for 15 seconds and then centrifuge at 16,000 g for 5 minutes. The supernatants (chromatin-bound nuclear extract) were then transferred to clean, pre-chilled 1.5 ml M/C tubes. Room temperature PEB containing protease inhibitors was added to the pellet, vortexed on the highest setting for 15 seconds and then incubate at room temperature for 10 minutes. Samples were then centrifuged at 16,000 g for 5 minutes. The supernatants (cytoskeletal extract) was then transferred to clean 1.5 ml M/C tubes. All subcellular extracts were stored at -80 °C.

2.2.9 Nuclear and Cytoplasmic Protein Extraction

This method allows the stepwise separation of cytoplasmic and membrane protein extracts from cultured HAC. The first two buffers led to the disruption of the cell membrane and the release of cytoplasmic contents, yet leaving nuclear membranes intact. The third buffer disrupts the nuclear membrane allowing the isolation of nuclear protein. Protein extracts were then used in SDS-PAGE and western blotting to assess changes in abundance following different cytokine stimulation duration.

Reagents:

- NE-PER Nuclear and Cytoplasmic Extraction Reagents

Method: HAC were cultured until 80-90 % confluent in 6-well plates. The cells were then subject to serum-starvation overnight and then stimulated the following morning. After appropriate stimulation duration, cytokine-containing medium was then aspirated off the wells and washed twice with ice-cold PBS. A cell scraper was then used to remove the cells from the tissue culture surface into 150 µl of ice-cold PBS. This was then transferred to a 1.5 M/C tube and centrifuged at 500 g for 3 minutes. The supernatant was the removed and discarded leaving a semi-dry pellet. A rough pellet volume was then established using known volume water comparisons. Pellet volume then governed the volumes of the buffers used throughout the procedure. For example, for every 10 µl packed cell volume, 100 µl of Cytoplasmic extraction reagent

(CER) I, 5.5 µl of CER II and 50 µl of Nuclear extraction reagent (NER) was used in later steps.

Ice-cold CER I containing protease inhibitors was then added to cell pellet and vortexed on the highest setting for 15 seconds and incubated on ice for 10 minutes. Ice-cold CER II was then added and vortexed for 5 seconds on the highest setting. Samples were incubated on ice for 1 minute, then vortexed on highest setting for 5 seconds and centrifuged for 5 minutes at 16,000 g. The supernatant (cytoplasmic extract) was then transferred to a clean, pre-chilled M/C tube. The pellet was then resuspended in ice-cold NER and vortexed on the highest setting for 15 seconds. Samples were incubated on ice for 40 minutes, vortexing every 10 minutes for 15 seconds. Samples were centrifuged for 10 minutes at 16,000 g and supernatant (nuclear extract) transferred to a clean, pre-chilled M/C tube.

2.2.10 Preparation of total cell lysates

For SDS-PAGE and western blotting protocols, cells within the sample must be fully lysed in order release the proteins of interest. In so doing the proteins are solubilised allowing them to be separated based on the size of each individual protein. In order to do this a lysis buffer which allows maximum cell lysis, supplemented with reagents that inhibit proteinases and phosphatases, is used to yield maximal protein levels whilst maintaining their stability and phosphorylation state.

Reagents:

- Whole Cell Lysis buffer: 50 mM Tris, pH 7.4, 10 % (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 5 mM NaF, 10 mM β glycerol phosphate, 5 mM $Na_4P_2O_7$, 1% (v/v) Triton X-100, 1 µM microcystin-LF and 1 Complete protease inhibitor Mini tablet/10 ml of buffer

Method: HAC were cultured until 80-90 % confluent in 6-well plates. The cells were subject to serum-starvation overnight and then stimulated the following morning. After appropriate stimulation duration cytokine-containing medium was then aspirated off the wells and washed twice with ice-cold PBS. Ice-cold Whole Cell Lysis buffer (150 µl/well) supplemented with 0.1 % (v/v) β-ME was then added. A cell scraper was then used to scrape the cells from the surface of the wells into the buffer

which was then transferred to a 1.5 ml M/C tube. Samples were vortexed for 10 seconds and incubated on ice for 20 minutes, followed by centrifugation at 10,000 g at 4 °C for 10 minutes. The supernatant was then removed, snap frozen on dry ice and stored immediately at -80 °C.

2.2.11 Non-Destructive Cytotoxicity Assay

In the assessment of *de novo* protein synthesis following pro-inflammatory cytokine stimulation, Emetine, a protein synthesis inhibitor was used. This allowed me to reveal whether or not new protein synthesis was required for transcription of *MMPs*. Before these studies could be carried out, it was important to assess the cytotoxicity of this compound on HAC. This was because a lack of protein production or gene transcription may be accounted for by cell death rather than the inhibition of protein synthesis. As such a bioluminescent assay was used to evaluate cell death. This works on the principle that a damaged cell will lose plasma membrane integrity. As such a phosphotransferase enzyme, adenylate kinase (AK) is released from these damaged cells into the medium. AK converts ADP to ATP. This ATP is then used to catalyse a bioluminescent reaction which is measured with a luminometer. Emitted light intensity is linearly related to AK concentration and percentage cell death can then be calculated from controls.

Reagents:

- ToxiLight BioAssay Kit: Non-Destructive Cytotoxicity Assay

Method: Prior the start of the assay 10 ml of Assay Buffer was added to the AK Detection Reagent and allowed to equilibrate for 15 minutes at room temperature with gentle agitation. HAC, cultured in 96-well plate, were removed from the incubator and 100 µl of the medium from the cells was transferred to a new 96-well plate. The remaining 100 µl of medium was then subject to repeated freeze-thaw cycles by freezing in the -80 °C freezer. Freeze-thawing leads to a loss in cell membrane integrity and so was used as a control for complete cell death. The 100 µl of normal culture medium and freeze-thawed medium was allowed to return to room temperature. A 20 µl volume was then taken from each sample and transferred to a luminescence compatible 96 well-plate. To this sample, 100 µl of reconstituted AK detection reagent was added and then incubated for 5 minutes at room temperature. The plate was then

placed in a luminometer and immediate 1 second integrated readings of appropriate wells were taken.

2.2.12 Protein Quantification

In order to more accurately assess protein quantity between samples following visualisation of western blot, equal amounts of protein from each sample must be added to the wells in SDS-PAGE. This means variation in protein loading can be discounted when assessing differences in protein quantity. Quantification is based upon maximal absorption changes of Coomassie dye upon protein binding. As the dye binds to proteins the dye is stabilised resulting in absorption change from 465 nm (un-bound dye) to 595 nm (bound dye) with a concomitant colour change from brown to blue. Changes in absorbance are proportional to the amount of bound dye and hence the amount of protein. Quantity is estimated by comparing unknown sample to serial dilutions of known protein concentration.

Reagents:

- Bradford ULTRA

- BSA

Method: In a flat bottomed 96-well plate a serial dilution of BSA (0.4 µg/ml) ranging from 0 µg/ml to 4 µg/ml in 0.4 µg/ml increments was made up to 10 µl with lysis buffer. A 3 µl volume of each unknown sample is then added in triplicate to the plate, which are also made up to 10 µl with lysis buffer. Room temperature Bradford ULTRA (150 µl) was then added to all samples and standards. The flat bottomed plate containing the standards and sample were then placed in a Tecan Sunrise microplate reader and absorbance read at 595 nm.

2.2.13 DNA affinity pull-down assay

An *in vitro* technique that allows the user to assess interaction between proteins isolated from cell lysates and their binding to oligonucleotide containing consensus binding sites found in gDNA. Bioyinylated oligonucleotides and the proteins bound to the consensus binding sites contained within them can then be extracted using streptavidin-coated agarose beads. Subsequent elution of these proteins allows identification of bound proteins using SDS-PAGE and western blotting. This allows the

user to identify factors that have the potential to regulate the genes that these consensus sites precede in the genome.

Reagents:

- Streptavidin-coated agarose beads

- Double stranded biotinylated-oligonucleotides (Sigma, UK): 5'-CGCTTGA*TGACTCA*GCCGGAA-3'; 3'-GCGAACT*ACTGAG*TCGGCCTT-5'

- BSA

- Poly deoxyinosinic-deoxycytidylic (dI-dC) (LiCor, USA)

- Binding Buffer: 12 mM HEPES pH 7.9, 4 mM Tris-HCl, 60 mM KCl, 5% (w/v) glycerol, 0.5 mM EDTA, 1 mM DTT and 1 mini protease inhibitor cocktail tablet/10ml of buffer

- 5 x 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-ME

Oligonucleotide annealing method: Oligonucleotide stock was first prepared by adding RNase and DNase-free water to lyophilised single stranded oligonucleotide making a 100 pmol/μl concentration (volume indicated in data sheet). A volume of 3.5 μl of both the sense and anti-sense oligonucleotide, along with 43 μl of RNase and DNase-free water were placed in a PCR tube and incubated to 100 °C and then left to cool to room temperature overnight. Complete annealing was checked by running annealed oligonucleotide on an agarose 3 % (w/v) gel.

Binding reaction method: For each pull-down reaction: 40 μl of streptavidin-coated agarose beads were blocked in 1 ml of binding buffer, supplemented with BSA (0.5 mg/ml), for 45 minutes at 4 °C with rotation; and 80 μg of nuclear protein extract incubated with 10 μl of the double-stranded biotinylated-oligonucleotides (35 pmol) in 500 μl of binding buffer, supplemented with poly (dI-dC) (2 μg/ml), for 45 minutes at 4 °C with rotation. The agarose beads were then briefly centrifuged to pellet, supernatant discarded and replaced with 500 μl of binding buffer containing protein-

oligonucleotide complex. Samples were then incubated for 2 hours at 4 °C with rotation. Agarose beads were then pelleted by briefly centrifuging samples, supernatant discarded and pellet washed three times with 1 ml of binding buffer for 5 minutes at 4 °C with rotation. Agarose beads were resuspended in 50 µl of final sample buffer and incubated at 100 °C for 5 minutes. Agarose beads were briefly centrifuges to pellet and supernatant removed for SDS-PAGE (as below).

2.2.14 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples containing protein, generated by subcellular protein fractionation, nuclear and cytoplasmic protein extraction or total cell lysis were subjected to SDS-PAGE, separating the proteins within these samples by their size. SDS plays a crucial role in ensuring that proteins are separated by size alone and not retardation through the gel due to other factors. SDS is an anionic detergent added to samples that binds to proteins at a ratio of 1 anion to 2 amino acids. This imparts a negative charge to the proteins which is proportional to unit mass. It also serves to prevent the formation of secondary and tertiary structures and linearize the proteins. β-ME is also added, thereby reducing disulphide bridges ensuring monomeric primary structure proteins. Denaturation of the proteins is also aided by heating of the sample to 100 °C in order to further denature proteins allowing comprehensive binding of SDS.

Once charged and linearized, the samples are placed into a stacking gel. Due to the larger pore size compared to separating gel, movement through the stacking gel is dependent on the electric field applied rather protein size. This sharpens loaded samples into a single band before it enters the separating gel. This is achieved by difference in migration speed of ions within the gel and the electrophoresis buffer. The proteins are trapped within a narrow band between these migrating ions. Once they reach the separating gel, which has a smaller pore size, a higher pH and a higher salt concentration, the voltage gradient is lost allowing the proteins to be separated based on size. Large proteins are retarded by the gel and so move more slowly through the gel whereas smaller proteins move more freely and so move further through the gel.

Reagents:

- 4 x Lower gel buffer (LGB): 1.5 M Tris-base, pH 8.8, 0.4 % (w/v) SDS

- 4 x Upper gel buffer (UGB): 0.5 M Tris-base, pH 6.8, 0.4 % (w/v) SDS

- Stacking gel: 40 % bis/acrylamide diluted to 4.5 % with water and 4 x UGB

- 10 x running buffer: 250 mM Tris-base, 2 M glycine, 10 % (w/v) SDS

Method: Electrophoresis was performed in a Bio-Rad Mini-Protean Tetra system with 1.0 mm spacers and combs. Polyacrylamide-bis-acrylamide was purchased as a 40 % (w/v) (37.5:1 acrylamide:bis) solution, diluted with water and 3 ml of 4 x LGB to the required percentage (Table 5). A spacer plate and short plate were combined within a casting frame, fixed within a casting stand and checked for a clean seal with water. Gel mixture (12 ml) was polymerised by the addition of 20 µl TEMED and 60 µl APS (0.2 % w/v) and immediately poured into gel cassette assembly. The lower gel was overlaid with propan-2-ol to exclude oxygen and allowed to polymerise. Once set, propan-2-ol was washed off the gel and a 4.5 % bis/acrylamide stacking gel laid on top, combs inserted and the stacker allowed to set. Upper gel (5 ml) was set with 10 µl TEMED and 30 µl 0.2 % (w/v) APS. The gel within the two plates was fixed into the electrode assembly which is then placed within the electrophoresis tank and surrounded with 1 x running buffer. Cell lysate samples were prepared as follows: 1 ml of 5 x sample buffer was mixed with 250 µl β-ME. Sample buffer (4 µl) was added to 20 µl of each cell lysate sample and heated to 105 °C for 5 minutes. PageRuler pre-stained protein standards were loaded alongside samples in order to visualise molecular weight on membranes. Proteins were electrophoresed at a constant 80 V in running Buffer for approximately 1 hour 30 minutes, until the dye front had run to the end of the separating gel.

Percentage gel (%)	40 % (w/v) acryl/bis solution (37.5:1) (ml)	dH$_2$O (ml)	4xLGB (ml)
10	3	6	3
7.5	2.25	6.75	3

Table 6: Volumes of reagents that constitute agarose gels at 10 and 7.5 % (w/v).

2.2.15 Western Blotting

Following SDS-PAGE, separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane. This transfer works on the same principle as SDS-PAGE: proteins that have a negative charge, imparted upon them by the binding of SDS, are transferred from the polyacrylamide gel onto to PVDF membrane by an electric current where they are tightly held in a mirror image of the proteins on the gel by electrostatic and hydrophobic interaction. This membrane is considerably more robust compared to the polyacrylamide gels and is therefore easier to handle during subsequent steps in the protocol. Before antibody incubation the PVDF membrane must first be "blocked". The membrane has a high affinity for proteins, and so, to ensure that no proteins bind that could lead to unspecific binding of primary antibody, free sites on the membrane are blocked with skimmed milk protein. The membrane is then subject to antibody incubation firstly with antibody that has affinity for the protein of interest and then an HRP-conjugated antibody that has affinity for the primary antibody isotype. HRP then cleaves a chemi-luminescent substrate, emitting light which is then detected in the vicinity of the protein of interest (Figure 9).
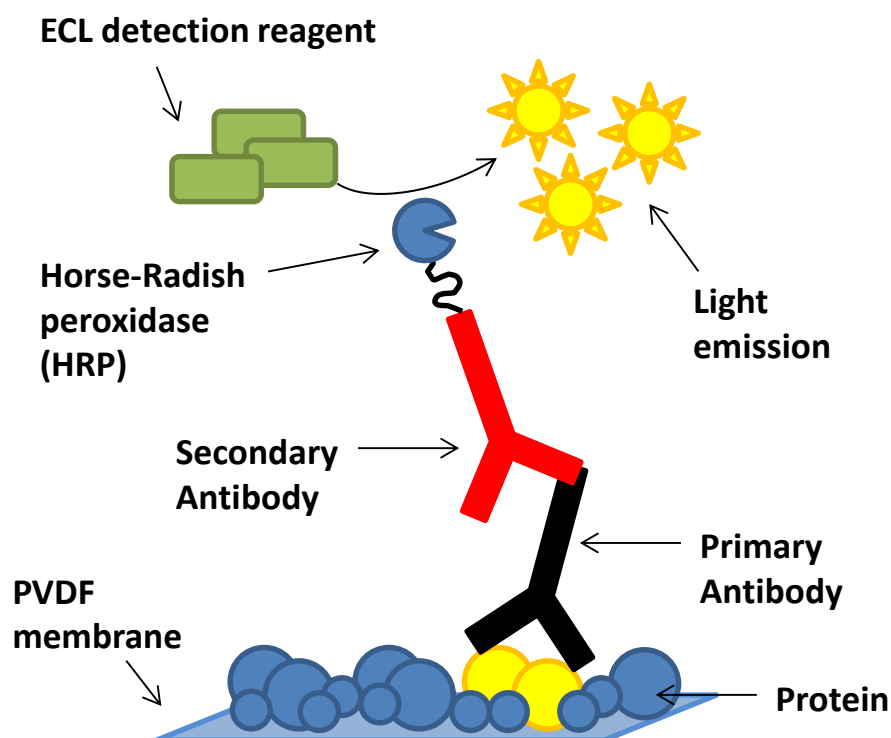
Figure 9: Schematic representation of western blotting. Primary antibody binds epitope on proteins that are hydrophobically bound to PVDF membrane. Secondary antibody, that are conjugated to HRP, bind to Fc region on primary antibody. HRP cleaves ECL detection reagent emitting light. Schematic adapted from [186].

Reagents:

- Transfer Buffer: 39 mM glycine, 48 mM Tris-base, 0.0375 % (w/v) SDS and 20 % (v/v) methanol

- Tris-buffered saline-Tween (TBS-T): 10 mM Tris-HCl pH 7.4, 0.15 M NaCl and 0.2 % (v/v) Tween-20

Method for BioRad Semi-dry Transfer Unit: PVDF membrane and 6 sheets of filter paper were cut to the size of the gel being transferred. The PVDF membrane was activated by placing in methanol for 5 minutes prior to transfer. PVDF membrane was then removed from methanol and along with the gel and filter paper, soaked in transfer buffer. A sandwich of 3 sheets of filter paper, PVDF membrane, gel and remaining filter paper was assembled within the transfer unit plates with the membrane closest to the anode plate and the gel closest to the cathode plate. Any bubbles within the sandwich were removed with a roller prior to sealing within the unit. Proteins were then transferred by running at 80 mA per gel (35 V, 200 W) current

across the sandwich for 1 hour 40 minutes where upon the gel and two sets of filter paper were discarded.

For some experiments, the iBlot gel transfer system (LifeTech) was used according to manufacturer's instructions to simplify and standardise blotting.

Blocking and Immunoblotting Method: Following transfer PVDF membranes were then placed in TBST-T containing 5 % (w/v) skimmed dried milk for 1 hour at room temperature to block. Blocking solution was then poured away and the blot briefly washed with TBS-T to remove any excess solution. The blot was then incubated with primary antibody diluted in TBS-T (containing 5 % BSA) overnight at 4 °C with agitation. Blots were then washed three times for 5 minutes in TBS-T. Blots were then incubated with secondary antibody diluted in TBS-T containing 5 % (w/v) non-fat dried milk for 1-2 hours at room temperature with agitation. The secondary was then discarded and the blots washed three times for 5 minutes in TBS-T. Proteins were then visualised using Amersham ECL Western Blot Detection Reagents or Millipore Immobilon Western Chemiluminescent HRP substrate.

2.2.16 Small interfering (si)RNA-mediated gene silencing

In order to assess the roles of certain transcription factors in the regulation of *MMP* expression, genes were transcriptionally silenced using double-stranded RNA sequences that bind through complementarity to mRNA of specific genes, targeting them for degradation.
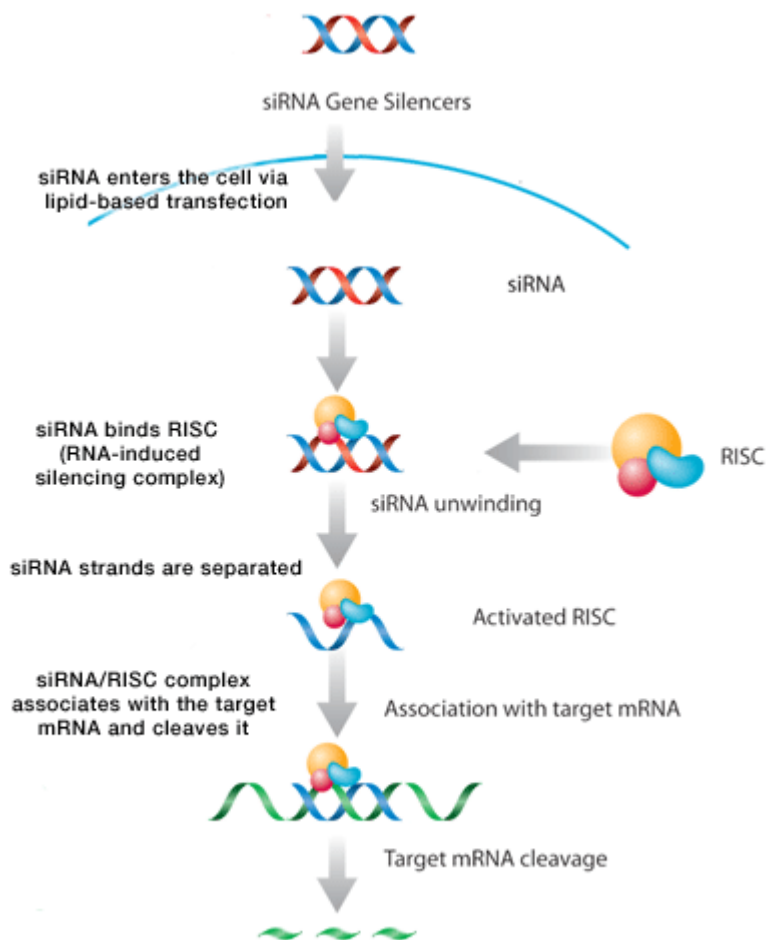
Figure 10: Schematic diagram of siRNA-mediated gene silencing. Taken from [187].

This process involves the initiation of the RNA interference pathway: dsRNA is delivered intracellularly using lipid vesicles which permeate the cell membrane. Once in the cell the dsRNA are cleaved into 21-23 bp small interfering (si) RNAs by Dicer which are then assembled into the RNA-induced silencing complex (RISC). This complex is targeted to a specific mRNA sequence of a specific gene leading to cleavage and degradation by exonucleases (Figure 10). This effectively silences the gene and its function within the cell. One is then able to assess the role of specific proteins in transcriptional regulation of *MMPs* via real-time PCR.

Reagents:

- Dharmacon ON-TARGET plus SMARTpools (ThermoFisher Scientific) or FlexiTube siRNA (Qiagen)GeneSolution of 4 specific siRNA duplexes (total of 100 nM siRNA)

- Dharmacon ON-TARGETplus Non-targeting Pool control siRNA

- DharmaFECT Transfection reagent (ThermoFischer Scientific)

Method:

HAC were cultured until 70-80 % confluent in T75 cm^2 tissue culture flasks. Culture medium was removed and the cell monolayer washed with PBS containing 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin. Trypsin-EDTA was then placed on the cells and incubated for 5 minutes at 37 °C until the cells had been released from the tissue culture surface. Cells were then suspended in serum-containing medium, cells pelleted at 300 g for 10 minutes, supernatant containing trypsin removed and pellet resuspended in fresh serum-containing medium. The cells were then counted using a haemocytometer and re-seeded at 10,000 cells/well in 96-well plates and/or 330,000 cells/well in 6-well plates. The cells were then allowed to adhere to the surface of the plate and cultured overnight until 50-70 % confluent, at which point the culture medium was replaced with 50 µl of serum-containing medium. Cells were then allowed to equilibrate for around 2-3 hours, during which time siRNA were prepared for transfection.

DharmaFECT transfection reagent (0.26µl/well of 96-well plate) was added to serum free medium (15µl/well of a 96-well plate or 1ml/well of a 6-well plate) in a sterile bijou container. siRNA including non-targeting siRNA control pool was made up at a final concentration of 100 nM from a 20 µM stock in serum-free medium (15 µl/well of a 96-well plate or 1 ml/well of a 6-well plate) in separate bijou container. An equal volume of DharmaFECT transfection reagent was then added to siRNA. Complexes of siRNA within lipid vesicles were then allowed to form over 15-20 minutes at room temperature within the tissue culture hood. Following this, 30 µl of the lipid vesicle-siRNA complexes were then added to the 50µl of serum-containing medium on the cells. Cells were then incubated with siRNA for 24-48 hours to allow transfection to occur. Following appropriate incubation, siRNA containing medium was removed, cells washed with PBS containing 200 IU/ml penicillin, 200 µg/ml streptomycin and 40 IU/ml nystatin and replaced with 100 µl of serum-free medium. The next day cells were stimulated with cytokines as previously described.

2.2.17 DNA microarray

The DNA microarray study was outsourced to Cambridge Genomic services (Cambridge University, UK). RNA from validated populations underwent quality control procedures in order to check for contamination with protein or phenols carried through from the RNA extraction that may have affected the hybridisation efficiency. Once satisfied with the quality of the samples, RNA was then reverse transcribed to cDNA and amplified by In Vitro Transcription to generate biotin-labelled cRNA. Samples were placed on a HumanHT-12 v4 Expression Beadchip from Illumina (San Diego, CA). This chip has 47,000 transcripts and known splice variants, known as features, spotted onto its surface. Complementarity between the cRNA and these features leads to hybridisation at specific regions on the chip. The beads are then washed in order to remove non-specific binding and blocked, thereby preventing any further hybridisation. The chip is then stained with streptavidin-Cy3 molecules which bind to any biotinylated-cRNA hybridised to the features. Emission of fluorescence by Cy3 is then quantitatively detected. The greater the amount of transcript, the more hybridisation and so more fluorescence emitted.

Data normalisation

Raw data generated in the DNA microarray study was subject to standard analysis carried out by Cambridge Genomic services. This included:

- Verification of quality controls using Illumina GenomeStudio software.
- Normalisation of raw data output using quantile normalisation (R package lumi software).
- Calculation of statistical significance using R microarray analysis of variance (maanova) software which corrects p-values for false positives of differential gene regulation that arise through multiple statistical tests.

Analysis of results

Following data normalisation I received the results which include raw and normalised data, a full report on the analysis and results of the comparisons with gene annotations, fold-change, p-value and corrected p-value. In order to further analyse this data with respect to my study I imported this data into GeneSpring 12 (Agilent).

This allowed for the exclusion of genes that did not have corrected p-values less than or equal to the user-specified p-value cut-off (p<0.05). The generation of an experiment with GeneSpring also allowed for improved visualisation of the data and comparisons of differentially expressed genes in one comparison group against those in another.

2.2.18 Statistical analysis

For ChIP analysis the difference between individual HAC population means was tested for statistical significance using ANOVA (analysis of variance): two-factor without replication with a post-hoc Bonferroni multiple comparison test. Real-time PCR data were normalised against 18S rRNA, then plotted as the fold induction of the target gene expression over control levels. Levels of statistical significance are shown as *p≤0.05, **p≤0.01 and ***p≤0.001. For clarity, only selected comparisons are presented in some figures.

3 Chapter 3: AP-1-dependent collagenase gene regulation in primary chondrocytes

3.1 Introduction

As mentioned previously, collagen degradation is a critical pathological process that once initiated leads to an irreversible loss of articular cartilage and subsequent loss of tissue function. The cleavage of type II collagen, the main structural component of articular cartilage, is effected by the collagenases MMP-1 and -13 [37, 44, 47]. Their aberrant expression in disease is, in-part, due to pro-inflammatory cytokine signalling via the chondrocyte. The gene regulation of these enzymes, that play a critical role in pathology, has been demonstrated to be controlled by a number of different signalling pathways as well as histone modification and DNA methylation. Activation of cis-elements in the gene promoter also represents a crucial point of collagenase gene regulation in response to pro-inflammatory cytokine stimulus. AP-1 homo/heterodimers are an example of one transcription factor family that play a significant role in IL-1+OSM-dependent collagenase gene transcription [82].

Understanding of AP-1-dependent collagenase gene transcription is limited [112, 113], and how AP-1 regulates collagenase gene expression in chondrocytes in response to complex pro-inflammatory cytokine stimulation remains to be determined. The following study was performed in order to improve our understanding of AP-1 gene expression, protein induction and its impact on collagenase gene expression in the chondrocyte.

3.1.1 Assessment of the functional role of c-Fos/c-Jun in collagenase gene regulation

Previous studies have demonstrated that c-Fos/c-Jun binds to MMP-1 promoter/luciferase reporter constructs and that the expression of this collagenase is dependent on AP-1 expression [82]. Further studies have shown that silencing of the c-Fos/c-Jun genes using small interfering(si) RNA led to a significant reduction in both *MMP1* and *MMP13* gene expression [97]. Therefore, I firstly wanted to confirm that

these AP-1 transcription factors were functionally important for IL-1+OSM-dependent collagenase gene expression, such that my subsequent research would be based on proven and reproducible data.

The AP-1 transcription factor *FOS* was silenced either alone, or in combination with the family member *JUN*. Silencing of *FOS*, either alone or in combination with *JUN* led to a significant decrease in *MMP1* and *MMP13* gene expression induced by IL-1+OSM stimulation, when compared to a non-targeting control siRNA (Figure 11). Silencing of *JUN* alone led to a slight decrease in gene expression of both collagenases but this was not statistically significant. There was no significant difference between *FOS* and *FOS+JUN* silencing on *MMP13* gene expression compared to the control, whereas silencing of both these AP-1 factors resulted in a more significant decrease of *MMP1* expression compared the control, than *FOS* alone. These data demonstrate that c-Fos is an important regulator of *MMP1* and *MMP13* expression. Moreover, silencing both *FOS* and *JUN* highlights the importance of these AP-1 members to *MMP1* expression.
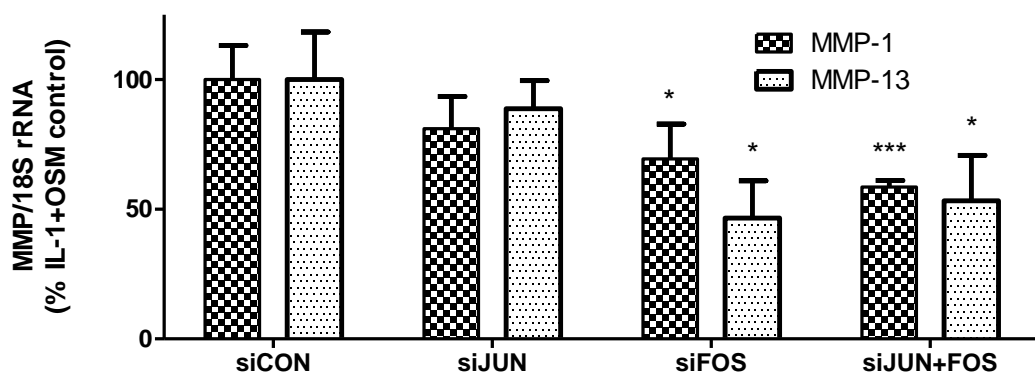
Figure 11: Real-time PCR analysis of the effect of *FOS* and *JUN* gene silencing on *MMP* gene expression in IL-1+OSM stimulated HAC. Following transfection with siRNA specific for *FOS*, *JUN* or non-targeting siControl (100nM), HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 24 hours. Real-time reverse transcription PCR of isolated RNA was performed for *MMP1* and *MMP13* and normalised to *18S* rRNA housekeeping gene. Data are pooled from three separate HAC populations (each assayed in sextuplet) and expressed as a percentage of stimulated control (specific siAP-1 transfected versus siCon-transfected; mean ± S.D.; *, p <0.05; ***, p < 0.001).

Having demonstrated that *FOS* and *JUN* silencing significantly reduced collagenase gene expression, I next wanted to confirm that the observations in Figure 11 were as a result of a reduction in AP-1 protein expression following gene silencing. Western blot analysis revealed that unstimulated HAC had no change in protein level between siControl and siRNA for either c-Fos or c-Jun (Figure 12). Stimulation of HAC using IL-

1+OSM led to an increase in both AP-1 proteins in the presence of siControl, confirming data from Litherland 2010 *et al* [97]. Importantly, the expression of both c-Fos and c-Jun protein, following IL-1+OSM stimulation, was markedly decreased following gene silencing. This confirms that the observed changes in collagenase expression (Figure 11) were due to a successful reduction of gene expression and protein production following siRNA gene silencing.
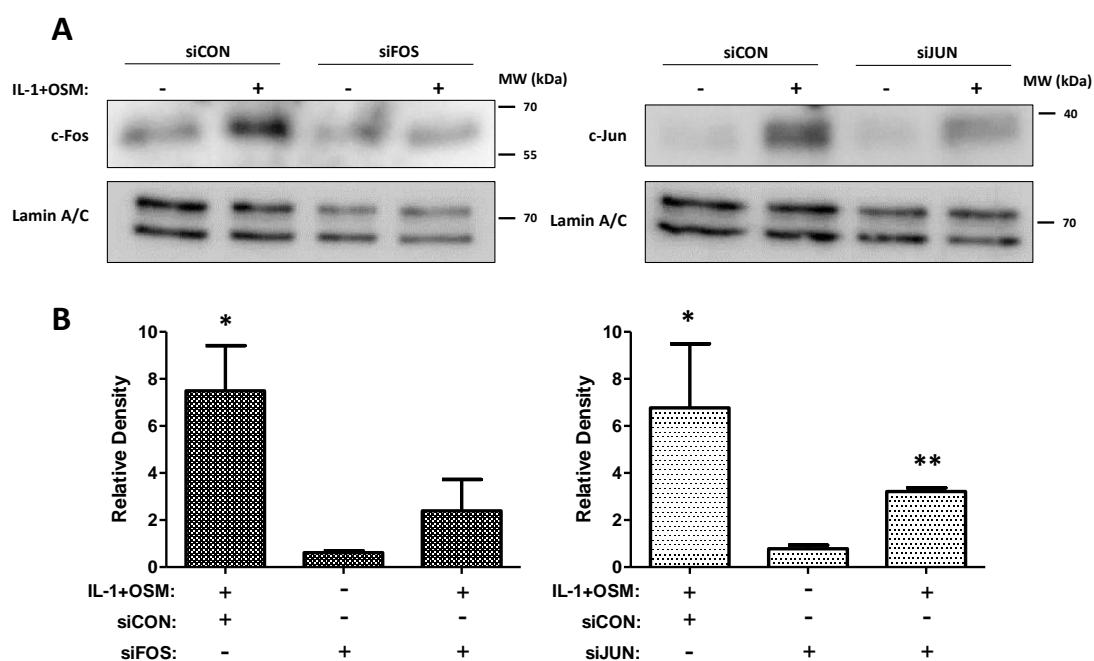
Figure 12: Western blot for c-Fos and c-Jun following gene silencing. A, HAC were transfected with siRNA specific for *FOS* and *JUN* or non-targeting siControl (100nM) prior to stimulation with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 1 hour as indicated. Nuclear extracts were isolated and proteins resolved by SDS-PAGE. Immunblots were probed using indicated antibodies. Blots shown are representative of three experiments using chondrocytes from different donors. Lamin A/C was used as a loading control. B, combined densitometric scans of three separate blots each from separate chondrocyte populations. Density is relative to unstimulated HAC transfected with siCON, where *, $p < 0.05$ and **, $p < 0.01$.

3.1.2 Investigation of AP-1 protein induction and cellular localisation

Having confirmed the functional importance of c-Fos and c-Jun in the expression of *MMP-1* and *MMP-13* expression, I next assessed the kinetics of AP-1 expression following IL-1+OSM stimulation. A greater understanding of this temporal profile would assist subsequent ChIP studies and allow comparison to the study by Martens *et al*. [158] which, using semi-quantitative PCR, demonstrated the kinetics of c-Fos and c-Jun binding to the MMP-1 promoter following PMA stimulation of a glioblastoma cell line. These studies revealed that there was an ordered recruitment of regulatory

proteins to the collagenase promoter which included c-Fos and c-Jun. The study suggested that binding of c-Fos and c-Jun was transient and preceded the recruitment of other regulatory factors like transcriptional co-activator, p300. Though intriguing, these results were generated without a disease context and under an extremely potent chemical stimulation, also in addition there was relatively little quantification in the study. I used time points found relevant by Martens *et al* and previous study in our group [82], as a basis for my own experiments to assess when AP-1 is expressed in response to IL-1+OSM stimulation by primary articular chondrocytes isolated from patient cartilage samples. The use of primary cells not only provided my study with a greater degree of disease relevance, but interestingly, the widely used chondrosarcoma cell line (SW1353 cells) does not express *FOS* (Rowan, personal communication), though IL+OSM stimulation still synergistically induces *MMPs* [72]. Furthermore, Gebauer *et* al [188] has demonstrated limitations of using SW1353 cells as a model for HAC. However, the use of primary cells has logistical limitations: cell yields from enzymatic digestion of cartilage samples are limited by the amount of residual, macroscopically normal cartilage. As such, some experiments are limited in their scope and so I was unable to perform as extensive a time course study for AP-1 enrichment on the collagenase promoters as described by Martens *et al* [158]. I therefore planned to limit the number of time points used within ChIP experiments by first investigating c-Fos/c-Jun protein localisation and temporal induction in order to then select pertinent IL-1+OSM stimulation durations for ChIP assays.

I first confirmed the suitability of the subcellular protein fractionation protocol by taking samples from all five subcellular fractions from cultured HAC and resolving them with SDS-PAGE. Western blotting using antibodies specific to protein markers only found in each specific fraction (e.g. Histone H3 is a member of the histone octamer that facilitates the condensation of DNA into chromatin and as such is found tightly associated with DNA and therefore an ideal marker for the chromatin-bound fraction). As shown in Figure 13, marker proteins were only present in the fractions to which they are associated indicating good fractionation efficiency for subsequent use.
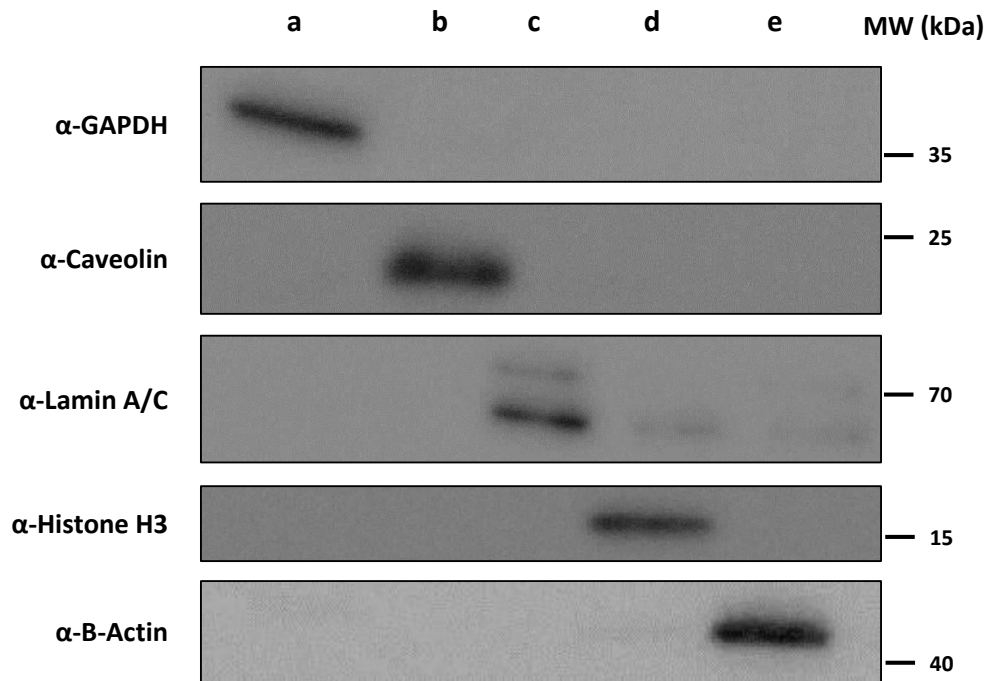
Figure 13: Confirmation of Protein subcellular fractionation. HAC were subject to subcellular protein fractionation using the Subcellular Protein Fractionation Kit (ThermoScientific). Proteins from cytosolic (A), membrane-bound (B), soluble nuclear (C), chromatin-bound, (D) and cytoskeletal (E) subcellular fractions were resolved using SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots shown are representative of three separate experiments each using chondrocyte cultures from different donors.

The expression and localisation of AP-1 transcription factors were then determined at 0,1,3,6, and 24 hours post IL-1+OSM stimulation. Expression of c-Fos was not observed under unstimulated, basal conditions, however at 1 hour post-stimulation there was a marked increase in protein levels which then decreased at 3 hours (Figure 14A). Expression of c-Fos was still detectable at 6 hours in some (Figure 14A), but not all HAC populations, but had diminished substantially by 24 hours (Figure 14B). The c-Fos binding partner, c-Jun also displayed similar kinetics of protein phosphorylation, but was more transient than c-Fos, not being detected 6 hours post-stimulation (Figure 14A and C). The localisation of both c-Fos and c-Jun appeared to be confined to the soluble nuclear fraction (lanes C, Figure 14) with IL-1+OSM duration having no obvious effect on AP-1 localisation.
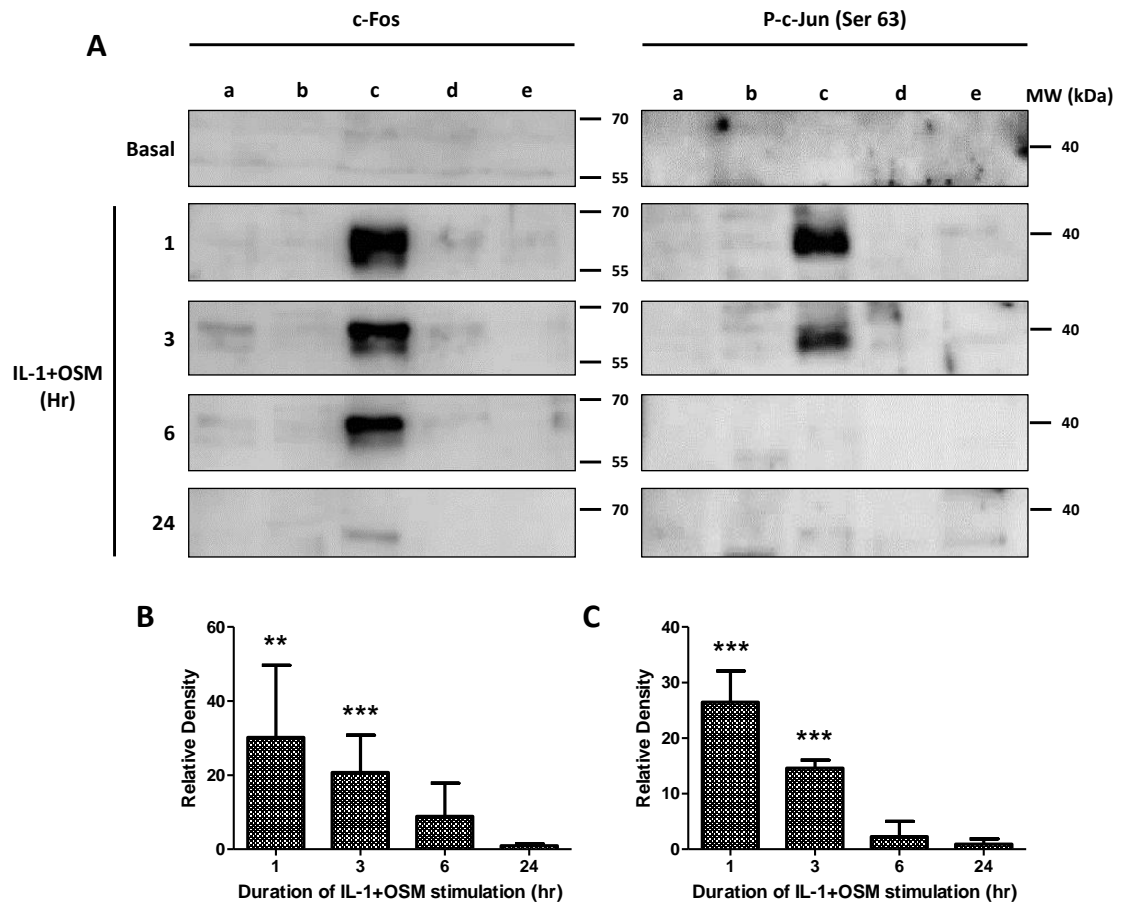
Figure 14: Western blot for c-Fos and c-Jun AP-1 factors in subcellular protein fractions from HAC stimulated with IL-1+OSM. A) HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to subcellular protein fractionation. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with the indicated antibodies. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed. Immunoblots shown are representative of five separate experiments each using chondrocyte cultures from different donors. Images in this figure are cropped from larger exposure image. Full-length blots/gels are presented in Appendix 7.1, figure 48. Combined densitometric scans of separate blots (from separate chondrocyte populations) immunoblotted with antibodies against c-Fos (**B**) and P-c-Jun (Ser63) (**C**). Density is relative to unstimulated control (mean ± SD; where **, $p < 0.01$; ***, $p < 0.001$).

The high abundance of c-Fos protein within the soluble nuclear fraction may have resulted in the masking of other, less abundant, protein levels within other fractions. AP-1 is a transcription factor capable of binding to DNA, a process essential for its function as a transcriptional regulator. I therefore diluted the soluble nuclear fraction, 1 in 5, in order to help 'unmask' proteins present within the chromatin-bound fraction that would otherwise be undetectable next to the robust signal observed in soluble nuclear fractions on western blot. In Figure 15 lane A, detection of both c-Fos and p-c-Jun in the soluble nuclear fraction confirmed previously observed changes in protein with time as described in Figure 14. Proteins within the chromatin-bound fraction were now observable in both the c-Fos and c-Jun immunoblots. Although not as robust as in

the soluble nuclear fraction, levels of c-Fos protein increased from basal to peak at 1 hour post IL-1+OSM stimulation, reducing thereafter. Low, but detectable levels of c-Jun were observed at 1 and 3 hours post IL-1+OSM stimulation in the chromatin-bound fraction. These observations are reflected in the densitometric scans for c-Fos and p-c-Jun (Ser 63) (Figure 15B and C). Together these data demonstrate that in the context of IL-1+OSM stimulation of HAC, c-Fos production and c-Jun activation occur rapidly and transiently, and binding of these factors to chromatin is relatively short-lived.
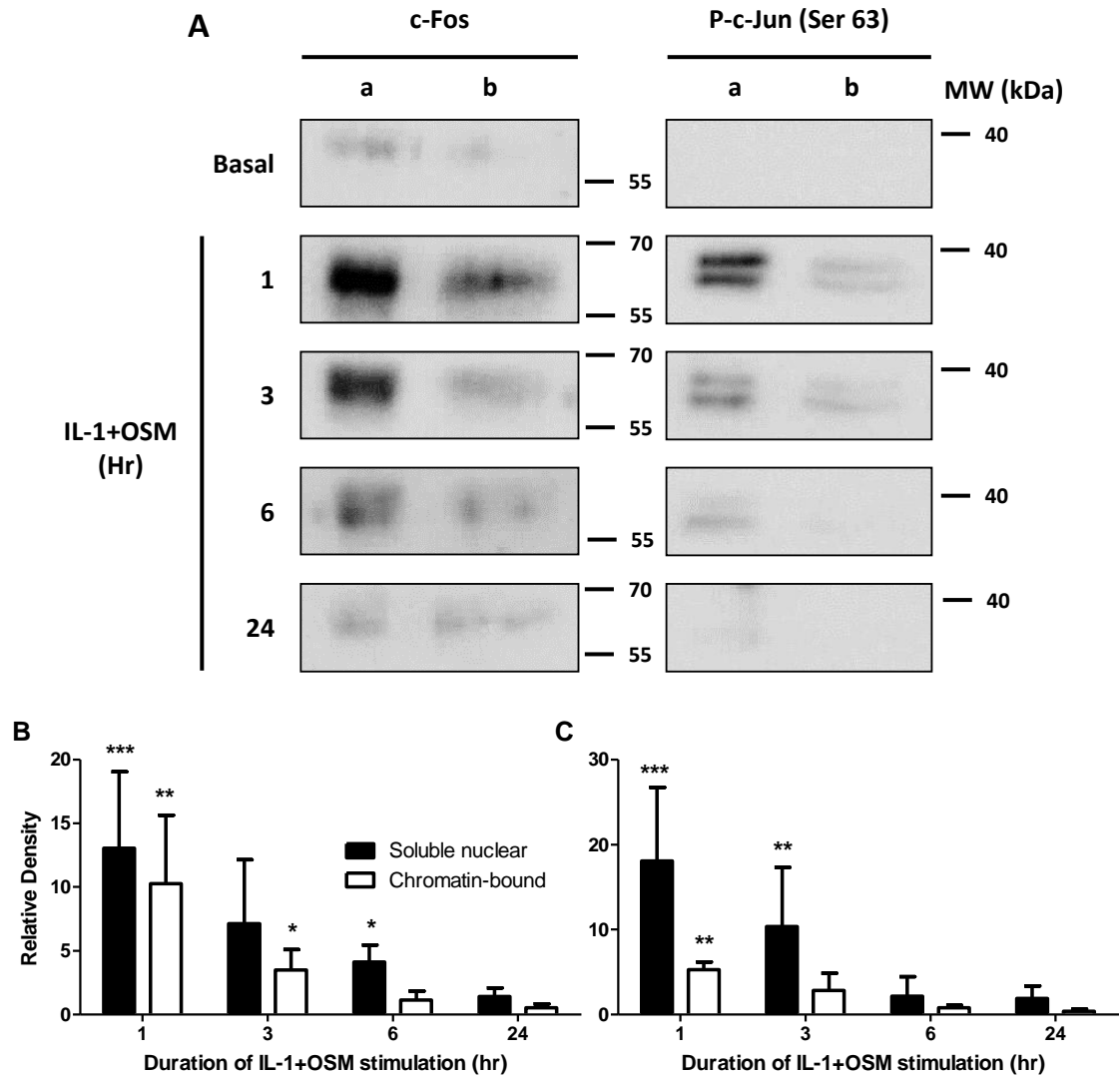
Figure 15: Western blot for AP-1 factors in soluble nuclear and chromatin-bound protein fractions from HAC stimulated with IL-1+OSM. A) HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to subcellular protein fractionation. Proteins from 1 in 5 dilutions of soluble nuclear (a) and neat chromatin-bound (b) subcellular fractions were resolved using SDS-PAGE and immunoblotted with the indicated antibodies. Immunoblots shown are representative of five separate experiments each using chondrocyte cultures from different donors. Combined densitometric scans of separate blots (from separate chondrocyte populations) immunoblotted with antibodies against c-Fos (**B**) and P-c-Jun (Ser63) (**C**). Density is relative to unstimulated control (mean ± SD; where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3.1.3 Investigation of temporal *FOS*/AP-1 gene expression in chondrocytes

The rapid induction of the c-Fos/c-Jun AP-1 factor (see Figure 14) confirmed the reported immediate early gene status for *FOS* and *JUN* [189]. [189]. I next wanted to correlate the kinetics of *FOS* and *JUN* mRNA expression with the concomitant kinetics of *MMP1* and *MMP13* expression. Figure 16A demonstrates that expression of *FOS* gradually increased from basal (unstimulated) up to 1 hour post IL-1+OSM stimulation, but at time points after 1 hour stimulation, gene expression levels decreased dramatically to levels comparable with basal gene expression. Both *MMP1* and *MMP13* expression was not observed to increase until 6 hours post IL-1+OSM stimulation. This expression then increased through to 24 hours (Figure 16B and C, respectively). Furthermore, the expression of *JUN* was also observed to increase rapidly following IL-1+OSM stimulation, with expression peaking slightly earlier at 45 minutes post stimulation (Figure 17). Though *JUN* decreased post-45 minutes IL-1+OSM stimulation, *JUN* expression remained significantly elevated above basal gene expression levels at all subsequent time points measured up to 24 hours. These data, taken together with the protein studies, show that *FOS* and *JUN* are transient and that both gene expression and c-Fos production/c-Jun activation peaks around 1 hour IL-1+OSM stimulation. In contrast, collagenase gene expression does not occur until 6 hours post-IL-1+OSM stimulation. This highlights a temporal deficit between AP-1 and the initiation of collagenase gene expression. As such I next wanted to determine if, and when, c-Fos was binding to the promoter regions of *MMP1* and *MMP13* during this deficit.
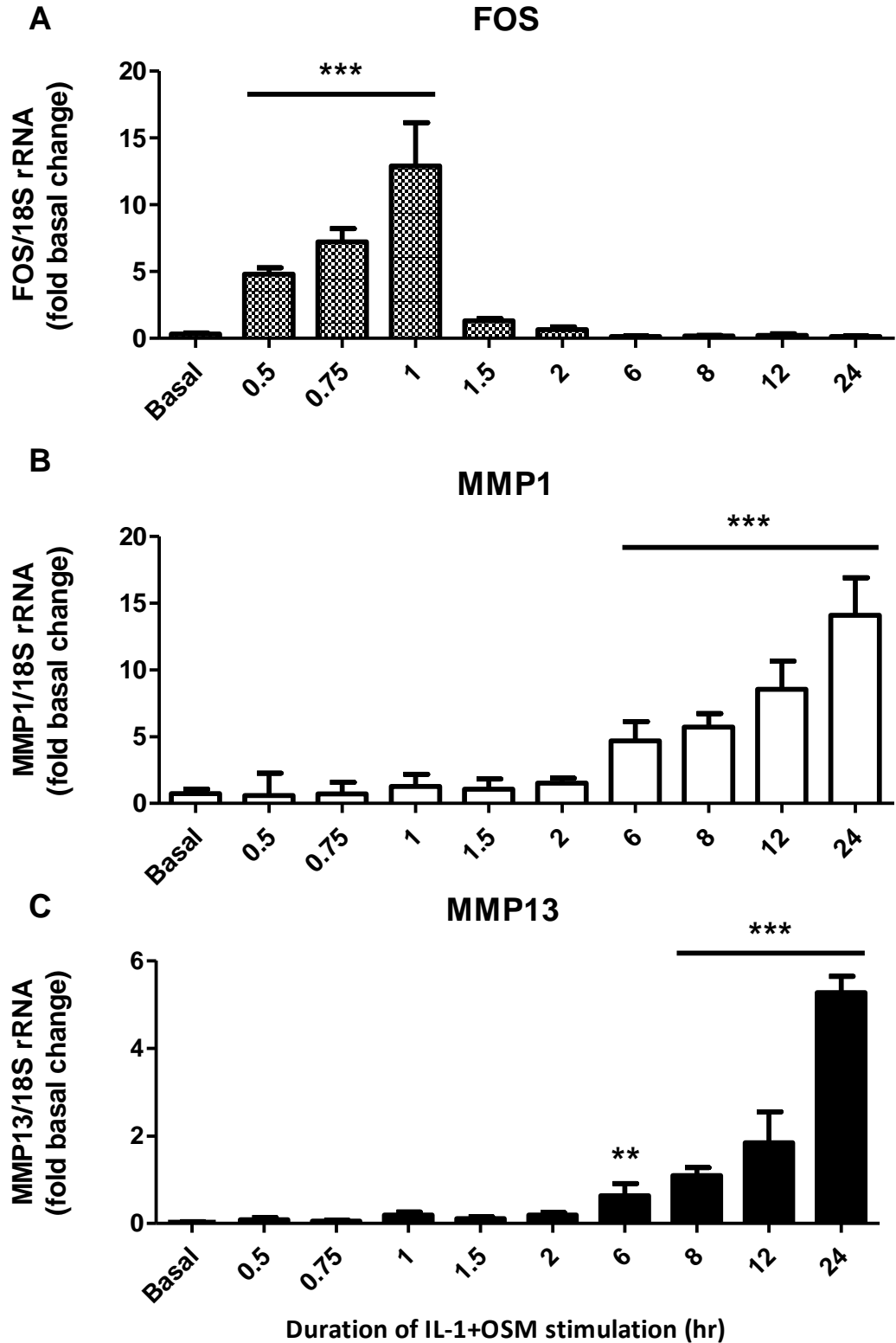
Figure 16: Real-time PCR analysis of gene expression following different durations of IL-1+OSM stimulation. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cell lysates were subjected to real-time PCR and relative expression levels of *FOS* (A), *MMP1* (B) and *MMP13* (C) mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Data are representative of three separate experiments each using chondrocyte cultures from different donors. Statistical analysis are shown for gene expression following indicated IL-1+OSM stimulation versus basal (mean ± SD; where **, p < 0.01; ***, p < 0.001).
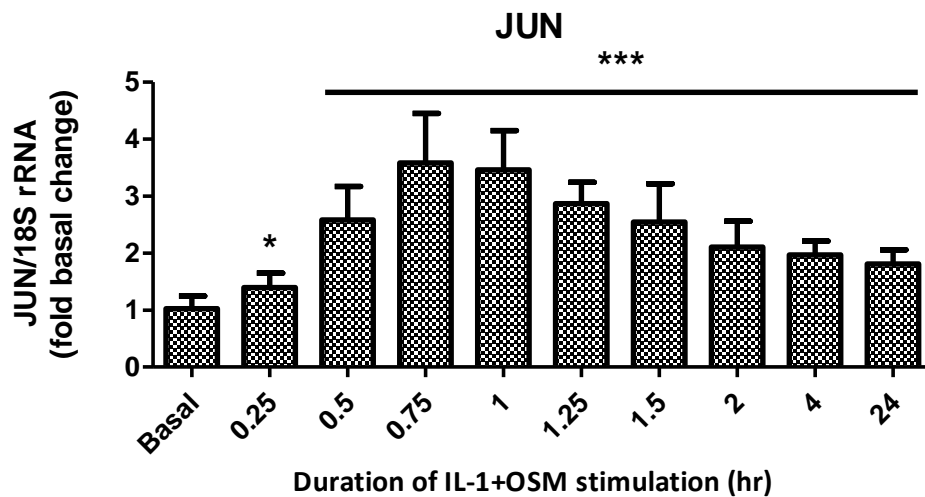
Figure 17: Real-time PCR analysis of *JUN* gene expression following different durations of IL-1+OSM stimulation. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cell lysates were subjected to treatment with DNase followed by real-time PCR and relative expression levels of *JUN* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Data are representative of three separate experiments each using chondrocyte cultures from different donors. Statistical analysis are shown for gene expression following indicated IL-1+OSM stimulation versus basal (mean ± SD; where *, $p < 0.05$; ***, $p < 0.001$).

3.1.4 Assessment of the enrichment of AP-1 and markers of transcription on collagenase gene promoters

With improved understanding of c-Fos/c-Jun AP-1 factor gene expression and protein production, it was evident that the functional effects of these transcription factors were almost immediate. The transient induction of both mRNA and protein indicated that the functional contribution was early and may not fully account for the temporal lag between c-Fos/c-Jun peak and *collagenase* expression. I therefore used ChIP to better assess the transcriptional regulation of collagenase genes by c-Fos. This allowed me to compare and contrast c-Fos gene expression and protein production data with its capacity to bind at the collagenase gene promoters. ChIP was also used to assess transcriptional activation of collagenase promoters, by assessing RNA polymerase II (pol II) phosphorylation which correlates with transcriptional initiation, allowing comparison between gene expression and c-Fos binding. This may also reveal if the regulation of pol II 'pausing' at the promoter [190] may be accounting for the temporal deficit. These studies allowed me to consider dynamic, functional changes that take place at the collagenase gene promoters in response to IL-1+OSM stimulation.

A key step in the ChIP technique is chromatin shearing which determines subsequent binding site prediction and primer design. Figure 18 confirms appropriate chromatin shearing at various concentrations of gDNA. At all three concentrations, chromatin shearing by high powered sonicating water bath produced fragments that were approximately 200 – 500bp in length. This meant that I could be confident that enrichment demonstrated in ChIP experiments was occurring within the promoter region. It also showed that I could sonicate concentrated sample volumes without compromising shearing efficiency and therefore multiple immunoprecipitations from the same sample could be sonicated at the same time.
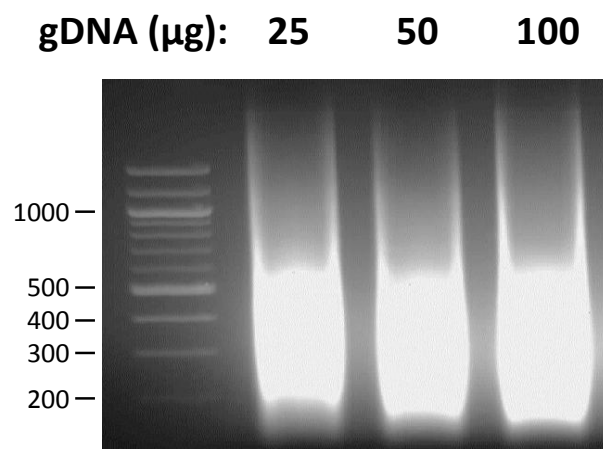
Figure 18: Agarose gel electrophoresis analysis of HAC gDNA fragment length following shearing. Three amounts of gDNA taken from an individual HAC population isolated from a singular patient sample was subject to cell lysis. The concentration of DNA was measured and split between 1.5 ml tubes at the indicated amounts made up to the same volume in lysis buffer. The samples were then subject to sonication, shearing the gDNA into small fragments. The product was then resolved on agarose gel (3%), supplemented with ethidium bromide, by electrophoresis. DNA fragments were visualised using syngene software. Markers indicate DNA fragment size.

Using ChIP, Figure 19A shows the temporal changes in c-Fos enrichment at the proximal promoter and 3'UTR of *MMP1* and *MMP13*. Following 1 hour stimulation, c-Fos binding to the proximal promoter was increased for both collagenases when compared to basal. Post 1 hour IL-1+OSM stimulation, enrichment of c-Fos decreased to levels comparable with basal. There was no observable increase in c-Fos binding in the 3'UTR following any duration of IL-1+OSM stimulation. A further series of experiments confirmed the significant but transient enrichment of c-Fos to the collagenase promoters at the early, 1 hour IL-1+OSM stimulation (Figure 19B). These ChIP data confirm that, along with *FOS* expression and protein production, enrichment to the collagenase promoter following stimulation is also rapid but transient.

Figure 19: ChIP analysis of *MMP* gene enrichment by c-Fos. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. Cells were then subject to DNA-protein cross-linkage, lysis and DNA shearing. Immunoprecipitation for c-Fos and isolation of complexed gDNA allowed the assessment of c-Fos binding to indicated regions on the *MMP1* and *MMP13* genes by real-time PCR. Data were pooled from three (A) and six (B) separate HAC populations. Statistical analysis are shown for enrichment following 1 hour IL-1+OSM stimulation versus 4 hours (mean ± S.D.; *, $p < 0.05$) and 1 hour versus unstimulated (mean ± S.D.; #, $p < 0.05$). For details on ChIP primer sequences and their position in relation to TSS see table 4 in chapter 2.2.4.2.

Pol II is the enzyme responsible for generating nascent mRNA from a gene. The phosphorylation of serine 5 (ser 5) in the carboxy N-terminal domain results in promoter escape and the initiation of active transcription [167]. I used pol II as a marker of active transcription at the collagenase proximal promoters. The fold enrichment of pol II (Ser 5 phos-CTD) over basal, on both the *MMP1* and *MMP13* proximal promoters, was observed to increase gradually from 1 to 24 hours post IL-1+OSM stimulation (Figure 20A). This observation was more striking for *MMP1* promoter enrichment. There was no observable increase in pol II (Ser 5 phos CTD) binding in the 3'UTR following any duration of IL-1+OSM stimulation. Repeating these data in 5 further HAC populations (Figure 20B) confirmed the significant enrichment of

pol II (Ser 5 phos CTD) on *MMP1* and *MMP13* proximal promoters at 24 hours compared to 1 hour of IL-1+OSM stimulation. These data taken with gene expression studies, show that collagenase gene expression correlates directly with recruitment of pol II (Ser 5 phos CTD). Therefore, pol II 'pausing' at the promoter does not appear to account for the time taken for collagenase gene expression to be initiated following the peak of c-Fos and c-Jun.
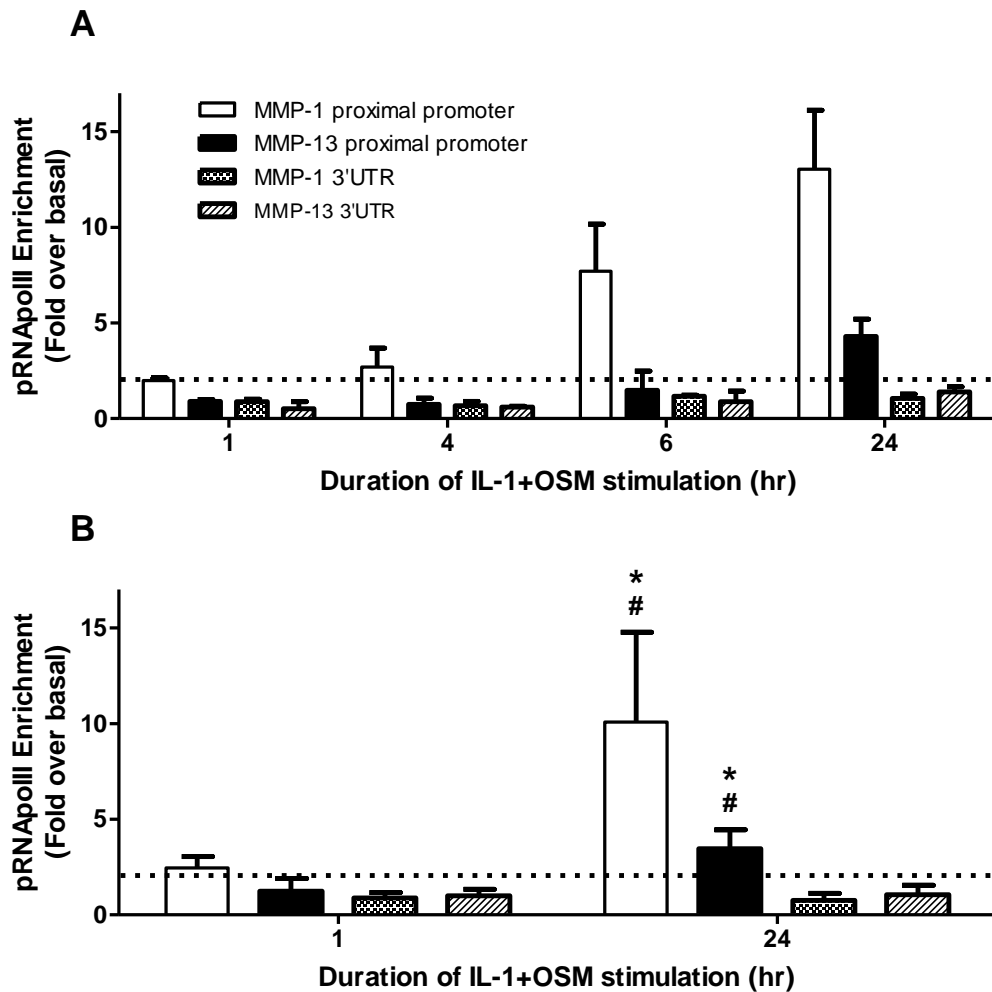
Figure 20: ChIP analysis of *MMP* enrichment by RNA polymerase II (Ser5 phosphorylation of carboxy terminal domain). HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to DNA-protein cross-linkage, lysis and DNA shearing. Immunoprecipitation for RNA polymerase II (Ser5 phosphorylation on carboxy-terminal domain) and isolation of complexed gDNA allowed the assessment of RNA polymerase II binding to indicated regions on *MMP1* and *MMP13* gene by real-time PCR. Data were pooled from three (A) and five (B) separate HAC populations. Statistical analysis are shown for enrichment following 24 hour IL-1+OSM stimulation versus 1 hour (mean ± S.D.; *, $p < 0.05$) and 24 hours versus unstimulated (mean ± S.D.; #, $p < 0.05$). For details on ChIP primer sequences and their position in relation to TSS see table 4 in chapter 2.2.4.2.

Acetylation of histone H3 leads to nucleosome charge neutralisation, thus removing attraction between histones and DNA. This process opens the chromatin, a process

crucial for the access of trans-activating transcription factors to cis-binding elements within gene promoters [163], allowing transcription to occur. Acetylated histone H3 is therefore a useful marker of transcriptionally permissive chromatin. Levels of acetylated histone H3 associated with the proximal promoters of both *MMP1* and *MMP13* were found to increase from 1 to 24 hours following IL-1+OSM stimulation. Acetylated histone H3 levels were not elevated above basal at the 3'UTR of either collagenase gene following any duration of IL-1+OSM stimulation (Figure 21). As with pol II (Ser 5 phos CTD) data, histone acetylation correlated directly with collagenase gene expression. This means histone modifications that govern how permissive chromatin is to transcription may be a rate limiting step in collagenase gene expression.

Observations from these ChIP data continually demonstrate higher fold enrichment at the *MMP1* promoter compared to *MMP13*. *MMP1* has been shown to be expressed at levels approximately 10 times greater than that of *MMP13* in primary chondrocytes [45]. This disparity in expression levels between the collagenases may well be manifested at the level of transcription initiation and histone acetylation thereby accounting for the higher *MMP1* promoter enrichment levels compared to *MMP13*.
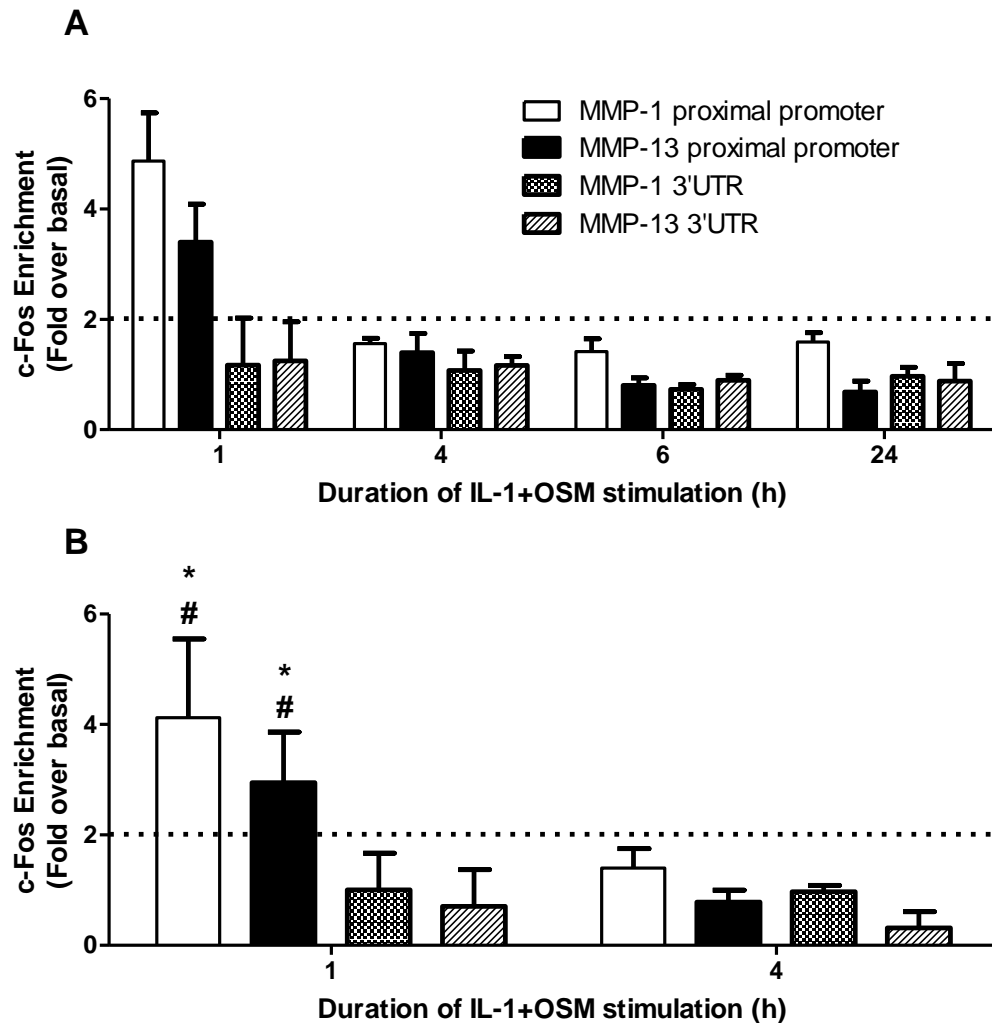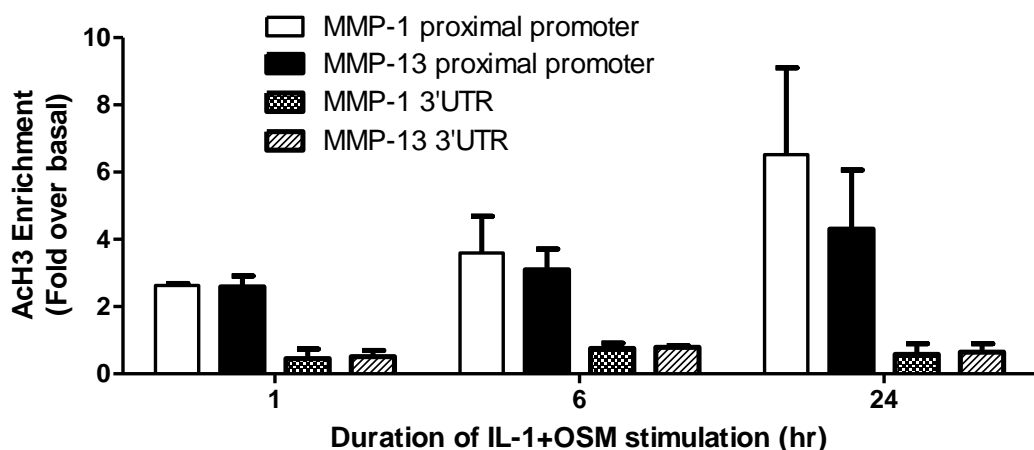

Figure 21: ChIP analysis of *MMP* enrichment by Acetylated Histone H3. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to DNA-protein cross-linkage, lysis and DNA shearing. Immunoprecipitation for acetylated histone H3 and isolation of complexed gDNA allowed the assessment of acetylated histone H3 association to indicated regions on *MMP1* and *MMP13* gene promoters by real-time PCR. Data were pooled from two separate HAC populations. For details on ChIP primer sequences and their position in relation to TSS see table 4 in chapter 2.2.4.2.

3.1.5 Investigation into the contribution of new protein synthesis on the regulation of collagenase gene transcription

My previous findings confirmed that following IL-1+OSM stimulation, *MMP1* and *MMP13* are dependent on both c-Fos and c-Jun (Figure 11), and that *FOS* gene and c-Fos protein induction were both rapid and transient. Together, these data indicate that there is a temporal deficit between c-Fos binding and initiation of *MMP* transcription. I therefore proposed that c-Fos was necessary, but not sufficient, to regulate collagenase gene expression. As such I carried out protein synthesis inhibition experiments to determine if newly produced or, constitutively active, regulatory factors were contributing to collagenase gene expression.

Emetine is a protein synthesis inhibitor which acts by binding to the 40S ribosomal subunit, preventing ribosome translocation along mRNA, therefore inhibiting protein synthesis. Emetine was added at sequential time points up to 24 hours post IL-1+OSM stimulation. Stimulation of HAC for 24 hours with IL-1+OSM led to a significant increase in *MMP1* gene expression over basal. Similarly, significant inductions of *MMP1* were observed when emetine was added at 6 and 8 hours post the initiation of IL-1+OSM stimulation. However, the addition of emetine within the first 4 hours post IL-1+OSM stimulation strikingly resulted in no significant increase in *MMP1* gene expression (Figure 22A). Furthermore, similar results were observed with *MMP13* with significant expression only being observed when emetine was added 8 hours post IL-1+OSM stimulation (Figure 22B). These data indicate that new protein synthesis is required post-c-Fos/c-Jun peak and that the expression of new factors that contribute to the regulation of collagenase genes are produced up to 6 and 8 hours (*MMP1* and *MMP13*, respectively) after the initial stimulation.

This above study suggests that, although important, c-Fos and c-Jun do not solely regulate collagenase transcription and that factors, expressed after c-Fos/c-Jun peak, are crucial in bringing about collagenase gene expression. Interestingly, this means that other contributing factors are yet to be revealed. I hypothesised that these factors are likely to be transcription factors themselves and that because these factors are

produced after c-Fos/c-Jun peak, they may too be regulated by AP-1. Further study was then undertaken to try and elucidate these factors.
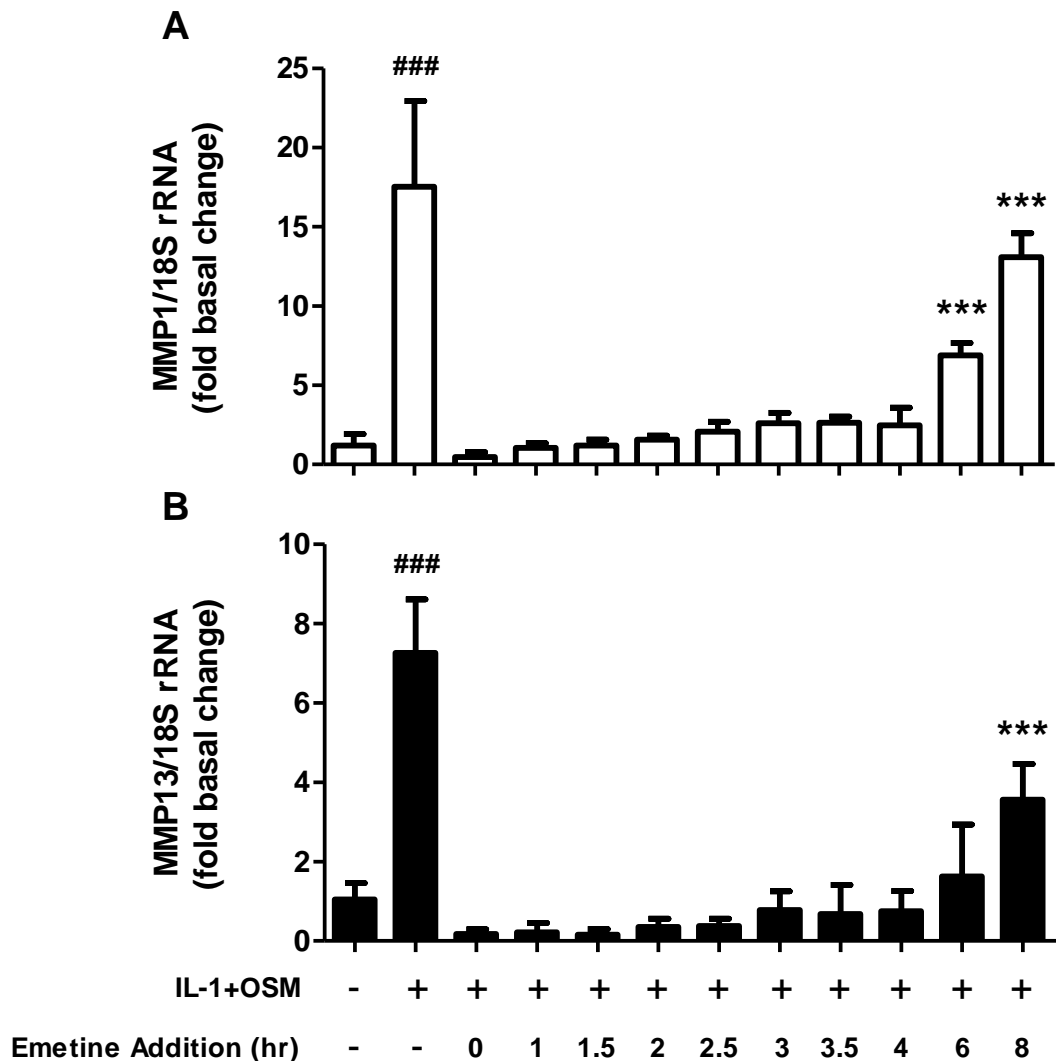
Figure 22: Real-time PCR analysis of IL-1+OSM stimulation of HAC in the presence of protein synthesis inhibition. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 24 hours. Emetine (10 mM), a protein synthesis inhibitor, was added at the indicated times following IL-1+OSM stimulation. Cell lysates were subjected to real-time PCR and the relative expression levels of *MMP1* (A) and *MMP13* (B) mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Data are representative of three separate experiments, each using chondrocyte cultures (assayed in hextuplet) from different donors. IL-1+OSM versus basal (mean ± SD; ###, $p < 0.001$) and IL-1+OSM+emetine versus basal (mean ± SD; *, $p < 0.05$; ***, $p < 0.001$)

3.2 Discussion

Cartilage degradation by collagenases as a consequence of pro-inflammatory cytokine stimulation is an important pathological process [37, 191, 192], that subsequently results in the loss of articular collagen and cartilage architecture contributing to patient pain and disability [193]. Within IL-1+OSM-mediated collagenase gene expression, an apparent role has been demonstrated for the transcription factor AP-1. My data reveal that although the c-Fos/c-Jun AP-1 heterodimer is crucial for *MMP1/MMP13* gene regulation there is a striking temporal deficit between c-Fos/c-Jun expression and collagenase gene induction. My investigations suggest that there are clearly additional *de novo* generated factors which are required to mediate collagenase expression.

A key finding within Chapter 3 was that there is a clear temporal deficit in AP-1 expression and collagenase induction following IL-1+OSM stimulation, highlighting somewhat of a discrepancy in the AP-1-dependent transcriptional regulation of collagenase paradigm. Silencing *FOS* and *JUN* affects collagenase expression highlighting their functional importance to this process. These AP-1 factors are also rapidly and transiently induced, yet, collagenase expression, transcription initiation and chromatin unwinding all begin to increase at times after the point at which AP-1 returned to unstimulated levels. This suggests that the AP-1 heterodimer of c-Fos/c-Jun could act as a transcription initiator and is the first step in a sequence of complex molecular events that ultimately culminate in collagenase transcription. The mechanism by which this occurs is unclear. One mechanism I proposed was that after the initiation of gene expression by c-Fos/c-Jun, transcription machinery was established on the collagenase gene promoter. This complex would build transcript levels through continual recycling and re-initiation of the transcription cycle accounting for the observed gradual increase in collagenase gene expression over time [194]. Another theory is that AP-1 heterodimers were required to initiate a cascade of transcriptional and post-transcriptional events that gradually bring about the full complement of molecules necessary for optimal gene transcription. The transcription cycle is governed by the ordered recruitment and replacement of various co-factors in a cascade of events that leads to formation of a pre-initiation complex at promoters

and productive elongation [171]. Martens *et al* [158] and Schmucker *et al* [115] both highlight the sequential recruitment of various regulatory factors to the promoter region of *MMP1* and *MMP13*, respectively. This includes c-Fos and c-Jun as well as pol II and chromatin remodelling factors and histone modifications. As such, there are many potential processes that could account for the temporal deficit.

Pol II pausing is a regulatory process that occurs after the formation of the pre-initiation complex on gene promoters. Studies have indicated that pausing is a key regulatory mechanism that acts to maintain the gene in a state ready for productive elongation to rapidly occur [190]. Two pause-inducing factors, DRB-sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) halt the transcription complex soon after transcription initiation. The kinase P-TEFb then phosphorylates DSIF, NELF and CTD domain of pol II allowing elongation to occur [195]. I hypothesised that pol II pausing may be occurring on the collagenase promoter with c-Fos/c-Jun initiating transcription only for it to be immediately paused. The release of the transcription complex was then dependent on factors that were expressed during the temporal deficit. However, direct correlation between collagenase gene expression and pol II enrichment showed that pausing was unlikely to be occurring as transcription initiation resulted in simultaneous gene expression. Moreover, genes controlled in this manner are normally IEG that require rapid induction upon stimulation, such as *FOS* and *MYC* [196, 197], rather than secondary response genes, like *MMP13* [198], that require activation by IEG.

Another explanation for the temporal deficit was that the chromatin structure surrounding the collagenase genes could be delaying the binding and recruitment of transcriptional machinery. Histone modifications act to tighten or loosen chromatin, either directly by removing the attraction between histones and DNA or by providing docking sites for chromatin remodelling complexes such as SWI/SNF [162]. These modifications are targeted events thus allowing distinct regions of chromatin to open allowing access to the gene. Previous studies have shown that histone modifications play a role in regulating MMPs [199-202] including the collagenases [158, 172] with one study showing binding of c-Fos/c-Jun dimer to a SWI/SNF subunit thereby governing trans-activation capacity [203]. My own data demonstrated a direct correlation between increasing histone H3 acetylation and pol II enrichment to

collagenase promoters, and collagenase expression. These data led me to hypothesise that following IL-1+OSM stimulation, histone modifications were gradually re-modelling the chromatin structure around the collagenase genes over a 24 hour duration, resulting in more transcriptionally permissive chromatin and therefore increasing pol II enrichment and increasing gene expression.

Histone modifiers such as, HDAC 1 and 2 and the histone acetylase (HAT), p300, are constitutively expressed in chondrocytes [158, 174, 204]. As such, histone modifications would be able to be carried out despite protein synthesis inhibition and therefore if collagenase gene regulation was dependent on histone modifications this inhibition would not affect *MMP1* and *MMP13* gene expression. However, my data demonstrate that protein synthesis inhibition abrogated collagenase gene expression for up to 8 hours following the initiation of IL-1+OSM stimulation. This was a crucial finding as it showed that histone modifications were not able to account for the observed temporal deficit. Furthermore, these findings also suggested that the basic building of transcript level over time was unlikely to account for the temporal deficit as transcription is unaffected by protein synthesis inhibition. Previous study is limited with regard to the effects of protein synthesis inhibition on *MMP* expression, with no literature in the context of pro-inflammatory stimulation of chondrocytes. Some literature corroborates my own findings in cancer cell lines following stimulation with TGF-β [198], while other studies in osteoblastic cell lines show that inhibition of *de novo* protein synthesis does not affect *FOS*, *JUN* or *MMP13* expression following mechanical stress and ultrasound [205-207]. This is to be expected in the case of *FOS* and *JUN* as they are both IEG, however, the lack of effect on the known secondary response gene, *MMP13,* which, by its definition, requires *de novo* protein synthesis for its expression is somewhat questionable.

An observation I found interesting was that *FOS* silencing had a greater effect on *MMP1* and *MMP13* expression than *JUN*. A reason for this may be attributed to c-Jun promiscuity. c-Jun is capable of dimerising with proteins from either Fos or Jun families whereas c-Fos can only dimerise with Jun family members [208]. Therefore, when *FOS* is silenced the induction of c-Jun by IL-1+OSM may lead to a high proportion of c-Jun homodimers, an AP-1 complex that is less capable of DNA binding than heterodimers [138, 209]. Conversely, *JUN* silencing could still result in heterodimers of c-Fos with

other Jun members that retain a slightly reduced DNA binding capacity [210]. This would also go some way to explain why there was no difference in *MMP13* expression between *FOS* silencing alone or in combination with *JUN* silencing. *MMP1* expression however does decrease more significantly when both *FOS* and *JUN* are silenced compared to *FOS* alone. This could be due to differential regulation of *MMP1* and *MMP13* that has been postulated previously by Catteral et al [82] and Litherland et al [97, 104]. Indeed, the component members of the AP-1 homo/heterodimer govern the induction of different genes, at different times in many cells types throughout the body [208]. The regulation of *MMP1* may be more strictly regulated by c-Fos/c-Jun heterodimer and so more susceptible to *JUN* silencing and altered heterodimer partners than *MMP13.* In-line with this, though not significant, *MMP1* expression decreased to a greater extent compared to *MMP13* following *JUN* silencing. Evidence for this differential regulation of functionally similar genes by different AP-1 dimer constituents is observed in the control of cell cycle progression [139].

siRNA gene silencing was employed to determine the role of AP-1 factors in collagenase expression. Although siRNA gene silencing is a useful tool to understand gene regulation it has limitations. Transfection efficiency is never 100%, and thus, the degree of gene silencing achieved is subject to the transfection efficiency and potency of the siRNA. Furthermore, gene knock-down occurs only transiently as the finite amount of siRNA transfected into the cell is degraded along with the target mRNA by RISC [211]. As such it is the cellular process hijacked by the *in vivo* administration of dsRNA that ultimately leads to siRNA degradation. This means that, over time, *FOS* and *JUN* silencing will diminish, allowing for new protein synthesis to occur and their function within the cell to be detected. However, in this context I demonstrate the rapid transient gene induction of *FOS* has returned to unstimulated levels following approximately 2 hours of IL-1+OSM stimulation. The loss of gene silencing, therefore, should not significantly affect knock down. Another potential concern is the difference in transfection efficiency within different HAC populations. The ECM surrounding cultured HAC severely affects transfection efficiency [212, 213]. Some HAC populations may be capable of synthesising ECM at a greater rate than others, a characteristic that may well be exemplified by the individual populations differing capacity for gene expression following cytokine stimulation [72]. As such increased ECM deposition

would impede transfection to a greater extent and therefore lead to less efficient gene silencing in that population of HAC. Another consideration when using siRNA gene silencing is that many commercially available gene-specific siRNA have a certain degree of non-specific, "off-target" gene silencing [214, 215]. Alongside the obvious unwanted effects of non-specific gene silencing, the specifically targeted genes may, consequently, be subject to less efficient silencing. Differences in *FOS* and *JUN* siRNA efficiency will therefore also contribute to the differences in protein induction and the expression of collagenases. There are many alternative methods that could be used including short hairpin (sh)RNA, antisense oligonucleotides and morpholinos. There are also inhibitors that selectively inhibit c-Fos binding to AP-1 elements in murine models [159]. However, these methods also have their own disadvantages and when considering the experimental questions being asked in my study, siRNA-mediated gene silencing was an appropriate technique that was succesfully used to answer them.

One of the more striking observations from the above study was that c-Fos, at the level of gene and protein induction, was rapidly and transiently induced. Other studies have demonstrated this characteristic of *FOS*: Pre-adipocytes stimulated with growth hormone [216], monocytes stimulated with TPA [217], and pulmonary smooth muscle cells stimulated with serotonin [218] all display this transient gene expression. The variety of stimuli and cell type that elicit the same response coupled with similar work carried out in chondrocytes stimulated with IL-1 alone or in combination with OSM [82] suggest that this rapid induction and equally rapid degradation curtails protein function. A rapid and robust onset acts like an 'on' switch resulting in a relatively instant abundance of mRNA and protein. Coupled with rapid protein degradation [219] and high mRNA instability (dependent on a 3'UTR mRNA destabilising element) [220, 221], c-Fos is only allowed to function in a very short period of time. The *FOS* gene is therefore termed an IEG in that there is no or very little expression occurring at basal conditions with rapid and transient induction following stimulus [189]. This is a very important level of regulation, as c-Fos is crucial in cell differentiation and proliferation [222]. Thus, c-Fos oncogenic function is well documented to play a role in both cancer growth and the down regulation of tumour suppressor genes [223] with over expression of c-Fos correlating to many types of cancers and their individual severities [223-226]. The speed with which the c-Fos signal is returned to unstimulated levels

therefore abrogates oncogenic potential. This rapid and transient induction thus confirms that c-Fos is an important transcriptional regulator immediately after the initiation of IL-1+OSM stimulation of HAC. It also shows that aberrant collagenase expression is not as a result of a loss in c-Fos regulation.

c-Jun also adheres to a similar temporal profile at the level of both gene and protein induction as c-Fos (supported in further studies [227-229]). Furthermore, *FOS* exhibited a very distinct transient peak in expression, whereas *JUN* expression, although decreased post 1 hour, remained significantly enhanced throughout the 24 hour IL-1+OSM time course. A potential explanation for this is that c-Jun activity is dependent not only on its mere presence (as with c-Fos) but also by post-translational modifications that improve its ability to bind to DNA. The JNK-mediated phosphorylation of serine 63 in the NH_2-terminal transactivation domain of c-Jun dramatically enhances transcription factor activity [230-232]. As such gene expression does not require the strict control enacted upon it through protein and mRNA degradation as seen with c-Fos and therefore no functional consequences of persistently high mRNA levels are seen. Previous experimentation has shown that use of pan-c-Jun antibody revealed no effect on protein levels following IL-1+OSM stimulation [97]. Accordingly, western blot analysis for c-Jun was carried out with an antibody specifically for endogenous serine 63 phosphorylated c-Jun and as such could explain why protein levels were more transient than c-Fos. The proto-oncogene capacity of *JUN* [233, 234] shows the need for strict regulation of its activity, like that required for *FOS*. As such, c-Jun effector function is as rapid and transient as c-Fos, however, this functional profile is imparted at the level of post-translational phosphorylation, whereas c-Fos is regulated at the level of gene expression and degradation. This demonstrates that although *JUN* expression was not as robust or as transient as *FOS*, it is still capable of contributing equally to gene regulation.

An interesting observation was that the decreases in *FOS* expression post 1 hour IL-1+OSM stimulation occurred in not only gene expression but also c-Fos protein production and DNA binding. However, changes in protein levels are not as transient as gene expression or DNA binding. In the case of c-Fos promoter enrichment, it would be expected that this would correlate with protein and not gene expression. The reason for this may simply be that as the transcription machinery is assembled at the

promoter post c-Fos binding, the epitope to which the c-Fos antibody binds is 'masked' preventing antibody binding and pull down of gDNA cross-linked to it [235]. Another explanation may be that due to the high amount of c-Fos generated in the chondrocyte following 1 hour IL-1+OSM stimulation, there may be non-functional binding to AP-1 elements on proximal promoters that otherwise would not readily allow factor binding. ChIP may therefore demonstrate a false positive at this stimulation duration. However, this is unlikely as protein levels (Figure 15) indicated that c-Fos binding to chromatin was more finite than its presence within the soluble nuclear fraction, perhaps, in-part, due to saturation of binding sites within the genome. This in itself is worth taking into consideration in that chromatin-bound levels are an indicator of total c-Fos binding, not collagenase promoter binding. This means that c-Fos enrichment may well be as finite as indicated in my ChIP studies and that soluble nuclear and chromatin-bound protein levels are misleading as they do not demonstrate binding to a specific gene. The assembly of transcriptional machinery is a dynamic system by which factors are recruited, modified and replaced rapidly [236]. c-Fos bound to the promoter may, therefore, be concealed within the growing transcriptional complex being assembled at the promoter [171] and therefore c-Fos is unable to be immunoprecipitated. As such I cannot be entirely sure that the binding of c-Fos to collagenase promoters is as transient as my ChIP data suggest. This does not call into question the need for other contributing factors but does suggest that c-Fos may have a less transient role in collagenase gene regulation.

Study in this chapter serves to highlight the false assumption that c-Fos/c-Jun AP-1 are solely responsible for the regulation of collagenase genes. It is now evident that other regulatory factors are being produced subsequent to c-Fos/c-Jun which significantly contribute to this complex mechanism of transcriptional regulation. Further study, therefore, will focus on trying to elucidate these factors.

3.2.1 Summary

- c-Fos/c-Jun gene expression, protein production and collagenase promoter binding occur rapidly yet transiently following IL-1+OSM stimulation in HAC.

- There is a temporal deficit between the transient gene induction/collagenase promoter enrichment of c-Fos and the initiation of collagenase gene transcription.

- Though important, c-Fos/c-Jun is not the sole transcriptional regulator of collagenase genes, with the induction of other contributing factors after the transient induction of AP-1 necessary in order to allow effective transcription.

4 Chapter 4: Identification of novel factors contributing to collagenase transcriptional regulation

4.1 Introduction

Observations within Chapter 3 demonstrated a temporal deficit between the transient induction of c-Fos/c-Jun AP-1 factors and the initiation of collagenase transcription. I hypothesised that new factors, critical for the transcriptional activation of collagenases, were being synthesised post-AP-1 induction in order to effect the subsequent IL-1+OSM-mediated collagenase gene expression in HAC.

The concept that collagenase transcriptional regulation is more complex than simply c-Fos/c-Jun promoter binding is rarely addressed. Long standing dogma states that only a very limited number of transcription factors are of critical transcriptional importance, other than AP-1 [112, 113]. Moreover, there is little reference to temporal recruitment of AP-1 members in chondrocytes [158]. As such, this level of regulation and the dynamic changes that occur over time in response to IL-1+OSM remain under-investigated. Greater knowledge about the transcriptional regulation of collagenases may highlight factors that display specificity toward the expression of the enzymes that drive collagen degradation in arthritis.

With its ubiquitous nature in orchestrating early gene regulatory events, AP-1 factors were also believed to be playing a role, not only in the regulation of collagenase gene expression but also in the expression of these other contributing factors. This hypothesis was based on the fact that rapid induction of *FOS* did not result in immediate collagenase gene expression, yet the AP-1 heterodimer, c-Fos/c-Jun, has proven functional importance in collagenase gene expression [97, 158, 159]. As such, I postulated that AP-1-dependent transcriptional regulation was due in-part, to indirect gene regulation of other factors that themselves contribute to collagenase gene regulation.

I therefore needed to determine what kinds of factors were governing this transcription, post-AP-1 peak; this would have implications on the method by which I elucidated these factors. I decided that assessing global gene expression with the use of DNA microarray would allow me to be able to observe changes in expression patterns of genes between certain time points. It would also allow for internal validations to be carried out, thereby allowing me to compare expression data with previous observations in order to confirm appropriate HAC gene expression in response to IL-1+OSM. This would highlight genes that could be potentially regulated by AP-1, or at least be expressed within the temporal deficit between peak AP-1 induction and the start of collagenase gene expression.

Using prior validation and quality controls, genome-wide gene expression changes were assessed using a DNA microarray of IL-1+OSM-stimulated HAC for durations intended to reveal genes contributing to collagenase transcriptional regulation. This was followed by rigorous assessment of genes and post-hoc control analysis.

4.1.1 Individual chondrocyte population validation prior to DNA microarray analysis

Before I could begin to extract RNA from HAC the durations of IL-1+OSM stimulation, intended to elucidate contributing genes, needed to be determined. This was necessary because stimulations for experimental validation and RNA isolation for the DNA microarray were carried out in parallel. This meant that I could not validate individual HAC populations prior to RNA extraction. Therefore, I had to treat each population as a potential candidate for DNA microarray study.

The decision as to which stimulation durations were used to assess changes in gene expression via the DNA microarray was of great importance. The HumanHT-12 v4 Expression Beadchip used for DNA microarray analysis contains 12 wells each containing 47,000 transcript probes dotted on to their surface. I decided that I would carry out the gene expression analysis in 3 HAC populations and pool the data. Consequently, I was able to stimulate each population at a maximum of 4 different durations. Enrichment of the collagenase gene promoter region by c-Fos, *FOS* expression and c-Fos protein induction all reached their maximal levels following 1 hour IL-1+OSM stimulation. Furthermore, *MMP1* and *MMP13* expression peaks

following 24 hour stimulation. As such 1 hour and 24 hours durations, as well as unstimulated condition, were chosen for use as controls and comparators for one other duration. I decided that this duration had to be after 1 hour in order to reveal gene expression that could be accounting for the temporal deficit. It also had to be very soon after 1 hour in order to improve the likelihood of elucidating AP-1-dependent gene expression. In Figure 23, the reduction in *FOS* expression, following 1 hour maximal expression to 1.25 hours was greater than that of 1.25 to 1.5 hours indicating the extremely transient nature of *FOS* and the potential for rapid induction of the genes being regulated by it. As such I decided that 1.25 hour stimulation may reveal genes that contribute to collagenase gene regulation and along with 1 and 24 hours, as well as unstimulated control, were used in subsequent experiments (as discussed in section 4.2).
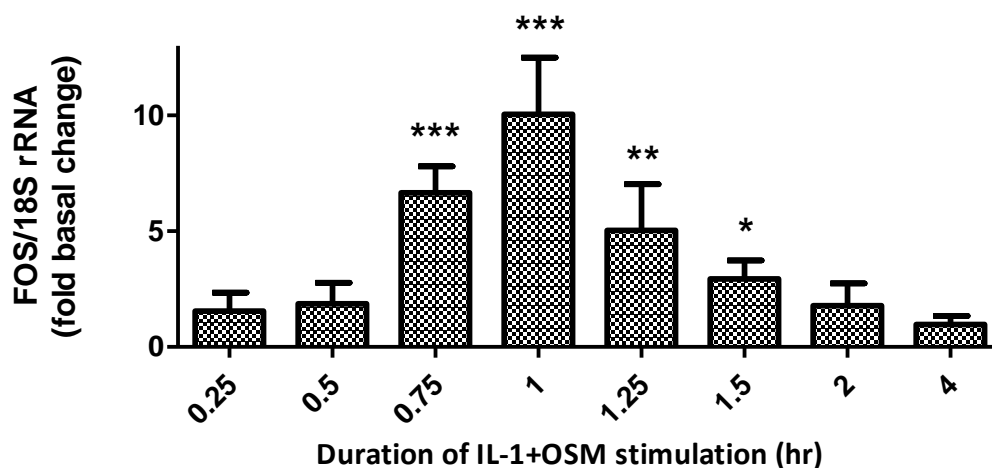
Figure 23: Real-time PCR analysis of *FOS* gene expression following different durations of IL-1+OSM stimulation. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. Cell lysates were subjected to real time PCR and relative expression levels of *FOS* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Data are representative of two separate experiments (each assayed in hextuplet) each using chondrocyte cultures from different donors (Mean ± SD; where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs basal). HAC populations were confirmed to demonstrate synergistic expression of *MMP1* following 24 hour IL-1+OSM stimulation versus IL-1 or OSM alone (data not shown).

4.1.1.1 Selection of HAC populations to be analysed via DNA microarray

Gene expression responses by HAC can be highly variable [72], in that some populations will respond poorly to enzymatic digestion and consequently cells can fail to grow correctly and/or have little response to synergistic IL-1+OSM stimulation. To

avoid the failure of microarray analysis based on erroneous HAC responses, I subjected isolated HAC populations to a strict series of validations and quality controls to confirm both gene expression and the ability of IL-1+OSM to synergistically induce collagenase expression.

Figure 24 to Figure 27 present the experimental evidence that was used to select HAC populations that demonstrated appropriate, previously described characteristics. I then selected samples for DNA microarray based on the following criteria:

1) Populations demonstrated a robust, high magnitude expression of *MMP1* and *MMP13* expression over basal levels [72, 82, 237].

2) The expression of collagenases by individual HAC populations, in response to IL-1 alone and in combination OSM, was synergistic [72, 82, 237].

3) Gene expression (*FOS*, *MMP1* and *MMP13*) and protein induction (c-Fos) at stimulation durations intended to elucidate factors contributing to collagenase transcriptional regulation in the DNA microarray corroborate with previous study in chapter 3.

4) RNA isolated from these populations met with the quality control standards required for use with DNA microarrays. This included my own spectrophotometric analysis of sample purity and external quality control checks (carried out by Cambridge Genomic services) using Agilent 2100 Bioanalyzer.

HAC were isolated from cartilage samples taken from 13 individual donors and assessed for *MMP1* gene expression. Figure 24 demonstrates the variation of *MMP1* expression in response to IL-1+OSM stimulation. Based on these results, the 6 populations that had the highest fold expression of *MMP1* (highlighted in red) were then subject to further validations of their gene expression. This was because HAC populations that demonstrated more robust gene expression, were more likely to better represent normal, IL-1+OSM-responsive chondrocytes.
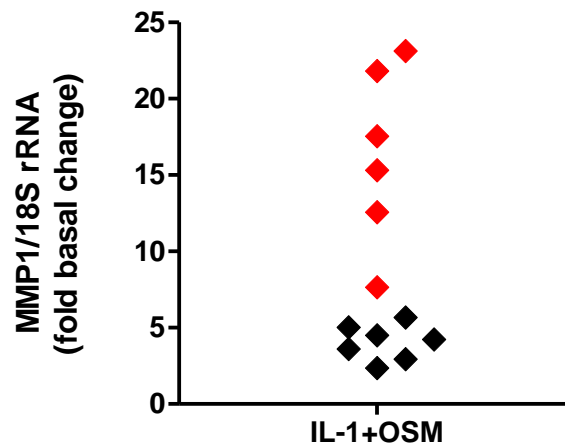
Figure 24: Real-time PCR analysis of IL-1+OSM stimulation response in 13 patient samples. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 24 hours. Cell lysates were subjected to real-time PCR and relative expression levels of *MMP1* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Each data point represents a separate chondrocyte culture from a different patient donor. Data points in red indicate donor populations used for further study.

Based on the synergistic response of chondrocytes to IL-1 in combination with OSM [76], Barksby *et al* [72] demonstrated that although the magnitude of collagenase expression changed between HAC populations, all the populations had a similar relative fold increase following stimulation with IL-1+OSM compared to IL-1 alone. As such, the expression levels of the 6 HAC populations selected from Figure 24 gave an indication of their validity for further study but did not confirm whether or not they demonstrated synergistic collagenase expression.

I next assessed if the 6 HAC populations exhibited enhanced collagenase expression following synergistic IL-1+OSM stimulation. Figure 25 demonstrates that *MMP1* expression, in all 6 populations, was significantly increased above basal conditions following 24 hour stimulation with IL-1 alone and in combination with OSM. Furthermore, in the majority of the HAC populations IL-1+OSM stimulation resulted in a significant increase in *MMP1* expression compared to IL-1 alone. In addition, the majority of the HAC populations exhibited increased *MMP13* expression following 24 hour stimulation with IL-1 alone and in combination with OSM. Only one population (donor 3) showed significant increase in expression following IL-1+OSM stimulation compared to IL-1 alone. As such, the lack of synergy was taken in to account when selecting the populations used for the DNA microarray.
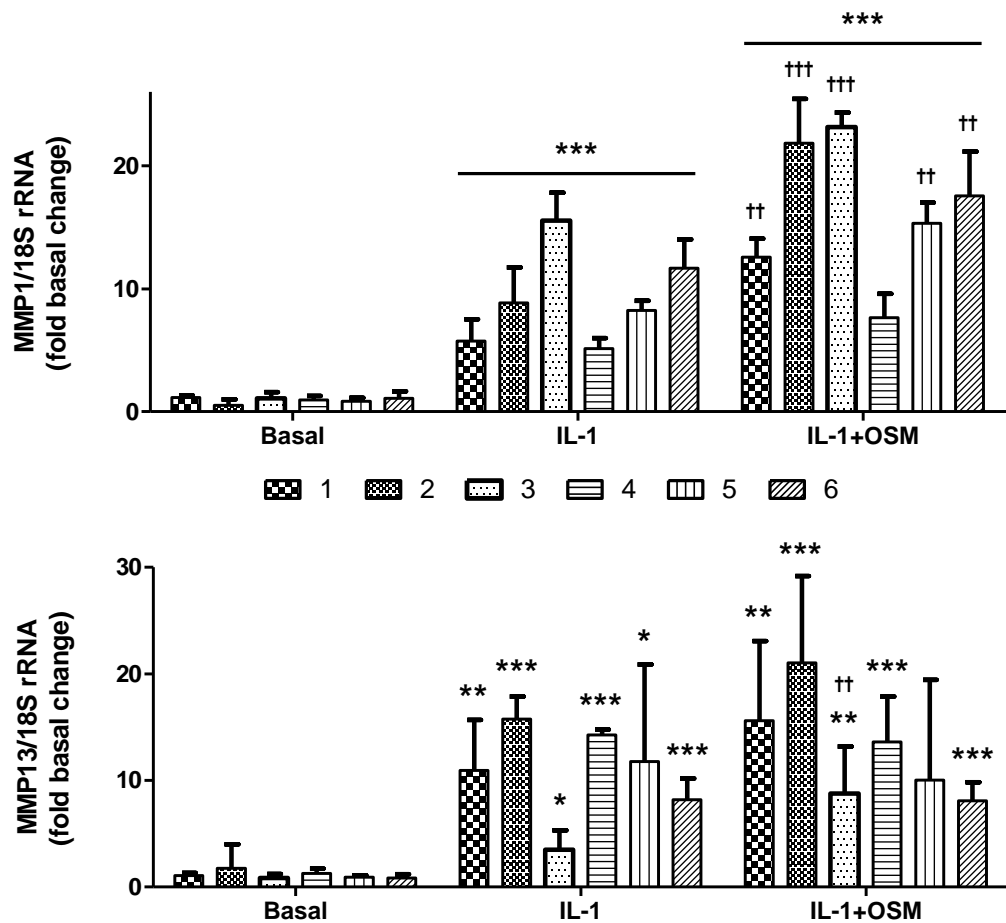
Figure 25: Real-time PCR analysis of *MMP1* and *MMP13* gene expression following IL-1 and OSM stimulation in 6 populations of HAC. 6 HAC populations taken from different donors (as indicated) were treated with IL-1 (0.05ng/ml), OSM (10ng/ml) alone and in combination for 24 hours. Cell lysates were subjected to real-time PCR and relative expression levels of *MMP1* and *MMP13* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Each population assayed in octuplet (stimulated vs basal; mean ± S.D. where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. I+O versus IL-1; mean ± S.D. where ††, $p < 0.01$; †††, $p < 0.001$)

To assess the IL-1+OSM-mediated induction of c-Fos in HAC population, protein was isolated, concomitantly with RNA, from the majority of the 13 HAC populations acquired. It must be noted that nuclear protein from only 4 of the 6 populations selected from Figure 24 was isolated because the nuclear localisation protocol failed to isolate protein and was changed during the time taken to acquire all the samples from different populations. In the 4 populations shown in Figure 26, c-Fos was not present in unstimulated conditions as previously observed. Following 1 hour IL-1+OSM stimulation c-Fos was present in all populations with levels returning to unstimulated levels (donor 2 and 3) or markedly decreasing (donors 4 and 6) following 24 hour stimulation. Levels of c-Fos protein remained comparable between 1 and 1.25 hours in donors 2, 4 and 6, with levels in donor 3 decreasing. This meant that of the populations tested, temporal c-Fos protein profile of donor 2 and 3 were in-line with previous

observations in chapter 3. These results were taken into account when selecting the populations to be used in DNA microarray study.
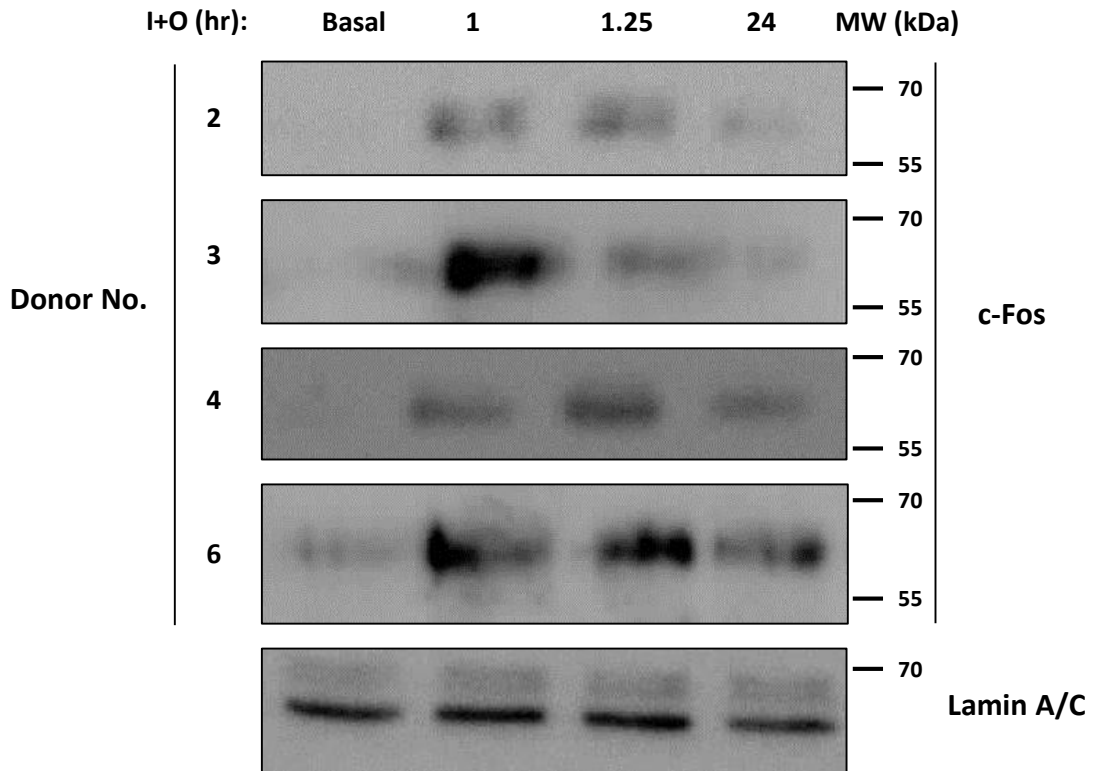
Figure 26: Western blotting for c-Fos in nuclear lysates generated from HAC stimulated with IL-1+OSM 4 HAC populations taken from different donors were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to cytoplasmic-nuclear protein fractionation. Proteins from nuclear fraction were resolved using SDS-PAGE and immunoblots probed using c-Fos antibody. Donor 2 blot was re-probed for Lamin A/C and used as loading control.

The six HAC populations that demonstrated the highest fold increase in *MMP1* expression, as shown in Figure 24, underwent final validation in order to determine the suitability for use in DNA microarray study (Figure 27). Control genes (*FOS*, *MMP1* and *MMP13*) were assessed following IL-1+OSM stimulation at 1, 1.25, or 24 hours. As previously demonstrated, *MMP1* and *MMP13* expression remained low at both 1 and 1.25 hours post IL-1+OSM stimulation, whereas 24 hours post stimulation resulted in a marked increase in expression of collagenase genes. Furthermore, each population exhibited the previously observed increase in relative *MMP1* expression compared to *MMP13* ([45] and in Chapter 3). In addition, the six HAC populations exhibited enhanced *FOS* expression following 1 hour IL-1+OSM stimulation compared to basal, which rapidly decreased after 1.25 hours stimulation duration (as observed in Figure 23) and were barely detectable following 24 hours stimulation. This confirmed that

expression of control genes, as assessed from the RNA extracted for use in the DNA microarray, were responsive to IL-1+OSM, as previously demonstrated.

Following experimental validations outlined in Figure 24 to Figure 27, I selected 3 populations (donors 1-3) that I believed to be good representations of synergistically responsive HAC, based on the combined selection criteria (as above). DNA microarray analysis was then performed on HAC populations 1-3 including the experimental conditions: basal, and 1, 1.25, and 24 hours post IL-1+OSM stimulation.
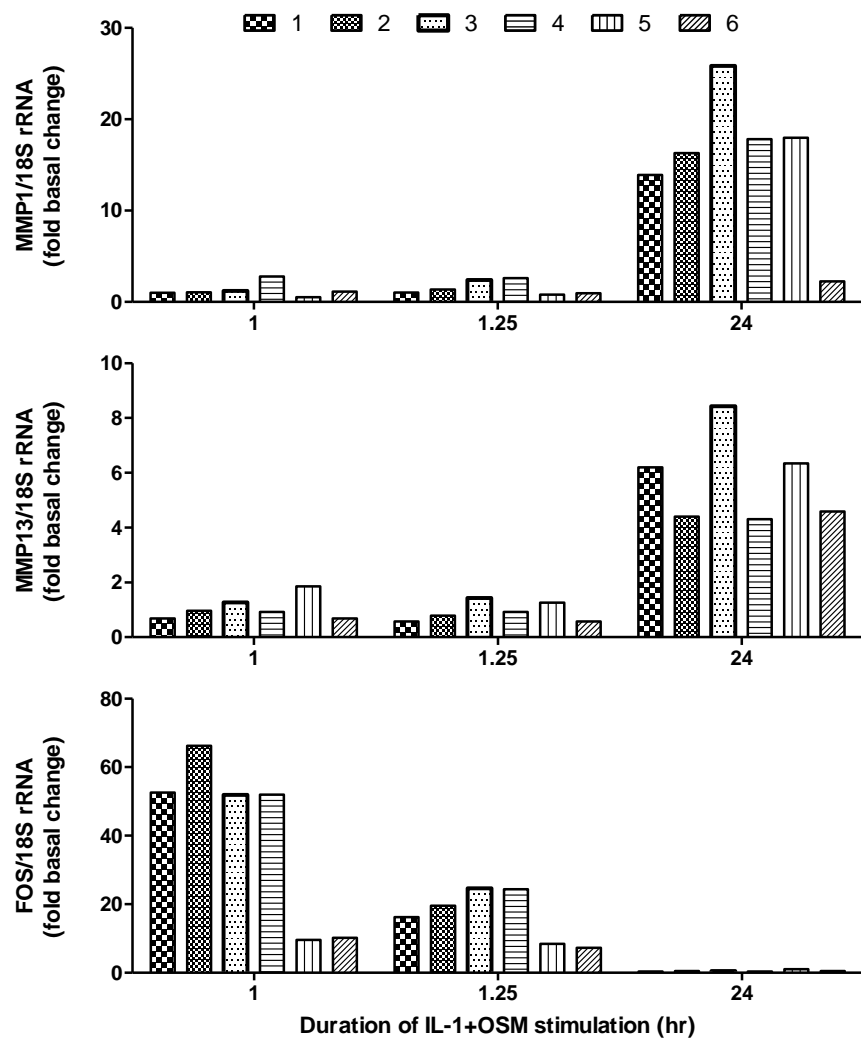
Figure 27: Real-time PCR analysis of *MMP1*, *MMP13* and *FOS* following IL+OSM stimulation of HAC. 6 HAC populations taken from different donors (as indicated) were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. RNA was isolated from cells and subject to real time PCR and relative expression levels of *FOS, MMP1* and *MMP13* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined.

4.1.2 DNA microarray

The RNA samples were then hybridised on to a HumanHT-12 v4 Expression Beadchip (Illumina) which has 47,000 transcripts and known splice variants, known as features, spotted onto its surface. Relative hybridisation to these features is then measured giving a raw data value. The data from all 3 populations at each time point was then subject to comprehensive analysis and data visualisation with GeneSpring software. This allowed a vast amount of raw, arbitrary hybridisation values to be compared and contrasted between the different IL-1+OSM durations. It also enabled the pooling of relative gene expression from the different populations and subsequent statistical analysis.

GeneSpring also allowed the integrated analysis of heterogeneous data. The degree of heterogeneity was evident from hierarchical clustering of the genome in the different populations at the different stimulation durations. Clustering in this manner identifies linkages and concordant expression patterns to cluster the genes in a dendrogram (tree diagram) as seen in Figure 28A and, specifically, for the 65 most significantly altered genes in Figure 28B. There are notable observations that came as a consequence of this hierarchical clustering. Firstly, all 24 hour stimulation durations from all three HAC populations clustered together, independently of other durations in those populations. This indicates that there is marked difference in global gene expression between very early and late time points. This could be due to the fact that very few genes respond with such immediacy to IL-1+OSM stimulation. It could also mean that few genes demonstrated any responsiveness to IL-1+OSM stimulation within the cell, which is entirely possible considering many genes are constitutively active in all cells and/or in differentiated cells, like chondrocytes. Also of note is that donor 1 samples (apart from 24 hour duration) cluster together, whilst donor 2 and 3 cluster together according to duration, not donor. This is evidence that, despite extensive validations in order to control for HAC population variability, difference in population gene expression had occurred. As such gene expression at early time points and basal controls in donor 1 was different to that of donor 2 and 3 which had similar temporal gene expression patterns to one another.

In Figure 29, the *FOS* gene expression for individual donors was taken from the raw data of the DNA microarray and re-plotted with separate histograms for each. This figure demonstrates that although the populations used for the microarray varied in the magnitude of *FOS* expression, the change in expression between durations in the same population is relatively similar in all three donors. I therefore decided that it was not necessary to omit data from donor 1, based on the fact that in Figure 28 the donors clustered differently, as all demonstrated similar relative *FOS* expression.
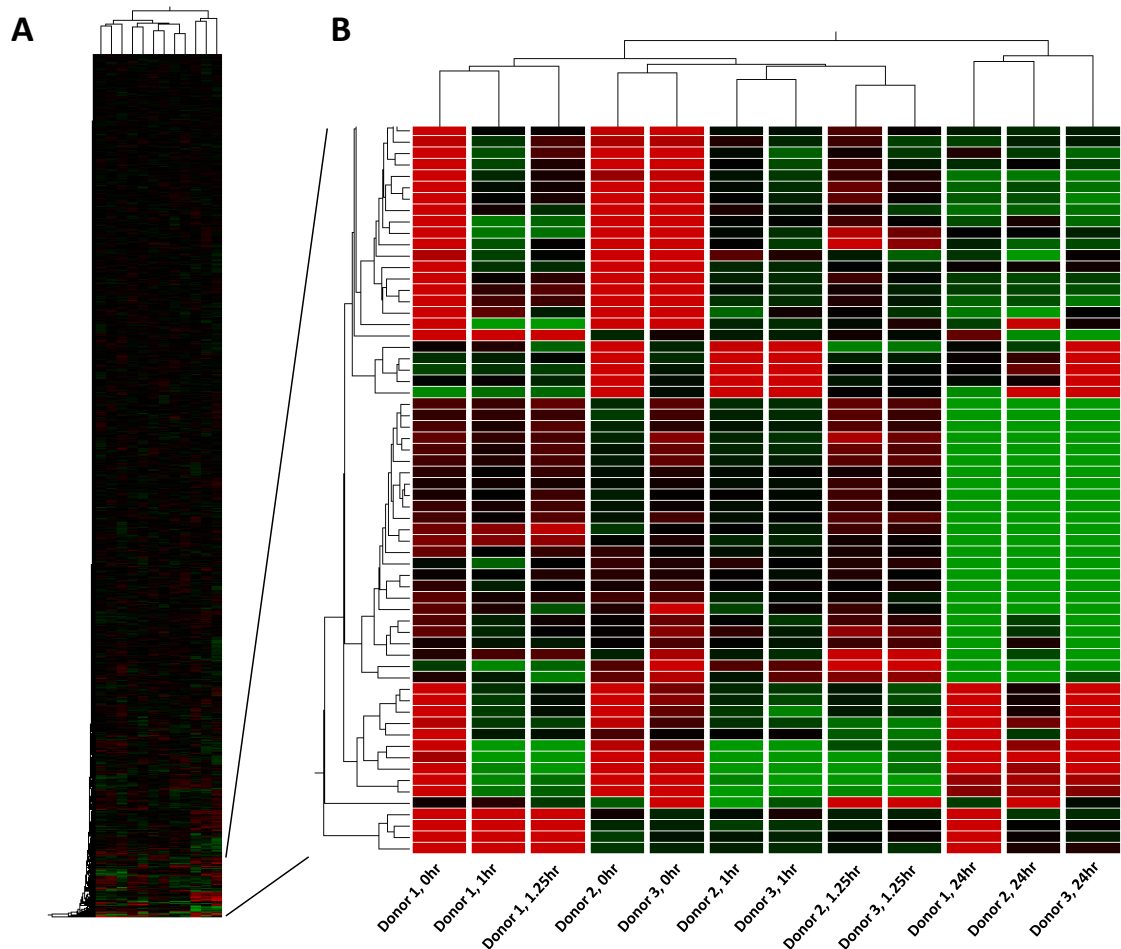
Figure 28: Genome-wide heat map analysis. HAC populations isolated from different donors (Donor 1-3) were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. RNA was isolated from cell lysates and hybridised on HumanHT-12 v4 Expression Beadchip kit to assess changes in gene expression. **A**, global representation of changes in gene expression within the genome of the 3 populations. **B**, representation of expression of the first 65 clustered genes. Green represents genes with increased expression, red for decreased expression and black for genes with no change in expression.
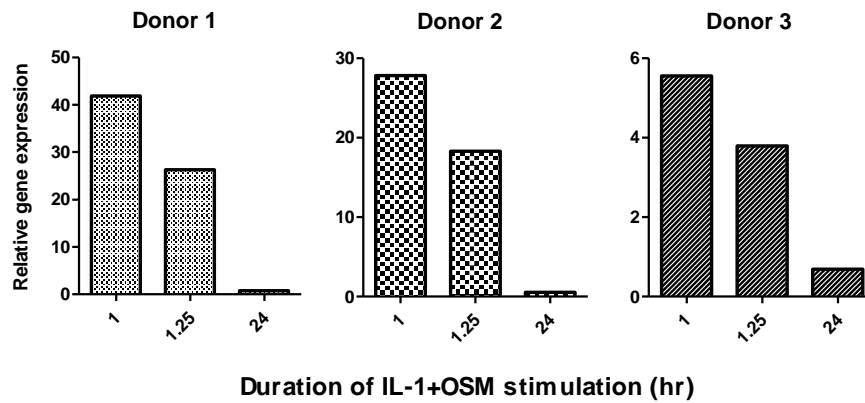
Figure 29: Relative *FOS* gene expression in HAC populations isolated from three separate donor samples. 3 HAC populations were taken from different donors (as indicated) and treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. RNA was then isolated from cell lysates and hybridised on a HumanHT-12 v4 Expression Beadchip to assess changes in gene expression. *FOS* expression is plotted relative to unstimulated control.

In Figure 30, the mean values of the fold change in expression of individual genes generated by the array for the three populations at each IL-1+OSM duration of stimulation were plotted on Volcano plots. As such every gene that changed in its expression between the indicated IL-1+OSM stimulations is represented as a single data point. Genes that exhibited a significant change in expression, with a corrected p-value ≤ 0.05, were plotted in combination with a two-fold change threshold demonstrating the cut-off point at which genes below this expression level would be omitted from the study. This was a higher threshold than other microarray studies in this research area [238-240] and so was intended to strictly limit the pool of genes carried forward for further study. It also meant that genes demonstrating a robust change in expression were more likely to be relevant to study than those with only moderate fold change. The change in threshold for 1 to 1.25 hours (Figure 30D) was due to the fact that only one entity from the DNA microarray was significantly elevated ≥2 fold and this entity did not code for a gene but for a miscellaneous RNA transcript. Lowering this relatively high threshold meant that I could compare the few genes that changed in their expression in this comparison group.

The genes of interest highlighted in red within these Volcano plots serve as controls for appropriate IL-1+OSM-dependent gene expression (*FOS*, *JUN*, *MMP1* and *MMP13*) and candidates for genes that either increase in their expression after *FOS* peak or remain up-regulated between early time points (*AXUD1*, *ATF3*, *EGR1* and *EGR2*). For example, in Figure 30A *FOS* is one of the most highly expressed genes following 1 hour IL-1+OSM stimulation. In Figure 30B it remains highly expressed but the fold change is

dramatically decreased compared to Figure 30A. This observation is complemented by Figure 30D, E and F which show that *FOS* is consistently one of, if not the most down-regulated genes at stimulation durations after 1 hour. The expression of *MMP1* and *MMP13* only increase in Figure 30C, E and F. These graphs all compare gene expression changes between early time points (C, basal; E, 1 hour; and F, 1.25 hours) and 24 hours. These observations indicate that the expression of *FOS* is early and transient and that collagenases are expressed at much later time points, in line with previous observations. With the lack of any substantial increase in genes between 1 to 1.25 hours none of the 11 genes, comprised of chemokines, receptors and non-coding RNA were deemed to be relevant to the study. The focus of the study then shifted to genes that increased from basal to both 1 hour and 1.25 hour. *EGR2* and *ATF3,* along with *FOS,* are significantly up-regulated following 1 hour stimulation (Figure 30A) however following 1.25 hours (Figure 30B) *EGR2* and *ATF3* remain up-regulated whereas *FOS* has significantly decreased (Figure 30D). This shows that these genes may well be candidates for further investigation.

As a result of these data, genes possibly contributing to collagenase regulation were selected from just two comparison groups: genes changing in expression from basal to 1.25 hour post IL-1+OSM stimulation and from basal to 1 hour. By comparing these sets of genes in combination with genes up-regulated from basal to 24 hours I was able to discount genes that had not returned to low levels following 24 hours stimulation and thus did not demonstrate transient gene expression. Moreover, by not including genes only increasing in expression from basal to 1 hour I was able to omit genes that were unlikely to be contributing to transcription at time points after AP-1 induction.
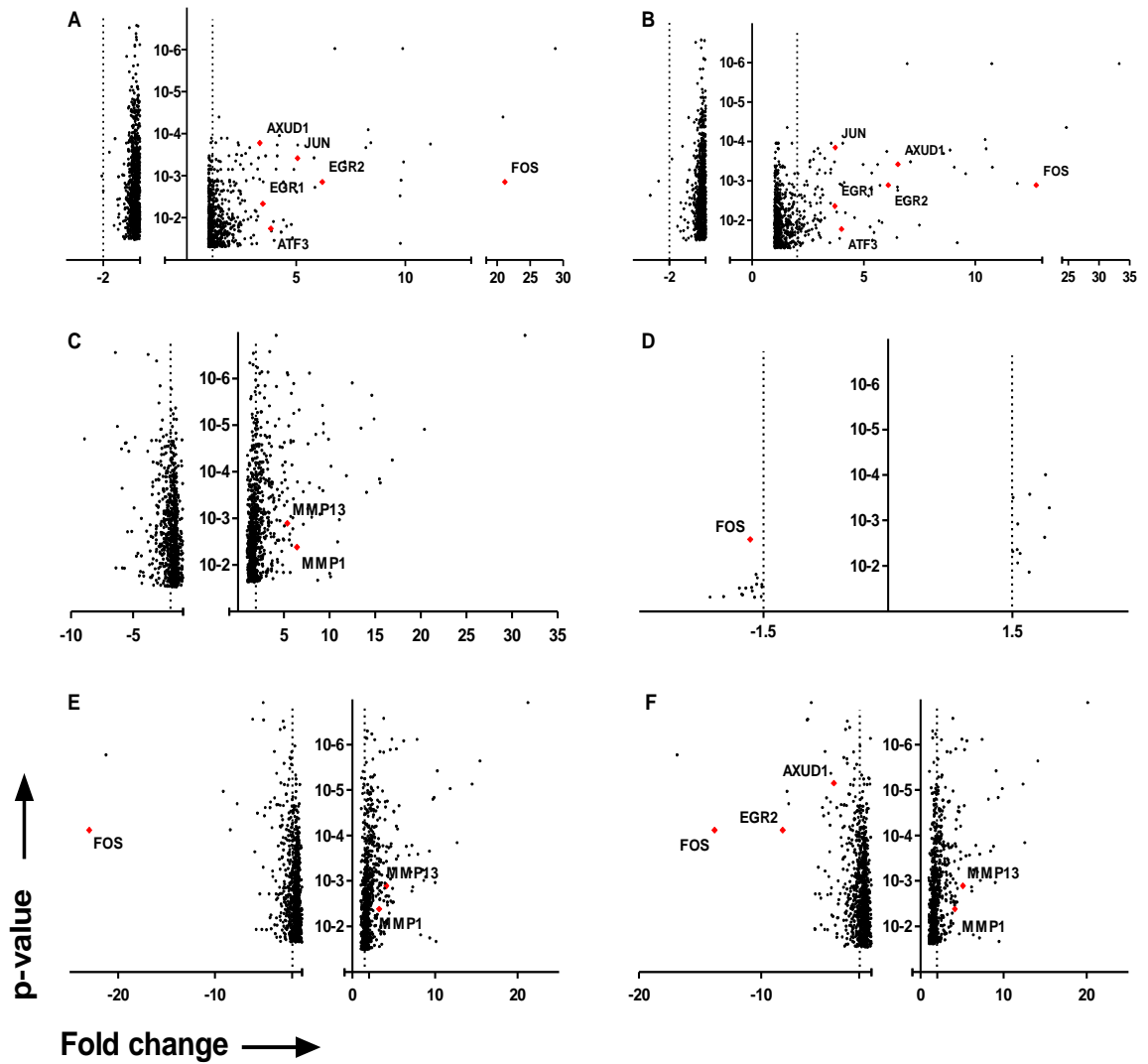
Figure 30: Volcano plots showing changes in gene expression between different durations of IL-1+OSM stimulation of HAC. Scatter plot diagrams showing fold up and down regulated gene expression changes between two IL-1+OSM stimulation durations and their significance. Data represent the average fold gene expression of 3 HAC populations. 2 fold (**A**, **B**, **C**, **E** and **F**) and 1.5 (**D**) fold threshold lines are indicated on individual plots. Gene expression comparison as follow: **A**, 1 hour IL-1+OSM vs Basal, **B**, 1.25 hour IL-1+OSM vs Basal, **C**, 24 hour IL-1+OSM vs Basal, **D**, 1.25 hour IL-1+OSM vs 1 hour IL-1+OSM, **E**, 24 hour IL-1+OSM vs 1 hour IL-1+OSM, and **F**, 24 hour IL-1+OSM vs 1.25 hour IL-1+OSM. Genes of interest have been highlighted in red.

4.1.2.1 Candidate genes selected for further study

The purpose of the microarray was to elucidate factors that may be contributing to the transcriptional regulation of collagenase genes soon after or as a consequence of c-Fos/c-Jun regulation following IL-1+OSM stimulation. By comparing the changes in global gene expression between early time points and control time points I was able to identify genes that may be relevant for further study. The selection process by which these genes were then chosen was based on several parameters that were intended to

highlight the most pertinent genes in the context of collagenase transcriptional regulation. These parameters were as follows:

1) Change in fold expression of genes was ≥2.

2) The change in gene expression had a significant corrected p-value.

3) Gene expression was induced after 1 hour IL-1+OSM stimulation and increased or remained high between 1 hour and 1.25 hours.

4) Literature search of genes adhering to parameters 1-3 indicated their possible relevance in the context of transcriptional regulation.

5) Assessment of the promoter of collagenases demonstrated that factors had the potential capacity to bind to specific sites within the promoter.

To assess the temporal expression of the individual candidate genes in each donor and assess whether these genes adhered to the third selection parameter (as above) I assessed the raw data values from the genome-wide expression analysis of the candidate genes. The majority of the genes in Figure 31 adhered to the third selection parameter; increasing from 1 to 1.25 hours and exhibiting reduced expression following 24 hours IL-1+OSM stimulation. This expression pattern was in-keeping with my previous hypothesis, whereby genes expressed after *FOS* peak may be contributing to the temporal lag that precedes the initiation of collagenase expression and that these may also be dependent on AP-1 for their own expression. *MMP1*, *MMP13, FOS* and *JUN* gene expression were also isolated from the genome-wide expression data to confirm validations carried out prior to the array (Figure 27). These selected candidates that may be contributing to collagenase regulation included the gene *ATF3*. ATF members can dimerise with members from the AP-1 family and bind to AP-1 elements [241] highlighting the possibility that cross-family dimerisation between ATF3 and AP-1 factors could contribute to the regulation of the collagenases. Similarly, there are many AP-1 factors other than *FOS* and *JUN* that increased in expression from 1 hour to 1.25 hours indicating that although important, c-Fos/c-Jun dimers may not be the only constituents of regulatory AP-1 dimers for collagenase gene regulation. It is also interesting that many members of the same transcription factor family also display the same gene expression profile (3 out of the 4 early growth response (EGR) and

immediate early response (IER) family members). This may simply be due to the fact they all have similar, overlapping function within the chondrocyte and so there is no differential regulation. Even so, they may all contribute to collagenase regulation. Although not adhering to parameter 3, Hypoxia-inducible factor-1alpha (HIF1A) is interesting as chondrocytes are subject to a hypoxic environment associated with OA progression [242] and so this induction may be of interest outside the postulated gene expression profile.

In Figure 32, a small proportion of the RNA extracted for the experimental validations for the 6 HAC populations (Figure 25 and Figure 27) was used to assess gene expression using real-time PCR with primers for the specific genes of interest in combination with probes from the universal probe library. One of these genes includes Axin-1-induced gene (AXUD1) which demonstrated notably similar gene expression profile in all 6 populations. Little study exists regarding this transcription factor and therefore it may prove to be a very interesting, novel candidate. As with HIF1A, bone morphogenetic protein (BMP)2 does not strictly adhere to parameter 3 but previous study has demonstrated it to be key to chondrocyte maturation [243], a process which leads to hypertrophy and aberrant collagenase expression [244]. The similar temporal changes in gene expression in the majority of the 6 populations for each of these genes allows for a greater confidence in the selected genes potentially playing a role in collagenase gene regulation.
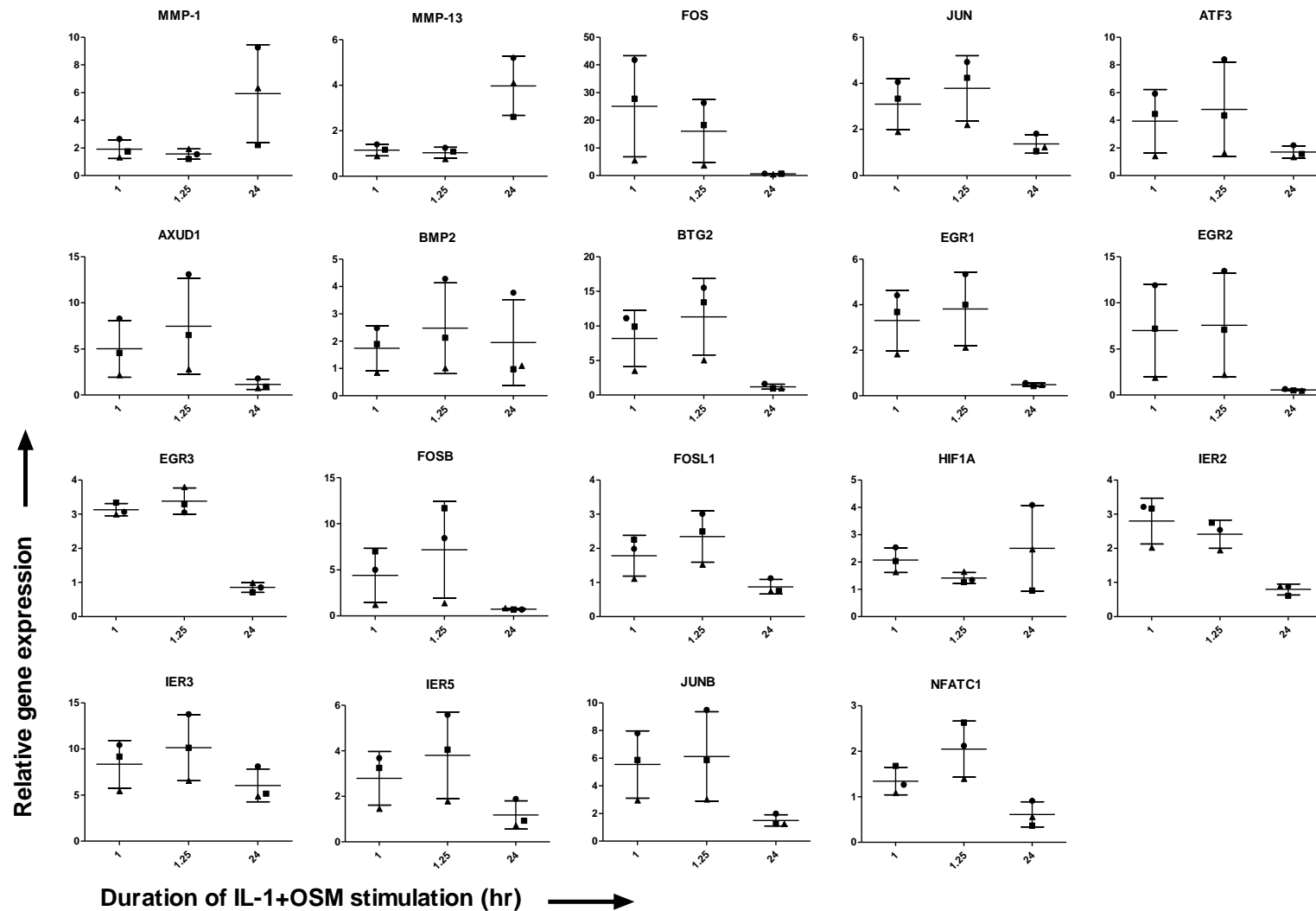
Figure 31: Relative gene expression generated from microarray 3 HAC populations were taken from different donors (1, ●; 2, ▲; 3, ■) and treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. RNA was then isolated from cell lysates and hybridised on HumanHT-12 v4 Expression Beadchip kit to assess changes in gene expression. Gene expression relative to unstimulated control of individual population was plotted for the indicated genes. Mean ±S.D.
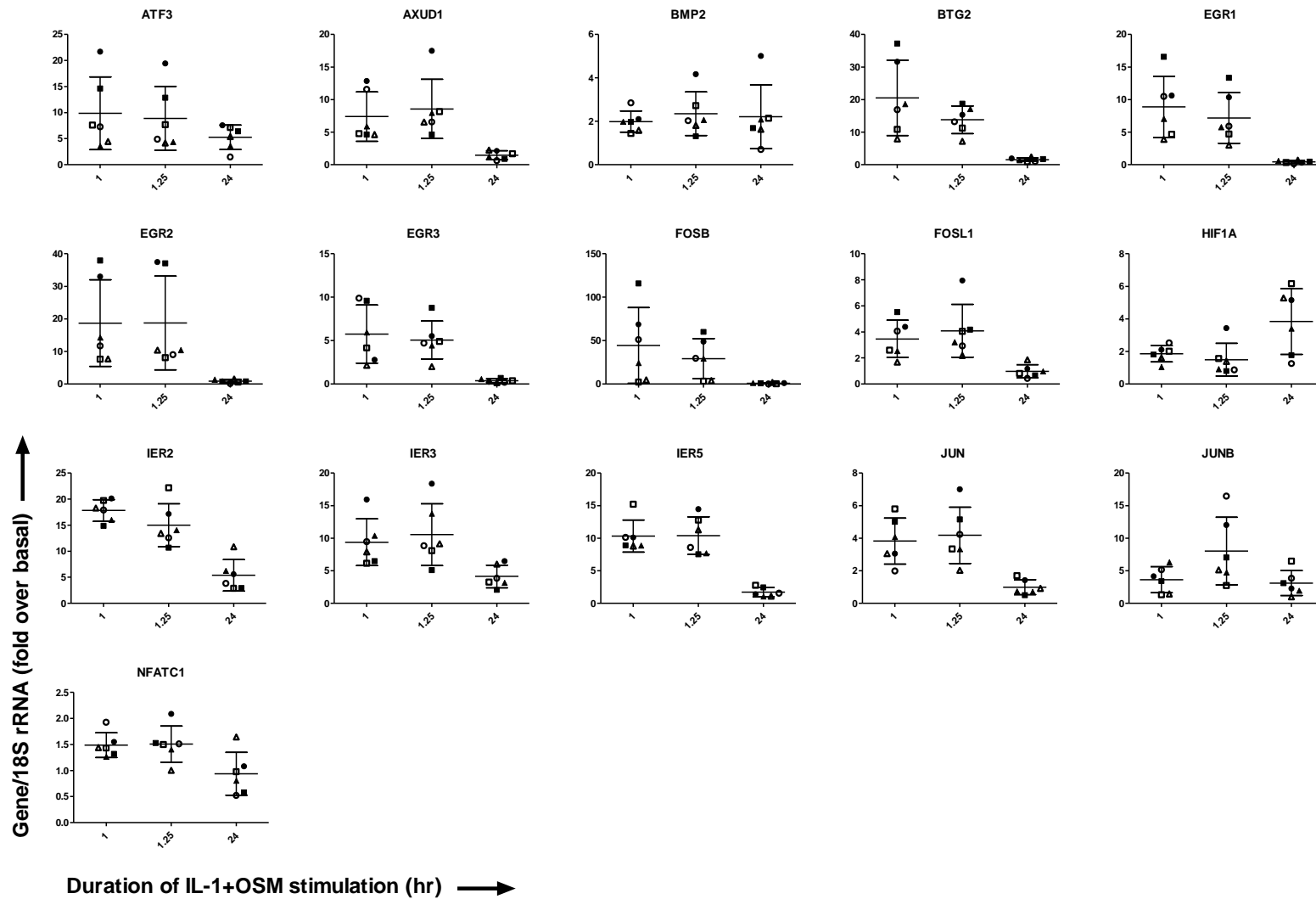
Figure 32: Real-time PCR analysis of gene expression. 6 HAC populations taken from different donors (1, ●; 2, ■; 3, ▲; 4, o; 5, □; 6, Δ) were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for indicated durations. RNA was isolated from cells and subject to reverse transcriptase-PCR and relative expression levels of indicated genes, normalized to the *18S* rRNA housekeeping gene, were determined. Mean ±S.D.

Genes that were deemed to be relevant and had trans-activating capacity were then subject to binding site prediction within the proximal promoters of *MMP1* and *MMP13* genes. The online software tool, PROMO [245], allows the identification of putative transcription factor binding sites within a designated section of DNA. A graphical representation is generated which combined with information on sequence specificity shows regions of transcription factor binding on the collagenase promoters (Figure 33). Analysis of one such transcription factor of interest, Nuclear factor of activated T-cells cytoplasmic 1 (NFATC1), showed that it had multiple binding sites within both the *MMP1* and *MMP13* promoter indicating that it may be involved in trans-activation of these genes. Calcineurin is a phosphatase that de-phosphorylates NFATC1 allowing it to shuttle into the nucleus [246]. The calcineurin inhibitors, cyclosporine and tacrolimus are used in combination with methotrexate to successfully treat active RA [247]. Other studies have also shown that the treatment of an OA mouse model with cyclosporine attenuates cartilage degradation [248]. These studies, taken with its potential to bind and trans-activate both the collagenase promoters means that *NFATC1*, as well as these other highlighted factors, is a very interesting gene candidate for further study.
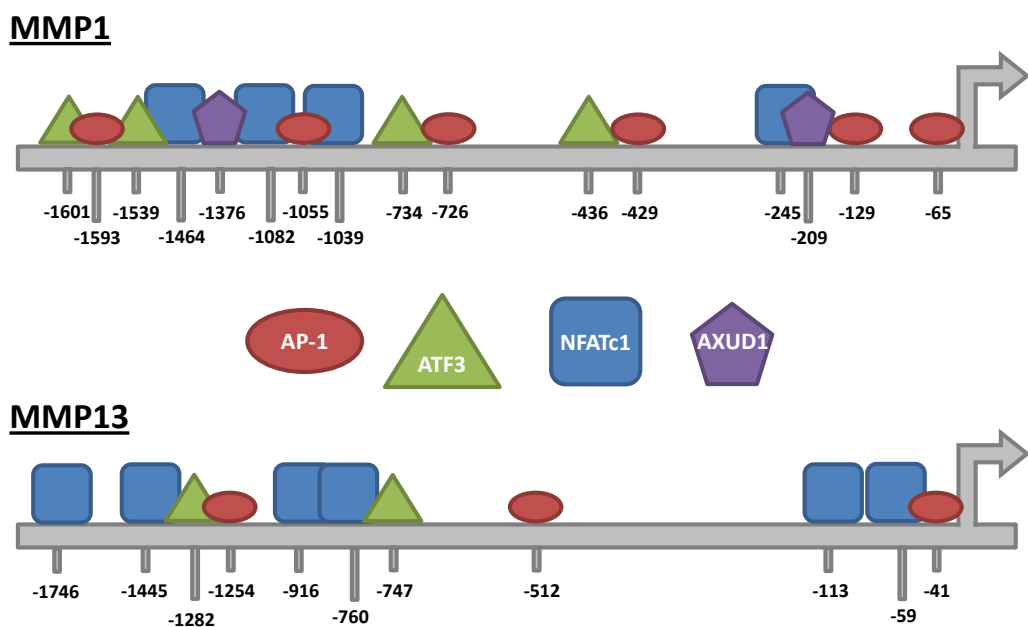
Figure 33: Diagrammatic representation of *MMP1* and *MMP13* proximal promoters and predicted transcription factor binding sites. Transcription factor binding site software was used to predict where certain indicated factors may possibly bind in the proximal promoter regions of *MMP1* and *MMP13*. The distance from the transcription start site for each binding site is denoted below.

4.2   Discussion

In chapter 4 I aimed to identify potential regulatory factors contributing to collagenase gene expression within the temporal deficit discovered in chapter 3. I assessed experimentally validated HAC populations by DNA microarray at 1, 1.25, and 24 hours post IL-1+OSM stimulation. The DNA microarray analysis revealed a number of exciting candidate genes, relevant to the transcriptional regulation of collagenases, that warranted further study.

The genes selected were all associated with compelling evidence indicating their potential role in collagenase gene regulation in HAC in response to pro-catabolic cytokine stimulation. This included many members of the AP-1 transcription factor family. This was an interesting finding as previous study carried out by our group and others had only focused on c-Fos/c-Jun dimers [82, 97, 115, 158]. As such, this represented a good opportunity to assess which of these AP-1 factors may contribute to collagenase regulation. Catterall *et al* [82] showed that AP-1 consensus sequences are bound in unstimulated chondrocytes demonstrating a potential capacity for AP-1 factors to repress gene expression. Previous study on the Jun family member JunB has indicated that it is capable of both activating and repressing collagenase gene expression. Deng and Karin [249] demonstrate that the JunB/c-Jun dimer is associated with transcriptional repression. Chiu *et al.* [250] also demonstrated JunB-mediated repression of c-Jun-dependent *MMP1* promoter constructs activation. However, when in the presence of c-Fos, JunB activated these promoter constructs. These studies suggest that in the context of my study, JunB may well be acting as a repressor. c-Jun has a positive feedback loop in which it activates its own expression [251]. JunB may be leading to repression of c-Jun-dependent *JUN* expression following stimulation. It may also be forming dimers with c-Jun at times after the 1 hour *FOS* peak, repressing the collagenase promoter thereby accounting for the time taken to initiate collagenase gene expression. Alternatively, JunB may be forming dimers with c-Fos which may contribute to the activation of collagenases or of other factors that are themselves capable of regulating the collagenases. Another of the AP-1 factors selected in this chapter, FosB is capable of dimerising with JunB inducing *MMP2* expression in cardiac myocytes [252]. This study also demonstrated that another AP-1 factor, Fra-1 (*FOSL1*)

highlighted in my study, could also form dimers with JunB and activate expression in the same way. Other studies implicate Fra-1 in the regulation of the collagenase *MMP8* through an interaction with STAT3 [253]. This is of interest as silencing of *STAT3* in IL-1+OSM stimulated HAC has been shown by our group to lead to the marked reduction of c-Fos and collagenase expression [97]. As such STAT3 may not only be regulating collagenase expression in-directly through c-Fos, but also directly in combination with Fra-1. These studies all serve to emphasise the need to better understand collagenase gene regulation and whether or not these AP-1 factors selected following the study carried out in this chapter are contributing to this regulation.

Another interesting set of candidate genes are *EGR1-3*. Previous study has shown that *EGR1* is an IEG expressed in chondrocytes in response to IL-1 [254]. This transcriptional regulator has also been shown to be co-regulated with *FOS* and has been demonstrated to have very similar expression kinetics to *FOS* [255]. Moreover, both *FOS* and *EGR1* expression are induced in collagenase-expressing RA synoviocytes [146, 256]. Taken together with studies implicating EGR1 in the regulation of *MMP14* expression [257], there is compelling evidence that EGR1 may well be regulating collagenase gene expression, possibly in collaboration with c-Fos, thereby leading to cartilage destruction in arthritic disease. *EGR2* has been shown to be activated in hypertrophic chondrocytes and is crucial in developmental pathways [258]. Although literature searches do not reveal any direct links to collagenase gene regulation or control of chondrocyte proliferation, EGR3 (as well as EGR2) has been shown to be involved in T and B lymphocyte proliferation [259]. This suggests that this family of transcription factors may potentially be playing a role in chondrocyte proliferation into a mature phenotype which is a process that underpins cartilage ossification into bone [260]. The re-initiation of these developmental pathways and the maturation of chondrocytes in OA is a key pathological step which leads to the aberrant expression of *MMP13* which degrades cartilage [244].

Other factors of interest selected from DNA microarray analysis are linked to chondrocyte proliferation. BTG2 for example, has been shown to have a potential role as a transcriptional co-regulator in cell growth and cell cycle control in neurones [261, 262]. Another such factor is BMP2, a member of the transforming growth factor (TGF) β superfamily, which also plays a critical role in developmental pathways in neuronal

cell phenotype development and also in orchestrating programmed cell death in embryonic limb formation [263-265]. However, it is perhaps the critical role of BMPs in cartilage and bone formation for which the majority of study of BMP2 has taken place. BMPs are crucial in the differentiation of mesenchymal stem cells to osteoblast and osteoclasts and the regulation of their cellular function, thus orchestrating bone formation and its remodelling [266]. A great deal of study has also highlighted the importance of BMP2 in all levels of chondrogenesis including chondrogenic lineage development [267], chondrocyte proliferation and maturation [243] and ECM deposition [268]. At odds with this anabolic function, BMP2 also leads to increased expression of *MMP13* by chondrocytes in response to IL-1 [269-271]. However, both collagen deposition and degradation are characteristics of OA chondrocytes. Interestingly, as well as *MMP13*, BMP2 also increases the expression of *SOX9* [272]. This is a transcription factor that is critical in the differentiation of mesenchymal stem cells to chondrocytes [273]. This is interesting because BMP2 has been demonstrated to lead to Sox-9 binding with CREB, promoting DNA binding [273]. Bui *et al* [102] demonstrated that CREB binding was necessary for induction of *MMP13* expression in OA chondrocytes. Therefore, autocrine/paracrine BMP2 signalling, induced by IL-1+OSM stimulation, may be facilitating CREB binding to the *MMP13* promoter accounting for elevated expression in OA. It must be noted that BMP2 is not a transcription factor and so did not meet the expression parameters for gene selection (as above). However, its association with aberrant collagenase production and its potential in-direct role in *MMP13* regulation made it a compelling gene for further study.

Similar to BMP2, other selected genes did not exhibit the gene expression profile outlined in parameter 3. However, evidence implicating these transcription factors in arthritic disease meant that further study would be of great interest; HIF1A was one such factor. This factor plays a critical role in the response of cells to hypoxia. It is a secondary response transcription factor and has been shown to be regulated by NFκB [274] and has been shown to cooperate with c-Jun/AP-1 in gene regulation [275]. A great deal of compelling evidence exists linking HIF1A to arthritis; this factor is up-regulated in patient samples taken from both RA and OA sufferers [276] and *HIF1A* transcripts correlate with areas of articular cartilage degradation [277]. With regards

to pro-inflammatory cytokine stimulus, the up-regulation of *HIF1A* expression in articular chondrocytes has been shown to be induced by IL-1 stimulation [277-279]. Moreover, IL-1-dependent *MMP1* and *MMP13* expression in synovial fibroblasts has been shown to be HIF1A-dependent [280]. The functionally related HIF2A has also been shown to induce *MMP1* and *MMP13* expression in chondrocytes. Moreover, HIF2a-deficiency in mice led to the abrogation of induced arthritis model [281]. Previous study therefore made HIF1A a very interesting factor in the context of IL-1+OSM-mediated collagenase gene expression in chondrocytes. Although this factor did not adhere to the experimental parameters as set out in this chapter, and since the transcriptional regulation of collagenases was proving to be highly complex, I deemed that discounting such an IL-1+OSM-induced factor that has been so heavily linked to arthritic disease and cartilage degradation would be unnecessarily limiting further study in this instance.

The decision as to how one might elucidate factors that were affecting the expression of the collagenase genes was crucial. It was evident from observations in chapter 3 that gene regulation, governed by factors that are constitutively expressed in the chondrocytes, were not as critical as those that required the induction of their own gene expression, subsequently or in parallel, with AP-1 expression. As such it was decided that a DNA microarray would identify novel genes that were induced by IL-1+OSM and that by comparing their expression pattern at certain time points, I would be able to deduce whether their expression could account for the observed temporal lag in the previous chapter. It must be considered that the induction of gene expression and the translation into protein are distinct processes and therefore raises the question as to the appropriateness of assessing changes in mRNA and not protein. There exist many post-transcriptional gene regulatory mechanisms that dictate the rate at which nascent pre-mRNA is processed and translated into protein. However, the majority of pre-mRNA generated through transcription of genes, is immediately spliced into mRNA [282]. The polyadenylation of the mRNA, protecting it from exonucleases and targeting it for transport out of the nucleus also occurs in relatively rapidly following splicing [283]. Moreover, the transport of mRNA to translation sites outside of the nucleus is carried out by transport factors that bind co-transcriptionally allowing immediate shuttling to ribosomes for translation [284]. In the context of a

substantial temporal deficit between AP-1 rapid, transient induction and the late-onset of *MMP* expression, these processes provide a negligible contribution to this lag and so, gene expression and protein production are considered to be closely correlated. Furthermore, considering the short stimulation durations used, gene expression and concomitant production of protein is maintained, supporting the use of DNA microarray. Short stimulations allow the induction of IEG, such as AP-1 factors to be observed. As mentioned previously, the transient nature of IEG governs the function of their corresponding protein [189]. In the case of AP-1, the rapid induction and equally rapid reduction means that the action of this transcription factor is finite and therefore is unable to elicit any unwanted oncogenic functioning [223]. As such any factors capable of being induced after relatively short stimulation durations are likely to be translated in a similarly rapid manner. Combined with prior knowledge of the technique I concluded that DNA microarray assessing genome-wide gene expression changes was an appropriate method for elucidating novel genes.

With this understanding I reasoned that by investigating gene expression changes at these early time points I would be able to establish which factors, that remained un-synthesised during protein synthesis inhibition studies, were contributing to the transcription of the collagenases. Alternatively, I could have assessed differences in actual protein profile by carrying out mass spectrometry studies between these different durations. Such quantitative proteomic techniques such as isotope-coded affinity tags (ICATs) would be very informative in terms of trying to account for the protein synthesis occurring in the temporal deficit between peak c-Fos and initiation of collagenase transcription that may contribute to this gene regulation. Whilst this was a viable option for the elucidation of contributing factors, I decided that assessing gene expression changes allowed me to validate the appropriate induction of control genes, such as *FOS* and *MMP1,* with greater ease and accuracy than by assessing protein. As such this allowed me to compare pre- and post-DNA microarray gene validations with data yielded from the DNA microarray itself thus providing confidence in the observed gene expression changes for potential contributing gene regulatory factors. The practicalities of assessing temporal gene expression of many different candidate genes were far easier to overcome than if I were trying to evaluate the same number of proteins. The fact that I had hypothesised that some of these contributing factors may

also be AP-1-dependent meant that the analysis of gene expression attributed to regulation by AP-1 more accurately than protein production. Finally, lower costs, time constraints and the presence of expertise within the group to prepare samples and analyse the data generated by DNA microarray meant that this analysis was the most viable option for factor elucidation in this instance.

Study in this chapter has provided me with a selection of factors that may be contributing to the regulation of collagenase genes at points within the temporal deficit between *FOS* peak and the initiation of collagenase gene expression. In the next chapter I will investigate these factors in greater detail by silencing these genes in HAC stimulated with IL-1+OSM and assessing the effect on *MMP1* and *MMP13* expression.

4.2.1 Summary

- Multiple HAC populations were experimentally validated for appropriate collagenase and AP-1 gene expression. Isolated RNA from those populations deemed suitable for further study was used for analysis of gene expression using DNA microarray.

- In-depth bioinformatic analysis of DNA microarray data and subsequent experimental validation highlighted a number of genes that were governed to be capable of contributing to collagenase gene expression in concert with and/or as a result of AP-1 regulation.

- The following genes were identified and deemed suitable for further study: *ATF3, AXUD1, BMP2, BTG2, EGR1/2/3, FOSB, FOSL1 (Fra-1), HIF1A, IER2/3/5, JUNB* and *NFATC1.*

5 Chapter 5: Investigation of novel regulators of collagenase transcription

5.1 Introduction

Chapter 4 revealed a number of genes potentially relevant to IL-1+OSM-dependent transcriptional regulation of the collagenases during the AP-1-collagenase temporal deficit. Within this Chapter I aimed to determine which of these genes were functionally relevant to collagenase expression and to also investigate if c-Fos/c-Jun AP-1 factors are responsible for the regulation of these factors.

5.1.1 Functional assessment of candidate gene

In order to ascertain the functional relevance of these genes in IL-1+OSM-mediated *MMP1* and *MMP13* expression, each gene was individually silenced using siRNA. Prior to siRNA transfection, the effect of transfection reagent alone and in combination with control siRNA upon expression of the collagenases was assessed. Figure 34 demonstrated that there was no significant difference in IL-1+OSM-dependent collagenase gene expression in HAC treated with transfection reagent alone or in combination with siRNA control. This therefore confirmed that in subsequent experiments, any changes in collagenase expression observed were as a result of siRNA knock-down. Furthermore, all three HAC populations from individual donors demonstrated synergistic *MMP1* and *MMP13* expression in-line with previous observations [72]. This response was used in subsequent experiments to ensure that inappropriate HAC responses to stimulation were unlikely to account for any changes in gene expression following knock-down.
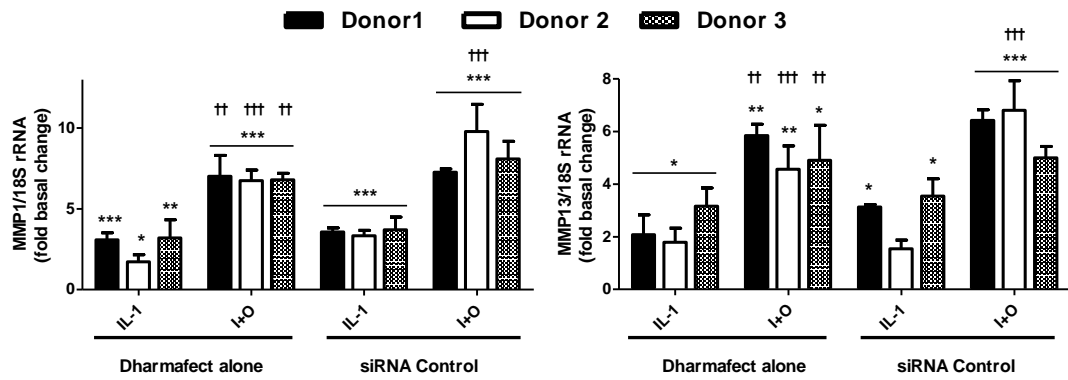
Figure 34: Real-time PCR analysis of the effect of transfection reagent, Dharmafect and siRNA control on *MMP* gene expression in IL-1+OSM stimulated HAC. HAC were treated with IL-1 (0.05ng/ml) alone or in combination with OSM (10ng/ml) for 24 hours. Cell lysates were subjected to real-time PCR and the relative expression levels of *MMP1* and *MMP13* mRNA, normalized to the *18S* rRNA housekeeping gene, were determined. Data are from three separate chondrocyte cultures from different donors. (Stimulated vs Basal; mean ± S.D. where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; I+O vs. IL-1; mean ± S.D. where ††, $p < 0.01$; †††, $p < 0.001$)

Collagenase gene expression was then assessed following 24 hour IL-1+OSM stimulation, in the presence or absence of individual siRNA against the genes identified by the DNA microarray (listed, for reference, in Table 7). Figure 35 shows the effect of this silencing on both *MMP1* (Figure 35A) and *MMP13* expression (Figure 35B) as a percentage of IL-1+OSM-stimulated control (denoted as siCON). The silencing of a number of genes such as *BMP2*, AP-1 factors *FOSB* and *FOSL1* and all three *IER* genes led to a significant reduction in the expression of both collagenases (Figure 35A and B, black bars). The silencing of factors that led to decreased expression of both collagenases showed that work performed in chapter 4 had elucidated a number of genes that in the majority played a significant role in transcriptional regulation within the chondrocyte. Conversely, some genes that were highlighted in chapter 4 study did not affect the IL-1+OSM-mediated expression of *MMP1* or *MMP13,* following their silencing (Figure 35A and B, open columns). *BTG2* and *EGR1* are two such transcriptional regulator genes that, despite playing a role in differentiation and cellular growth [261, 262, 285, 286] , a process key to chondrocyte hypertrophy in arthritic disease [244], do not affect gene expression when silenced. This was also the case for *HIF1A*, a promising candidate transcription factor which has been shown to be associated with areas of articular cartilage degradation [277] and is up-regulated in both RA and OA [276]. These results indicate that although these transcription factors adhered to gene expression profile parameters outlined in chapter 4, they were not functionally relevant in the context of collagenase gene regulation in chondrocytes. Interestingly, a number of these selected genes, when silenced, only led to the

reduced expression of one collagenase and not the other. These included *ATF3*, *AXUD1*, and *EGR2*. Previous studies have shown that *MMP1* and *MMP13* are differentially regulated [82, 97, 104, 112, 113]. As such, further investigation into these genes represents an important opportunity to better understand how these catabolic enzymes from the same protease family are regulated independently of each other. For this reason I decided to focus on these genes, deeming them worthy of more directed and thorough investigation for the remainder of my studies.

JUNB also demonstrated differential regulation (significant reduction observed in *MMP1* expression, Figure 35A, but no effect on MMP13 expression, Figure 35B) but was not investigated further. This was because it is an AP-1 member which is usually associated with repression of collagenases [153, 249] and so I decided that there were other genes of greater interest to investigate. I decided that along with *ATF3*, *AXUD1* and *EGR2*, *NFATc1* was also worthy of further investigation. This is because *NFATc1* regulates the expression of ADAMTS-9 in IL-1-stimulated HAC [287] and its inhibition attenuates IL-1-mediated expression of *MMP1* in OA chondrocytes [248]. Moreover, silencing data shows that silencing has more of an effect on *MMP1* expression than *MMP13* suggesting at possible role in differential regulation.

siRNA-silenced gene	Protein name
FOS (c-Fos)	FBJ murine osteosarcoma viral oncogene homolog
JUN (c-Jun)	Jun proto-oncogene
ATF3	Activating transcription factor 3
AXUD1	Axin-1-induced gene
BMP2	Bone morphogenetic protein 2
BTG2	B-cell translocation gene 2
EGR1/2/3	Early growth response factor 1/2/3
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FOSL1 (Fra-1)	Fos-related antigen 1
HIF1A	Hypoxia-inducible factor 1 alpha
IER2/3/5	Immediate early response gene 2/3/5
JUNB	Jun B proto-oncogene
NFATC1	Nuclear factor of activated T-cells cytoplasmic 1

Table 7: The fifteen potential transcriptional regulators, plus FOS and JUN, identified by the DNA microarray: Each gene was silenced by specific siRNA to assess the impact on IL-1+OSM-mediated *MMP* gene expression.
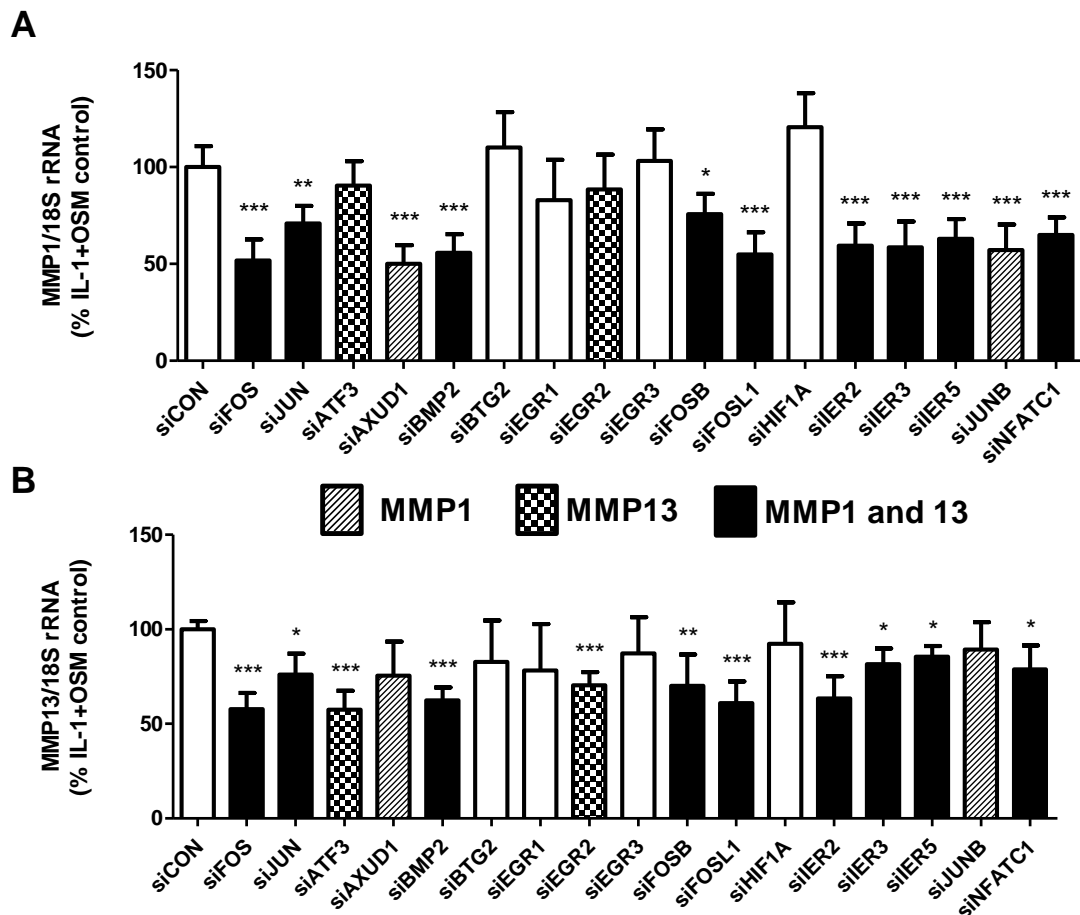
Figure 35: Real-time PCR analysis of the effect of gene silencing on *MMP* gene expression in IL-1+OSM stimulated HAC. Following transfection with siRNA specific for indicated genes or non-targeting siControl (100nM), HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 24 hours. Real-time PCR of isolated RNA was performed for *MMP1* and *MMP13* and normalised to *18S* housekeeping gene. Data are pooled from three separate HAC populations (each assayed in octuplet) and expressed as a percentage of stimulated control (specific siRNA transfected vs. siCon-transfected; mean ± S.D.; *, p <0.05; **, p <0.01; ***, p < 0.001). Diagonal lined columns denote significant reduction in *MMP1* expression alone following the silencing of the indicated genes; chequered for *MMP13* alone; and black for both *MMP1* and *MMP13*. Open columns indicate no significant inhibition.

Having confirmed that siRNA gene silencing of *ATF3*, *AXUD1*, *EGR2* and *NFATc1* led to a significant reduction in collagenase expression, I next aimed to confirm that siRNA knock-down also resulted in a decrease in protein expression. In Figure 36, all 4 proteins, following IL-1+OSM stimulation were observed to decrease in expression compared to siCON. Reduction in AXUD1 and EGR2 protein was also observed between siCON and specific gene knock-down at basal levels. ATF3 and NFATc1 levels were unaffected in unstimulated HAC transfected with specific siRNA. This confirms that the observed changes in collagenase expression (Figure 35) were due to a successful reduction of gene expression and protein production following siRNA gene silencing.
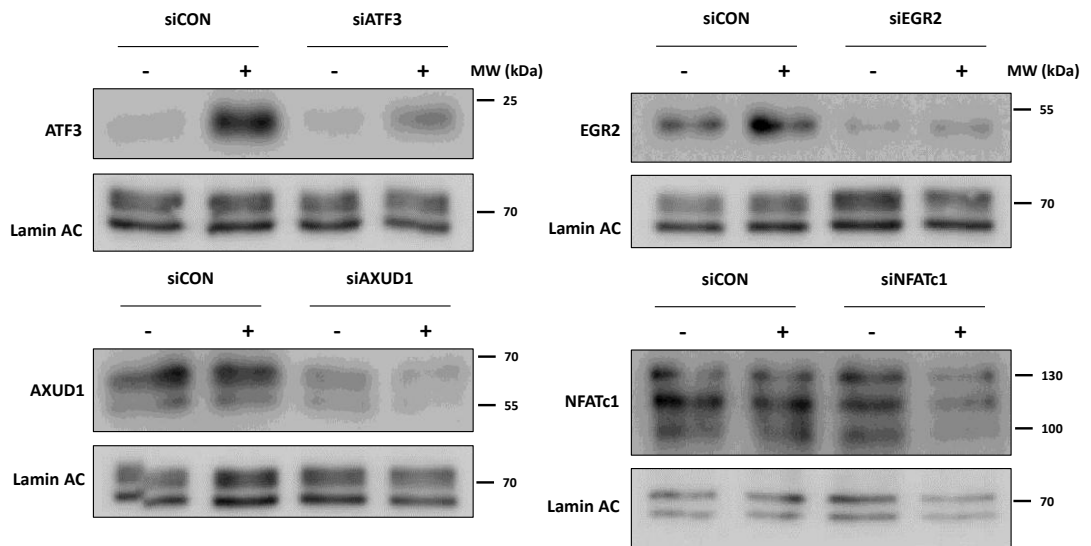
Figure 36: Western blot for ATF3, AXUD1, EGR2 and NFATc1 following gene silencing. HAC were transfected with siRNA specific for *ATF3*, *AXUD1*, *EGR2*, *NFATc1* or non-targeting siControl (siCON, 100nM) prior to stimulation with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 3 hours as indicated. Nuclear extracts were isolated and proteins resolved by SDS-PAGE. Immunblots were probed using the indicated antibodies. Blots shown are representative of three experiments using chondrocytes from different donors. Lamin A/C was used as a loading control.

5.1.2   Temporal assessment of functionally relevant genes

I next investigated the expression kinetics of the candidate genes. Having generated mRNA from three independent HAC populations I firstly confirmed the expression of *FOS* and *MMP1* and *MMP13* expression over a detailed time course. As previously shown, data in Figure 37 confirm that *FOS* is expressed both rapidly and transiently following IL-1+OSM stimulation and that expression of both *MMP1* and *MMP13* expression did not significantly increase until 6 hours post IL-1+OSM stimulation. Increased gene expression was then maintained through to 24 hours. This confirmed that the HAC populations used to assess candidate gene expression kinetics demonstrated previously observed temporal gene expression profiles.

Figure 37: Real-time PCR analysis of control gene expression following different durations of IL-1+OSM stimulation. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cell lysates were subject to real-time PCR and relative expression levels of mRNA, normalized to the *18S* rRNA housekeeping gene, were determined for the indicated genes. Data are representative of three separate experiments each using chondrocyte cultures from different donors. Statistical analyses are shown for gene expression following IL-1+OSM stimulation versus basal (mean ± SD; where ***, $p < 0.001$).

I then assessed the changes in *ATF3*, *AXUD1*, *EGR2* and *NFATc1* gene expression over the same time course (Figure 38) allowing for direct comparison of *FOS* and *MMP1/13* expression with newly identified regulatory factors. *ATF3*, *AXUD1* and *EGR2* gene expression increased from basal levels up to 1.25 hour IL-1+OSM stimulation. *ATF3* expression decreased after 1.25 hour yet remained significantly elevated above basal levels for the remainder of the time-course. *AXUD1* and *EGR2* gene expression also decreased after 1.25 hours remaining significantly elevated above basal levels until 4 hours post IL-1+OSM stimulation but then decreased to levels comparable with basal gene expression. *NFATc1* expression significantly increased from 1 to 1.25 hour stimulation, remained significantly increased following 2 hour stimulation and subsequently decreased to levels comparable with basal gene expression. These data indicate that, similar to *FOS*, expression of these factors is rapid, reaching a peak following short durations of IL-1+OSM stimulation. However, this expression is not as transient suggesting that these genes may not be conventional IEGs. These data also confirm DNA microarray data that show peak expression following *FOS* and *JUN,* suggesting a possible role for AP-1 factors in regulating these genes that contribute to collagenase expression. It must be noted that in Figure 38 each plot has a different scale on the Y axis meaning that each gene has different magnitudes of expression. Whilst *ATF3*, *AXUD1* and *EGR2* have robust gene expression compared to unstimulated levels, *NFATc1* expression, though significant, is not nearly as robust as the other genes studied.
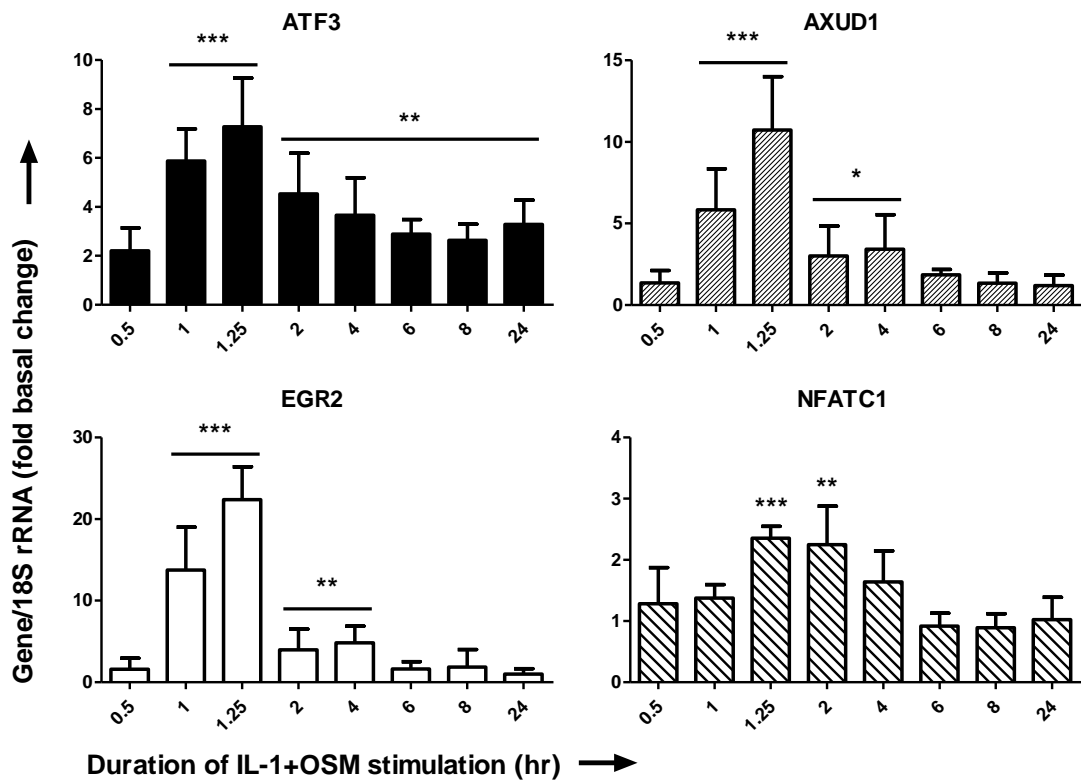
Figure 38:Real-time PCR analysis of gene expression following different durations of IL-1+OSM stimulation. HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cell lysates were subject to real-time PCR and th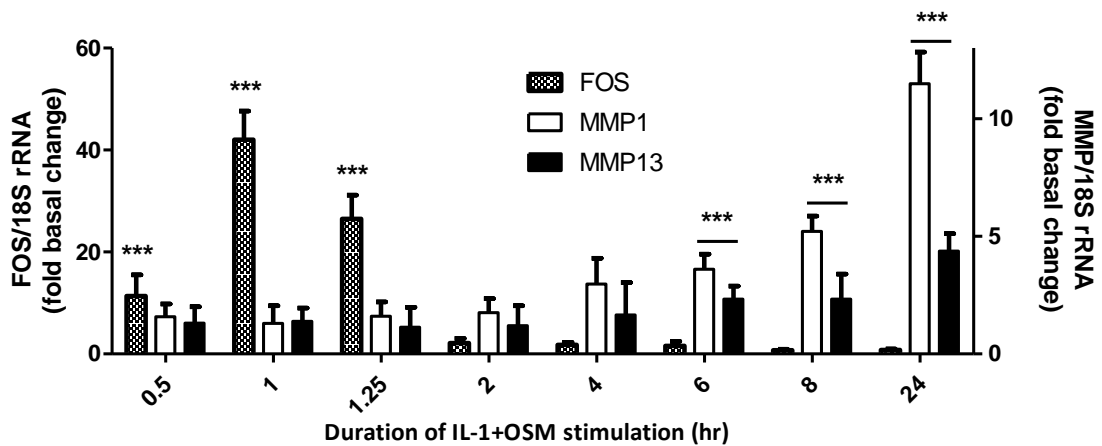e relative expression levels of mRNA, normalized to the *18S* rRNA housekeeping gene, were determined for the indicated genes. Data are representative of three separate experiments each using chondrocyte cultures from different donors. Statistical analysis are shown for gene expression following indicated IL-1+OSM stimulation versus basal (mean ± SD; where *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Having established the kinetics of gene expression, I then assessed candidate protein induction and the cellular localisation following IL-1+OSM stimulation time course used in previous study (chapter 3, Figure 14). As with gene expression in Figure 38, I was able to compare and contrast known changes in c-Fos and c-Jun protein levels with those of the newly identified contributing factors. Assessing cellular localisation would also give an indication if these factors were being regulated at the level of nuclear localisation [288, 289] which could explain the lack of robust changes in NFATc1 expression observed in Figure 38.

Figure 39A demonstrates that ATF3 protein was only found in the soluble nuclear fraction and was at very low level in basal conditions. Upon IL-1+OSM stimulation protein levels increased up to 3 hours and remained high until 6 hours post-stimulus. However, by 24 hours post IL-1+OSM stimulation protein levels had decreased to levels comparable with 1 hour post stimulations. In Figure 40A, EGR2 protein was only found in the soluble nuclear fraction with levels increasing up to 3 hours stimulation.

Expression of EGR2 marginally decreased by 6 hours post IL-1+OSM stimulation and had also returned to levels comparable with basal conditions following 24 hour stimulation. In Figure 41A, AXUD1 protein was observable in both soluble nuclear and chromatin-bound fraction. Protein levels in the soluble nuclear fraction increased following 1 hour stimulation and slightly increased from 1 hour to 3 hour IL-1+OSM stimulation where levels were maintained up to 6 hours post stimulation. Following 24 hours IL-1+OSM stimulation AXUD1 protein levels had returned to levels comparable with basal. In the chromatin-bound fraction AXUD1 is present following all stimulation durations as well as basal control (Figure 41A). Protein levels all modestly increase above basal conditions following 1 hour IL-1+OSM stimulation (Figure 41C). NFATc1 protein was present in cytoplasmic, membrane-bound and soluble nuclear fractions. There was no significant change in protein level of NFATc1 following any stimulation duration in any of the fractions in which NFATc1 is present (Figure 42A and B). Densitometric analysis of repeated HAC populations statistically confirms these observations following Western blot analysis for ATF3 (Figure 39B), EGR2 (Figure 40B), AXUD1 (Figure 41B and C) and NFATc1 (Figure 42B).

These data indicate that these factors are unlikely to be regulated at the level of nuclear localisation as ATF3, EGR2 and AXUD1 are not observed in the cytoplasm and once translated are seemingly shuttled rapidly into the nucleus. Although NFATc1 is regulated at the level of nuclear localisation in response to increasing intracellular calcium signalling [246, 290] there are no observable changes in localisation between cytoplasmic and soluble nuclear fractions suggesting that NFATc1 may not be IL-1+OSM-dependent. The observation that ATF3 and EGR2 protein levels increase over basal, peaking at stimulation durations later than the c-Fos peak, suggest that the time taken for their transcription and translation may partially account for the temporal deficit between c-Fos peak and initiation of collagenase expression. The robust and transient changes in *AXUD1* gene expression are not reflected at the protein level indicating that there may be some other level of regulation occurring that may explain the lack of marked protein level changes.

Figure 39: Western blot for ATF3 in subcellular protein fractionation and densitometry from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. **A**) Cells were subject to subcellular protein fractionation. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunobloted with ATF3 antibody. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed. Images in this figure are cropped from larger exposure image. Full-length blots/gels are presented in Appendix 7.3, figure 50. Immunoblots shown are representative of three separate experiments each using chondrocyte cultures from different donors. **B**) Combined densitometric scans of three separate blots each from separate chondrocyte donor populations. Density is relative to unstimulated control (mean ± SD; where *, p < 0.05; **, p < 0.01; ***, p < 0.001).
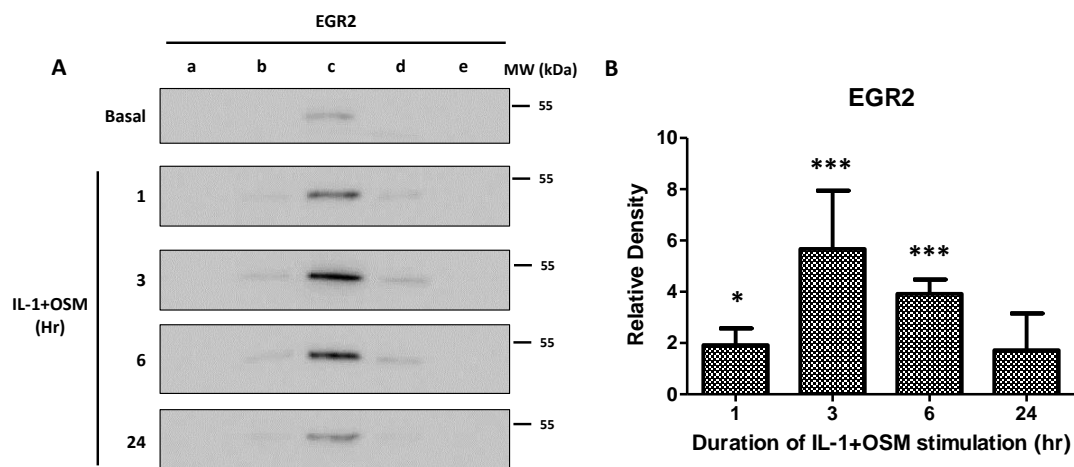
Figure 40: Western blot for EGR2 in subcellular protein fractionation and densitometry from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. **A**) Cells were subject to subcellular protein fractionation. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotting with EGR2 antibody. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed. Images in this figure are cropped from larger exposure image. Full-length blots/gels are presented in Appendix 7.3, figure 52. Immunoblots shown are representative of three separate experiments each using chondrocyte cultures from different donors. **B**) Combined densitometric scans of three separate blots each from separate chondrocyte donor populations. Density is relative to unstimulated control (mean ± SD; where *, p < 0.05; **, p < 0.01; ***, p < 0.001).

121
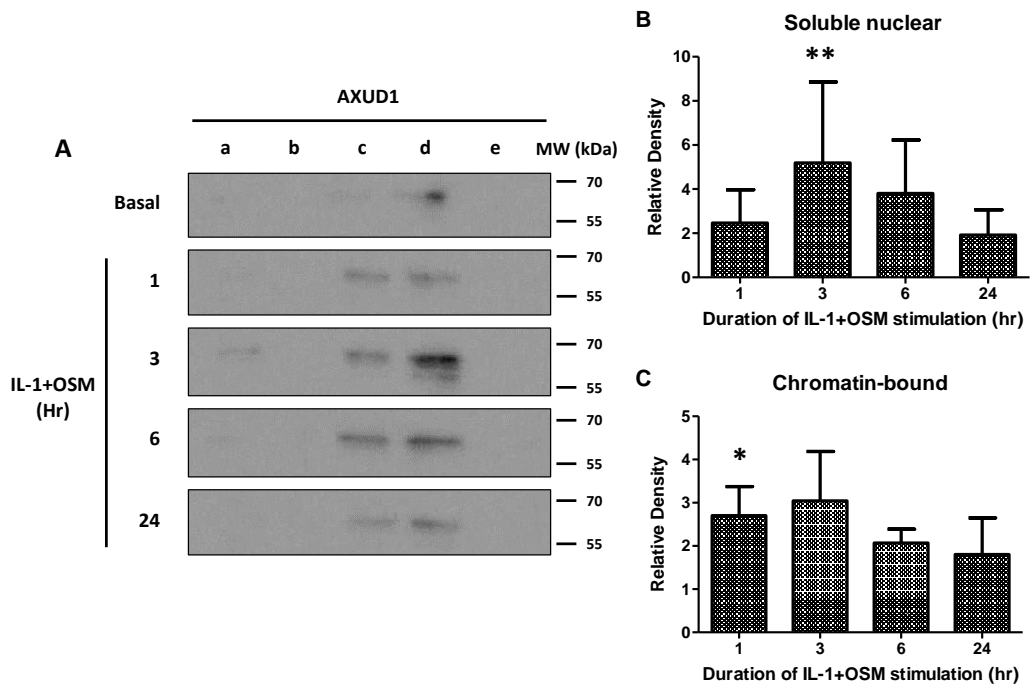
Figure 41: Western blot for AXUD1 in subcellular protein fractionation and densitometry from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. **A)** Cells were subject to subcellular protein fractionation. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotting with AXUD1 antibody. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed. Images in this figure are cropped from larger exposure image. Full-length blots/gels are presented in Appendix 7.3, figure 51. Immunoblots shown are representative of three separate experiments each using chondrocyte cultures from different donors. **B)** and **C)** combined densitometric scans of indicated subcellular protein fractionation of three separate blots each from separate chondrocyte donor populations. Density is relative to unstimulated control (mean ± SD; where *, p < 0.05; **, p < 0.01; ***, p < 0.001).
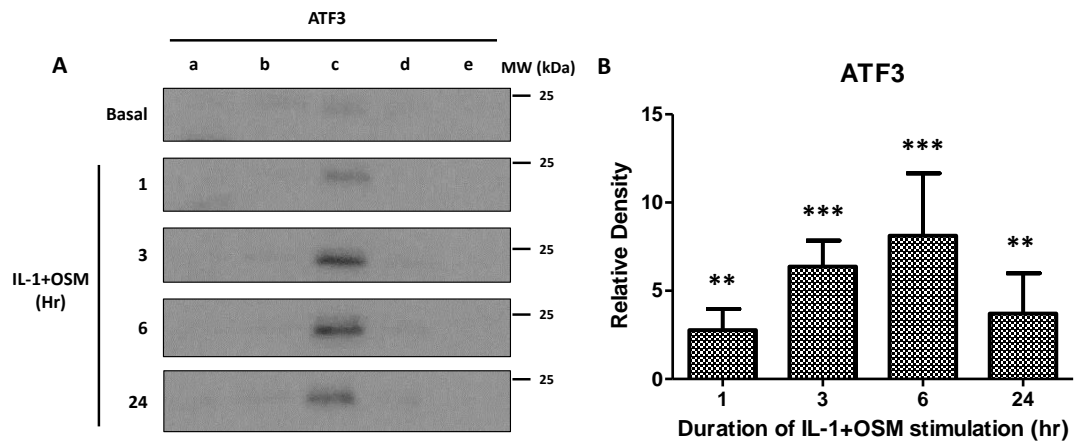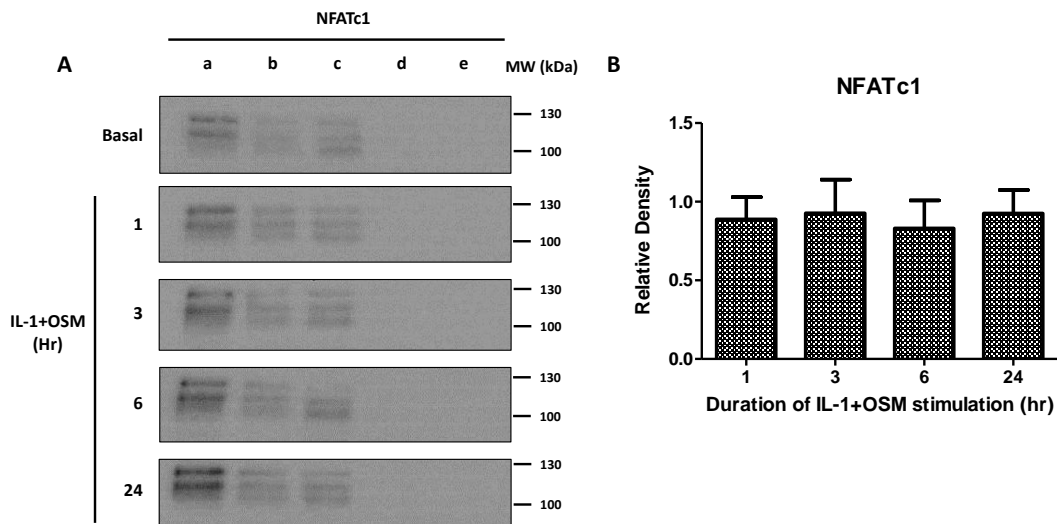

Figure 42: Western blot for NFATc1 in subcellular protein fractionation and densitometry from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. **A)** Cells were subject to subcellular protein fractionation. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotting with NFATc1 antibody. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed. Images in this figure are cropped from larger exposure image. Full-length blots/gels are presented in Appendix 7.3, figure 53. Immunoblots shown are representative of three separate experiments each using chondrocyte cultures from different donors. **B)** Combined densitometric scans of soluble nuclear fraction of three separate blots each from separate chondrocyte donor populations. Density is relative to unstimulated control (mean ± SD; where *, p < 0.05; **, p < 0.01; ***, p < 0.001).

5.1.3 Assessment of AP-1 regulation on genes contributing to collagenase transcription

Having demonstrated that as with *FOS* and *JUN*, gene silencing of *ATF3* led to a similar reduction of collagenase expression, I then wanted to identify whether or not ATF3 was bound to the promoter region of *MMP13* using ChIP. It was decided that ATF3 would be investigated and not the other contributing factors as primary cell numbers were limited and a "ChIP-grade" ATF3 antibody was readily available. Prior to ATF3 ChIP experiments, *in vitro* binding of ATF3 to AP-1 consensus sites present within the *MMP13* promoter was demonstrated (Figure 43). Although these oligonucleotides did not contain consensus ATF3 binding sites, the AP-1 consensus is bound with equal avidity [241]. Only ATF3 was bound to this AP-1 oligonucleotide sequence following 6 hour IL-1+OSM stimulation, unlike following 3 hour stimulation where c-Fos and p-c-Jun (Ser 63) were also bound. This suggests that ATF3 is potentially capable of regulating the expression of collagenase genes subsequent to, and in the absence of, AP-1 factors. However, this may well be an observation that is dependent on protein abundance in the nuclear lysate which is mirrored in AP-1 pull-down and not necessarily due to regulated changes in transcription factor binding. This observation also reveals a potential mechanism for ATF3-dependent regulation of *MMP13*. ATF3 can dimerise with members of the Jun family and activate gene expression [241, 291]. As such the constituents of the dimer bound on AP-1 binding sites on the *MMP13* promoter could be changing over time; as new genes are expressed these factors may then replace those previously bound to the promoter e.g. ATF/Jun dimer replacing c-Fos/c-Jun dimer.

In highlighting this binding capacity I gained some assurance that ATF3 protein levels were sufficiently high enough in order to bind following this IL-1+OSM duration. It also allowed me to keep subsequent ChIP experiments succinct without the need for extensive time course studies as in chapter 3, thereby reducing the number of HAC that I required. However, repeated attempts failed to show ATF3 binding to either collagenase promoters following 3 hours IL-1+OSM stimulation using ChIP. This could be accounted for by three possible scenarios: the first is that ATF3 does bind to one or both of these proximal promoters but the practicalities of carrying out ATF3 ChIP in primary cells is too difficult to overcome; secondly, ATF3 could be bound to an

enhancer element that is not encompassed within the resolution of my *MMP13* proximal promoter primer sets; and thirdly, ATF3 may not be bound to the promoter of either collagenase following IL-1+OSM stimulation.
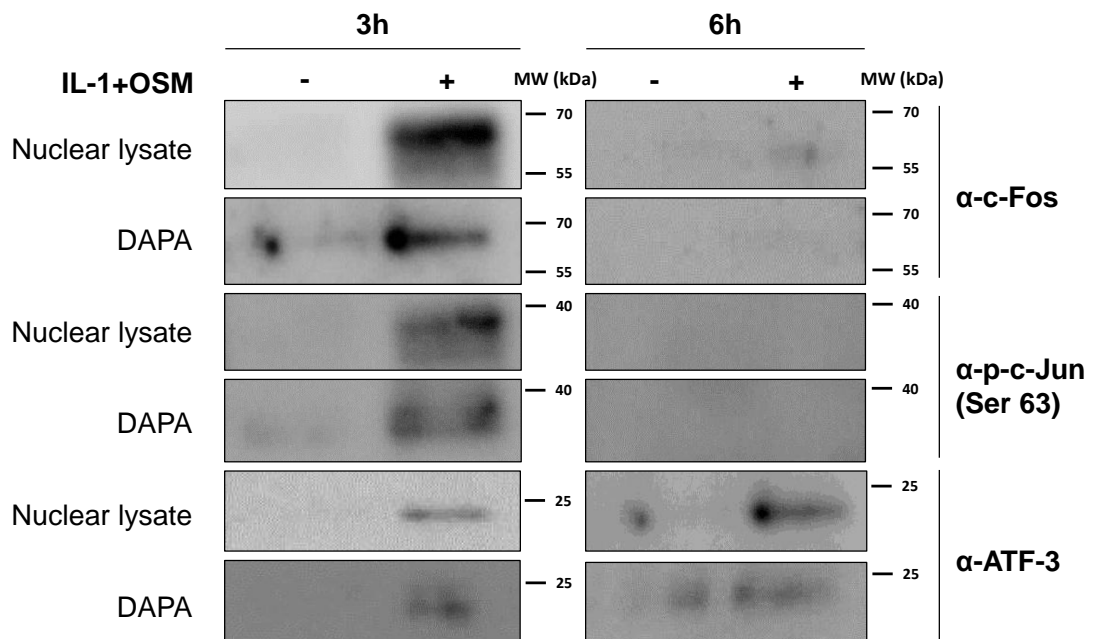
Figure 43: Western blot for indicated proteins following affinity pull-down assay using an AP-1 consensus site. HAC populations taken from different donors were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated duration. Cells were then subject to cytoplasmic-nuclear protein fractionation. A portion of nuclear lysates were incubated with double-stranded biotinylated oligonucleotide containing the AP-1 consensus binding site. Bound proteins were then subject to streptavidin coated agarose bead pull-down. Proteins were resolved using SDS-PAGE and immunoblotted with the indicated antibodies.

In the final stages of this study, I investigated the validity of the hypothesis which proposed that factors contributing to collagenase gene expression may themselves be regulated by AP-1 factors. Given that AP-1 factors play a role in regulating a large number of genes [292], I suggested that not only do they regulate collagenase expression but also these regulatory genes. Preliminary investigation using binding site prediction software [245], demonstrated that the three genes, with markedly induced gene expression, all contain multiple consensus AP-1 binding sites within the 2 kbp proximal to their TSS (Figure 44). This indicates that these genes were capable of being regulated by AP-1 through direct trans-activator binding to cis-elements.
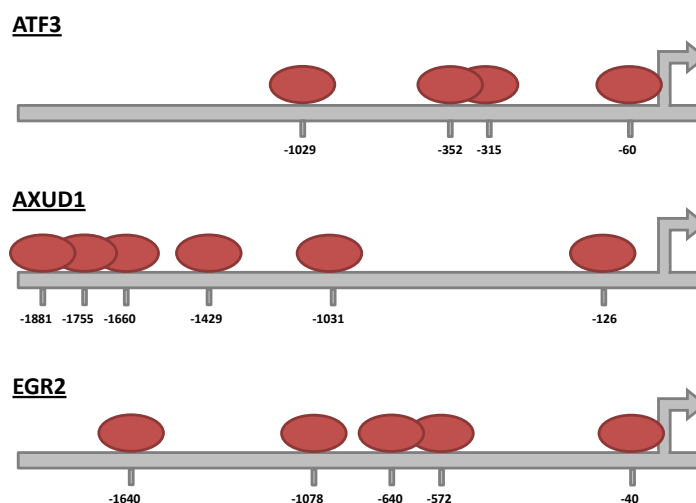
Figure 44: Diagrammatic representation of ATF3, AXUD1 and EGR2 proximal promoters and predicted AP-1 binding sites. Transcription factor binding site software was used to predict where AP-1 factors (denoted by red oval) may possibly bind to the proximal promoter region of indicated genes. The distance from the transcription start site for each binding site is indicated below the predicted binding site.

Figure 45 demonstrates that *ATF3* gene expression following 1.25 hour IL-1+OSM stimulation significantly decreased following *FOS* and *JUN* silencing indicating the importance of these AP-1 factors in the expression of this transcription factor. Interestingly, *AXUD1*, only exhibited a significant decrease in expression upon *FOS* silencing alone, whereas *EGR2* expression was only reduced when *JUN* alone was silenced implying a degree of differential regulation. Together these data confirm that c-Fos and c-Jun indirectly regulate collagenases by regulating other transcription factors expression which themselves contribute to collagenase gene regulation.

Study within this chapter has confirmed a role for ATF3, AXUD1 and EGR2 in the transcriptional regulation of collagenases. These findings have gone some way toward accounting for the IL-1+OSM-dependent gene regulatory events that occur following loss of c-Fos/c-Jun expression and the initiation of *MMP1* and *MMP13* expression in chondrocytes.
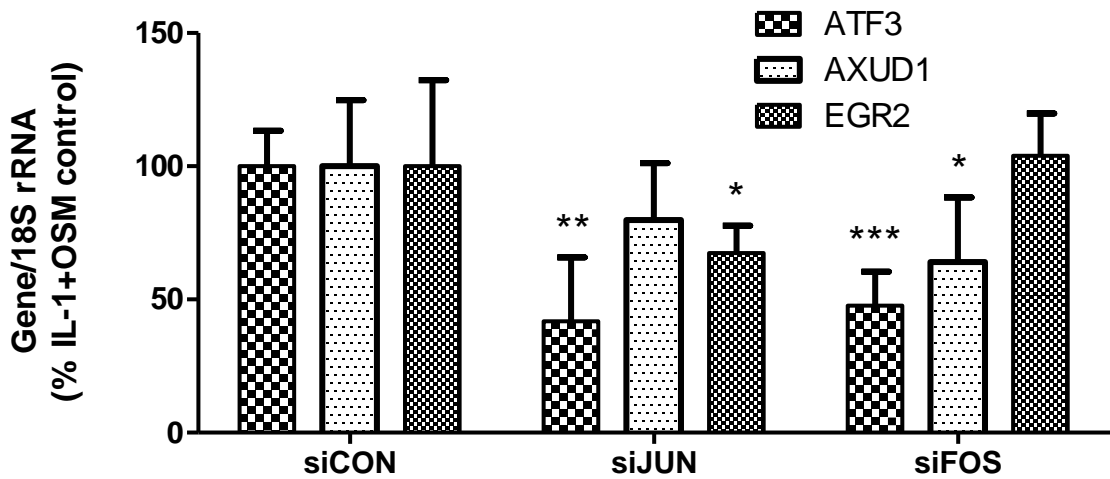
Figure 45: Real-time PCR analysis of the effect of *FOS* and *JUN* gene silencing on contributing factor gene expression in IL-1+OSM stimulated HAC. Following transfection with siRNA specific for *FOS*, *JUN* or non-targeting siControl (100nM), HAC were treated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for 1.25 hours. Real-time reverse transcription PCR of the isolated RNA was performed for *ATF3*, *AXUD1* and *EGR2* and normalised to *18S* rRNA housekeeping gene. Data are pooled from two separate HAC populations (each assayed in octuplet) and expressed as a percentage of stimulated control (specific siAP-1 transfected vs. siCon-transfected; mean ± S.D.; *, p <0.05; **, p < 0.01; ***, p < 0.001).

5.2 Discussion

In this chapter I assessed if the genes identified by DNA microarray analysis had a functional role in the regulation of IL-1+OSM-mediated collagenase expression in HAC. I demonstrated that ATF3, AXUD1 and EGR2 are key factors regulating *MMP1* and *MMP13* expression.

The finding that ATF3 is playing a role in regulating *MMP13* expression is a novel and exciting one. Previous study has not demonstrated this role in chondrocytes before now. However, there has been a great deal of study carried out for the family member ATF2 in chondrocytes. Many studies highlight its role in proliferation and maturation. Beier *et al* [293] showed that cyclin D1, which is crucial to chondrocyte proliferation, is regulated by ATF2, with ATF2$^{-/-}$ mice exhibiting defective cartilage development. Other studies have shown that ATF2 in combination with Smad are key regulators of chondrocyte maturation following stimulation with TGF-β [294, 295], a common precursor for development of OA [296]. ATF2 has also been shown to have a role in JNK and p38-mediated *MMP1* expression in fibroblasts. There is one study however, that demonstrated that ATF3 has a similar function to ATF2 in chondrocytes. James *et al* [297] showed that ATF3 induction correlated with terminal differentiation of

chondrocytes. The study went on to suggest that this was due to ATF3-dependent increase in Runx-2. This factor not only contributes to chondrocyte hypertrophy [298, 299] but also directly regulates *MMP13* expression [113, 300, 301] which is itself a marker for chondrocyte hypertrophy [244]. This re-initiation of developmental signalling pathways (e.g. Wnt [302, 303]) in the chondrocyte is a key pathological process in OA [244]. Processes that are characteristic of OA, including cartilage remodelling, vascularisation and focal calcification of joint cartilage forming osteophytes, are also characteristics of normal skeletal development by endochondral ossification. These data in combination with my own findings demonstrate a credible possibility that ATF3 has a critical role in this key pathological process that underpins OA. Moreover, this observed differential regulation of the collagenases complements this observation as MMP-13 is highly associated with OA, whilst MMP-1 production correlates with RA [304]. I therefore suggest that ATF3 may well be a valid candidate for further study in to providing therapeutic intervention for OA. ATF3 knock-out mice [305] could be utilised in combination with arthritic mouse models to identify if the loss of this factor attenuates disease. Inhibitors which prevent DNA binding, similar to those generated for c-Fos [159], could then be developed. In the consideration that this intervention would be administered at a time where development pathways would only be occurring as a consequence of disease, ATF3 inhibition represents specificity as well as potential efficacy.

Another interesting result from study carried out in this chapter is that ATF3 is bound to AP-1 consensus sequence following 6 hour IL-1+OSM stimulation duration and c-Fos and c-Jun are not. This suggests the potential involvement of other regulatory factors and/or co-activators being recruited to AP-1 binding site within the *MMP* promoters when AP-1 factors are not. It must be said that although informative, DAPA is an *in vitro* technique which does not reflect specific promoter binding or account for complex regulatory mechanisms involved in gene expression. However, it does provide clues as to how ATF3 could contribute directly to *MMP13* regulation. The ATF family consist of transcription factors that contain a leucine-zipper motif which allows promoter binding. The different members of this family are able to dimerise with one another and themselves, a characteristic which imparts varying DNA binding capacity and effector function [306]. Interestingly, the ATF binding element is almost identical

to that of AP-1. Moreover, ATF and AP-1 can bind to one another's binding element with equal avidity [241] and they can also form dimers between the different members of the two families [128, 307-309]. These studies also demonstrate that dimers between ATF members and Fos family members bind to DNA less efficiently than ATF/Jun family dimers. I therefore suggest that ATF3 may be forming heterodimers with Jun family members at times following stimulation where c-Fos is no longer present i.e. at 6 hours-post stimulation as shown in DAPA study. Shortly after stimulation c-Fos/c-Jun dimer binds to the *MMP13* promoter initiating gene regulation processes. Over time this dimer dissociates and is replaced with ATF3/Jun dimer which then contributes further to orchestrating gene expression. ATF homodimers are unlikely to contribute as they are transcriptional repressors [310]. I propose that JunD could be a possible binding partner as my own studies show that JunB regulates *MMP1* expression not *MMP13*, active c-Jun is not present following 6 hour IL-1+OSM stimulation and previous study has shown that ATF3/JunD dimer is a positive regulator of gene expression [311, 312]. The contribution of ATF3 could therefore account for the c-Fos-collagenase temporal deficit either by taking over trans-activation from Fos/Jun dimers during this time or by regulating transcription factors key to chondrocyte maturation.

With the aforementioned limitations associated with DAPA I repeatedly attempted to establish whether or not ATF3 was enriched on the *MMP13* promoter using ChIP. However, I could not do so. This may have been due to a number of reasons. One of them may simply be that I could not get this technique, which is renowned for its logistical difficulties, to work appropriately resulting in isolated gDNA that did not reflect actual *in vivo* enrichment. Another practical issue may be that the antibody used in immunoprecipitation was not ChIP validated, despite being 'recommended for Gel Supershift and ChIP applications'. As such the epitope on ATF3 to which this antibody binds may be hidden within a complex of transcriptional machinery and higher-order chromatin structure. Fragments of gDNA that are enriched by ATF3 could therefore not be pulled-down by agarose beads due to a lack of antibody binding. A negative result does not necessarily mean that there was error in the technique. Furthermore, ATF3 may not be bound to the promoter following 3 hours IL-1+OSM stimulation investigated. Enrichment may well be occurring at other time points. This

would require time-course ChIP studies as in chapter 3. In the case of c-Fos, enrichment was very transient with binding only demonstrated at 1 hour stimulation. This may, therefore, be the case for ATF3 and the correct stimulation duration was not used to elucidate this binding. Alternatively, ChIP experiments were carried out correctly and ATF3 simply does not bind to *MMP13* promoter following IL-1+OSM stimulation of chondrocytes. Yet, with evidence demonstrating that ATF3 plays a role in the expression of *MMP13,* as discussed above, it could be suggested that ATF3 indirectly contributes to collagenase gene regulation by directly regulating contributing factors like *RUNX2* expression. This hypothesis would account for the extended temporal lag that would be only partially accounted for by AP-1-dependent *ATF3* expression and protein production.

I propose that the absence of detectable ATF3 on *MMP13* promoter may be because it is bound elsewhere on the gene. Enhancer elements are functionally well defined regions present within a great number of genes. These cis-acting regions contain a number of transcription factor binding sites, which act to increase the level of transcription of a gene found on the same molecule of DNA. However, enhancer elements can be found up to 50 kbp up- and down-stream of the TSS. They exert their function by looping of the DNA in order to bring these distal regions into close proximity with trans-activators bound close to the TSS, thus contributing to the assembly of the regulatory complex necessary for transcription [313]. Early trans-activation studies of *MMP1* identified a TPA-responsive enhancer element relatively close to the TSS [314]. However, recent study has identified an IL-1-responsive enhancer element for *MMP13* in chondrocytes. Schmucker et al. [115] demonstrated the presence of a distal enhancer element 20 kbp, 5' of the TSS of *MMP13*. This study showed the enrichment of c-Fos and c-Jun to this enhancer. The study also showed that this enhancer was associated with transcriptionally permissive chromatin and RNA polymerase recruitment. Chromatin capture also showed that this distal element interacted with the proximal promoter a process critical to the optimal expression of *MMP13*. I therefore suggest that ATF3 could be binding to this enhancer region following stimulation. As such, by using primers for the proximal promoter I was unable to detect the distal binding of ATF3.

An interesting finding from the assessment of the temporal gene expression of the contributing factors was that *ATF3*, *AXUD1* and *EGR2* were not nearly as transient as *FOS*. Unlike *FOS* and other IEG, the expression of these three genes did not return to levels comparable with basal conditions. It could be suggested, therefore, that these genes are not conventional IEG. Freter et al. [127] proposes that IEG can actually be subdivided into "fast" and "slow" groups; fast IEG, for example *FOS*, are rapidly induced following stimulus and are capable of reaching peak induction following only 30 minutes of stimulation with levels returning to basal levels within 2 hours [315, 316]. Slow IEG, such as *MYC*, exhibit a 60-90 minute lag prior to the induction of the gene [317]. Moreover, the expression of these genes persists for much longer compared to the fast IEG which rapidly return to basal levels [318]. This expression pattern mirrors observation in ATF3 expression. I therefore suggest that following IL-1+OSM stimulation of HAC both subclasses of IEG are contributing to collagenase expression; fast IEG, *FOS* followed by induction of slow IEG, which include the candidates, *ATF3*, *AXUD1* and *EGR2*. It must be stressed that if ATF3 and EGR2 are indeed regulated by AP-1, as my study has suggested, then this means that they are not strictly IEG. IEG require no *de novo* synthesis in order to be expressed [117], however previous study indicates that *ATF3* and *EGR2* are IEG [319-321]. Previous study has shown that AP-1 factors demonstrate auto-activation [251, 322, 323]. One specific study demonstrated that c-Jun required both itself and ATF2 for full auto-activation [324]. Therefore evidence suggests that IEG can be regulated by other IEG. As such, I suggest that following IL-1+OSM stimulation, AP-1 contribute to the expression of *ATF3* and *EGR2* rather than governing whether or not they are induced.

Unlike ATF3 and EGR2, AXUD1 protein was present at high levels in the chromatin-bound fraction in both stimulated and unstimulated chondrocytes. Moreover, there were only modest changes in chromatin-bound levels over the IL-1+OSM time-course. This means that the IL-1+OSM-mediated induction of gene expression and protein production only serves to slightly increase chromatin binding rather than initiating it. A potential explanation for this may be that AXUD1 can both activate and repress gene transcription, a characteristic demonstrated by other IEG like *MYC* [325]. In unstimulated chondrocytes, AXUD1 may be bound to some gene promoters, repressing them. Upon stimulation, the AXUD1 induced binds to *MMP1* promoter

positively regulating expression which is observed as a modest increase in the chromatin-bound fraction. It is difficult to assess specificity when looking at chromatin binding throughout the genome with Western blotting and so investigating the role of AXUD1 on *MMP1* regulation in this way is not as informative as ChIP studies for example. Although continuous chromatin binding by AXUD1 is intriguing it is difficult to say how this relates to *MMP1* regulation.

A great deal of previous study has implicated NFATc1 in arthritis and cartilage degradation; It has been shown to be expressed in the synovium of RA patients [326] and that increases in the expression of ADAMTS-9 in IL-1-stimulated HAC was dependent on NFATc1 [287]. Moreover, Yoo *et al* [248] showed that inhibition of the NFAT activation led to decreased IL-1-mediated collagenase gene expression in OA chondrocytes and protected against experimentally induced arthritis in mice. Despite the lack of robust gene expression, these studies warranted the inclusion of NFATc1 in siRNA screening. However, following the assessment of temporal protein induction and gene expression, NFATc1 showed very little change following IL-1+OSM stimulation. The data therefore suggest that, although important in collagenase gene regulation, NFATc1 is not induced and that what minor effect on gene expression there is by stimulating with IL-1+OSM does not correspond to increased protein induction. Previous studies have demonstrated an IL-1-dependent induction of *NFATc1* by chondrocytes that peaks following 3 hour stimulation [287]. I suggest that with the addition of OSM to the stimulus there may be a change in signalling pathways rather than simply initiation of additional pathways. Such OSM-dependent changes in signalling are observed in Litherland et al. [97] where the addition of OSM to the an IL-1 stimulus leads to a change in the PKC isoforms regulating collagenase expression. As such the calcium (Ca^{2+})/calcineurin/NFATc1 pathway is surpassed by a more robust, alternate signalling pathway. It must also be highlighted that study demonstrating IL-1-dependent induction of *NFATc1* [287] used human chondrocytes after at least 3 rounds of passage. The effect of this process in primary cells leads to phenotypic changes and altered gene expression which could potentially account for differences in induction between this study and my results [327]. The fact that *NFATc1* is not induced does not necessarily mean that it is not contributing to the regulation of the collagenases, with my own gene silencing data suggesting it is. However, this means that *NFATc1*

expression and protein production do not account for any part of the temporal lag between peak c-Fos/AP-1 and the time in which all the constituents of effective transcriptional machinery have been produced (as demonstrated in protein synthesis inhibition studies, chapter 3). This means that when selecting NFATc1 from the DNA microarray study, I may have chosen a gene that rather than increasing in expression following modest induction at 1.25 hours actually peaked at that time. Previous studies have demonstrated that IL-1 stimulation of chondrocytes leads to the release of intracellular calcium stores and a transient increase of calcium [290, 328]. This should then lead to the binding of calcineurin to calmodulin and which then dephosphorylates cytoplasmic NFATc1 which can then translocate to the nucleus binding to gene promoters and regulating their expression [246]. As such, *NFATc1* may be constitutively expressed in chondrocytes, as demonstrated in the isoforms NFATc2 and 3 in T and B lymphocytes [329]. Therefore, IL-1-dependent Ca^{2+} increases could potentially control NFATc1 activation and subsequent collagenase gene regulation as opposed to increases in *NFATc1* gene induction.

5.2.1 Summary

- siRNA screening of a number of genes selected from DNA microarray were found to have a significant effect on collagenase gene expression.

- Assessment of temporal kinetics at the level of protein and mRNA indicated that certain selected genes, including *ATF3*, *AXUD1* and *EGR2*, were reaching peak induction after 1 hour IL-1+OSM stimulation, a duration that corresponded to peak AP-1 induction.

- The silencing of *FOS* and *JUN* in HAC stimulated with IL-1+OSM indicates that these contributing transcription factors are themselves regulated by AP-1.

6 Chapter 6: General Discussion

Cartilage degradation due to the aberrant expression of proteolytic enzymes (specifically the collagenases MMP-1 and MMP-13) is a key pathological process that occurs in arthritic disease [37, 41, 43-45]. It has been demonstrated that the combination of pro-inflammatory cytokines, IL-1 and OSM, which are both significantly elevated in disease [65, 76, 84] can elicit a robust induction of collagenase gene expression, protein production and subsequent enzyme-dependent collagenolysis, all orchestrated by the chondrocyte [57, 73, 75, 76]. The initiation of this process brings about the irreversible loss of articular cartilage architecture which leads to an impairment of tissue function, resulting in pain and disability in those suffering from the disease [25]. The present study was carried out in order to improve the understanding of the transcriptional regulation of collagenases in HAC following stimulation with IL-1 in combination with OSM which models the complex pro-inflammatory cytokine milieu found in disease.

My study began by investigating the AP-1 transcription factor family members, c-Fos and c-Jun. These factors form heterodimers that have been shown to regulate the transcription of a number of genes within the genome [292]. Previous study demonstrated that these AP-1 factors play a key role in the transcriptional regulation of collagenases in the chondrocyte following pro-inflammatory cytokine stimulation [330, 331] and specifically IL-1+OSM [97]. However, detailed knowledge of AP-1 in the context of this disease model is poor, with most studies perpetuating the dogma of c-Fos/c-Jun-dependent collagenase expression in the chondrocyte. I therefore utilised time-course studies looking at temporal changes in the level of c-Fos/c-Jun mRNA, protein and collagenase promoter recruitment. This led to the discovery of a temporal deficit between the transient peak in *FOS* induction and the initiation of *MMP1* and *MMP13* gene expression. Protein synthesis inhibitor studies then demonstrated that *de novo* synthesis of protein was required after the peak in *FOS* in order to elicit collagenase gene expression, showing that although important, c-Fos/c-Jun is not solely responsible for the transcriptional regulation of the collagenases.

Through the use of DNA microarray, investigation into changes in global gene expression following IL-1+OSM stimulation for durations immediately after the peak in *FOS* expression identified a number of genes that could potentially regulate collagenase gene expression. Subsequent siRNA screening highlighted a subset of these genes that encode factors that were functionally relevant to collagenase gene expression. Analysis of the temporal gene expression and protein production levels of the factors ATF3, AXUD1 and EGR2 indicated that their peak induction was within the temporal deficit. It was also demonstrated, through siRNA-mediated FOS and JUN silencing, that these factors were being regulated by c-Fos and/or c-Jun. This study has provided evidence that the transcriptional regulation of the collagenases following potent synergistic cytokine stimulation is a complex process that involves a number of factors that contribute to the aberrant expression of collagenases by the chondrocyte.

The *in vitro* model which I used to study cartilage degradation as a consequence of complex pro-inflammatory cytokine stimulation is a well-established and robust indicator of potential processes that occur within the articular cartilage of patients suffering from arthritis. *In vitro* models have their obvious failings however these *in vitro* findings form the basis of subsequent work that extrapolates to ever more biologically relevant studies. However, this does not mean that this *in vitro* work could not be further improved upon in order to provide a greater understanding of collagenase regulation in the disease context. Other cell types that reside within synovial joints have the capacity to contribute to the aberrant expression of collagenases within the joint in disease pathology. The expression of *MMP13* is mainly limited to chondrocytes, however, macrophages and fibroblasts also demonstrate expression [36, 37]. Conversely, *MMP1* is ubiquitously expressed throughout many cell types residing within the synovial joint which, as well as chondrocytes, includes macrophages, fibroblasts, osteoclasts, endothelial and epithelial cells [36, 37, 151]. As such, it would be incorrect to assume that chondrocytes were the sole contributors of collagenase production in arthritic disease. It would therefore be interesting to substitute chondrocytes for these other cell types within this model of inflammation-dependent *MMP* expression in order to assess whether or not transcriptional regulation of collagenase is common to all cell types or is in fact cell specific. The potential for conserved gene regulation between different cell types in response to

these cytokines would suggest that therapeutic intervention may result in a more comprehensive reduction in collagenase production in the joint. Alternatively, it may also suggest that targeting collagenase gene regulation may not be specific enough to mitigate side effects. If the differential gene regulation demonstrated in this study occurs exclusively in chondrocytes then therapeutic intervention could be highly specific and therefore only attenuating collagenase activity at the site of cartilage degradation.

The substitution of IL-1 and/or OSM with other pro-inflammatory cytokines that are capable of synergistic collagenase induction [75-77], would also be highly informative. The limitation of only using two cytokines to model a complex cytokine milieu is that the effect of other significantly elevated cytokines found in disease are unaccounted for. Though IL-1 in combination with OSM are valid candidates for this model, other pro-inflammatory cytokines may well be bringing about the regulation of collagenases in a manner distinct from IL-1+OSM. Conversely, a myriad cytokines, growth factors and cell surface receptor activation have all been shown to signal through MAPK pathways which culminate in the induction of AP-1 gene expression [111, 152, 332, 333]. Therefore, substitution with other combinations of cytokines may not affect the way collagenases are regulated in the chondrocyte and so provide a point of common convergence in response to all pro-inflammatory cytokines which could be targeted therapeutically.

Despite the focus of this study being on the gene regulation of the collagenases *MMP1* and *MMP13*, the findings presented may contribute equally to the gene regulation of other *MMP*s involved in arthritis. Previous study has mapped a number of putative binding sites for various trans-activating factors on the proximal promoter region of *MMP*s [2, 111, 114]. A great many *MMP* promoters have been shown to contain multiple AP-1 consensus binding sites found close to the TSS. The gelatinase, MMP-9 and stromelysin, MMP-3 are two such MMPs. Both are elevated in arthritic disease [11, 54, 334-336] and in response to IL-1, their expression has been shown to be dependent on AP-1 in chondrocytes [107, 337]. As well as MMP-3, other *MMP* containing AP-1 binding sites within their promoters have increased expression in chondrocytes following IL-1+OSM stimulation [72]. This includes MMPs that have direct collagenolytic function (MMP-1, MMP-9, MMP-13 and MMP-14 [338, 339]) and

those which cleave pro-collagenases to their active form (MMP-3 and MMP-10 [340, 341]). These studies indicate that many *MMP* gene products that are capable of either directly, or indirectly degrading cartilage, may be regulated in a very similar manner in response to pro-inflammatory cytokine stimulus. As such my study may also improve our understanding of how *MMPs*, other than the collagenase MMP-1 and MMP-13 which are typically associated with arthritis are regulated in the context of disease.

Although collagen cleavage is a key pathological process in arthritic disease, the functions of MMPs are also crucially implicated in the pathology of a number of other diseases that, similar to arthritis, are driven by inflammatory responses, including: chronic inflammatory airway disease (e.g. asthma) [342] and chronic pancreatitis [343] as well as cardiovascular disease [344] and brain disorders [345]. However, the role of MMPs in different diseases is not as definite as in arthritis. Chronic liver disease and the resulting hepatic fibrosis are associated with chronic inflammation and the activation of hepatic stellate cells within the liver. This activation leads to the production and deposition of excess ECM [346]. However, the distinct role of MMPs in fibrogenesis and fibrolysis is not clear. MMP can degrade the excess ECM [347], however, they have also been shown to be down-regulated in disease [348] allowing fibrosis to perpetuate. Equally, MMP have been shown to regulate the inflammatory process underpinning stellate cell activation and fibrogenesis [349]. The conflicting roles of MMP in liver fibrosis is demonstrated in de Meijer *et al* [350]. Following broad-spectrum MMP inhibition in a murine model of chronic CCl_4-induced hepatic injury there was a decrease in hepatic injury and down-regulation of pro-fibrogenic factors. However, pan-MMP inhibition also led to increased fibrosis in the liver demonstrating that a lack of MMP inhibition specificity can be as detrimental as it is beneficial. This contradiction in pathophysiological roles is also evident in tumourgenesis. Increased AP-1-dependent MMP-3 and MMP-9 levels are associated with both arthritis and cancer development. However, in tumourgenesis these MMP have rather contradictory roles, with MMP-3 shown to have a protective function [351] whereas MMP-9 is associated with tumour development and angiogenesis [352]. Interestingly, the collagenase MMP-8 which is generally shown not to contribute to cartilage degradation [53], has a protective role in metastasis [353, 354]. These studies therefore highlight the myriad of functions of MMPs and therefore the necessity for

their differential regulation in the body. Targeting of AP-1-mediated MMP for the treatment of arthritic disease, without specificity, may well prevent cartilage degradation but may also lead to the removal of homeostatic mechanisms that prevents fibrosis and/or tumour development. The insight gained through my study into the differential regulation of collagenases may be the first step toward targeted therapy. For example, ATF3 inhibition could lead to reduced MMP-13 production and cartilage degradation whilst leaving other MMP-dependent processes unperturbed. Although my study demonstrates differential regulation between two key collagenases in arthritic disease, it is yet to be shown whether this specificity at the level of transcriptional regulation is also the case in comparison to other MMPs.

A key piece of evidence that validated my early hypothesis of post-c-Fos/c-Jun production of factors necessary for the complete transcriptional activation of collagenases came from protein synthesis inhibition studies (chapter 3). I went on to reveal novel factors that contributed to collagenase gene expression, whose production may account for the time taken for gene expression to be initiated. However, of the factors elucidated in this study, their gene expression peaked immediately after 1 hour stimulation (maximal *FOS* expression and c-Jun activation). Furthermore, their protein levels also reached maximal levels before the point at which the gene expression of collagenases no longer required the *de novo* synthesis of proteins. This means that other factors produced after ATF3, EGR2 and/or AXUD1 may be required to bring the about complete initiation of transcription. Therefore, this study, although going some way to explaining the aforementioned temporal deficit, still does not account for it in its entirety. This present study may serve to improve our knowledge of the sequence of events which culminate in the initiation of collagenase transcription, yet may still not account for all the factors in this process. As previously referred to, tissue specific factor Runx-2 regulated the expression of *MMP13* [113] and is itself regulated by ATF3 in the chondrocyte [297] and as such Runx-2 could be at the end of this sequence and therefore potentially fully capitulate this cascade of gene induction thereby accounting for the full temporal deficit.

In order to refine my search for contributing factors that could regulate collagenase transcription I established criteria that allowed me to identify only the most pertinent genes in the context of my study. One of these criteria was that factors corresponding

to these genes had the capacity to trans-activate genes. In retrospect I would now go back to the gene expression data generated by the DNA microarray and widen my search criteria. This would therefore allow me to assess the role of other factors that increase in their gene expression following short IL-1+OSM stimulations. Though important, trans-activation of gene promoters is not the only gene regulatory mechanism. For example, DNA methylation is a key repression process exhibited throughout the genome. Bui et al. [102] demonstrated that osteoarthritic HAC have decreased DNA methylation at the proximal promoter of *MMP13* which correlated with increased transcription factor binding (CREB) and increased *MMP13* gene expression. This study demonstrated that methylation state is altered in disease. It could therefore be suggested that other factors highlighted in gene expression analysis could be contributing to this regulation (e.g. through expression of enzymes that control DNA methyl-transferases) and that these were not highlighted due to the alternate research focus. Similarly, histone modifications in order to bring about transcriptionally permissive chromatin [172], the re-structuring of chromatin to bring enhancers into closer proximity to proximal elements [115] and assembly and activation of the transcriptional machinery at the TSS are all potential levels of regulation that could be controlled by factors elucidated by DNA microarray but which were initially overlooked as a consequence of gene search criteria.

The differential regulation of the collagenases has been highlighted in previous studies [93, 97, 104, 355]. These show that the differential expression of *MMP1* and *MMP13* is manifested at the level of intracellular signalling pathways in the chondrocyte. However, some of these studies also highlight the fact that many other cell types that do not produce collagenases exhibit similar induction of these signalling pathways following the same pro-inflammatory stimulus. As such, they suggest that "expression must be due to other factors that enable tissue-specific expression of the gene" [93]. I suggest that not only are collagenases differentially regulated at the level of intracellular signalling but also by gene trans-activation in response to stimulus. Data in the present study demonstrate that silencing of specific genes in chondrocytes stimulated with IL-1+OSM lead to the decreased expression of one collagenase and not the other. Moreover, I suggest that these factors could act as a more appropriate target for therapeutic intervention. In disease there are a number of elevated pro-

inflammatory cytokines present which can be attributed to the aberrant expression of collagenases [356, 357]. These cytokines signal via a great number of signalling pathways, which are capable of "cross-talk" between different pathways resulting in very complex signalling in the chondrocyte [2, 358, 359]. However, many of these pathways still converge upon transcriptional activation of collagenases. As such greater understanding and therapeutic targeting of factors regulating individual collagenase expression would target the hub of pathological signalling in the chondrocyte.

6.1 Future work

During the course of these studies a number of key issues have been highlighted that require further investigation. This would serve to further improve the understanding of transcriptional regulation of collagenases following IL-1+OSM stimulation and the process of cartilage degradation in arthritis.

Initially I would silence AP-1 factors in combination with novel contributing factors, *ATF3*, *EGR2* and *AXUD1* in order to assess the effect on collagenase expression. By silencing these factors in combination with AP-1 factors I would be able to understand if their regulation is entirely dependent on AP-1 regulation or if other factors also contribute to their regulation. For example, if there was no further reduction in collagenase expression following AP-1+*ATF3* silencing compared to AP-1 silencing alone, this may suggest that *ATF3* is entirely dependent on AP-1 and therefore there is no ATF3 present to silence further. Conversely, if there were a greater reduction in collagenase expression when AP-1 and *ATF3* were both silenced then this may suggest that *ATF3* is still being expressed in order to be silenced and though important, AP-1 is not solely regulating *ATF3*. As *ATF3* and *EGR2* have both been previously demonstrated to be IEG [319-321] like *FOS* [315, 360], I would hypothesise that AP-1 are enhancing the expression of these factors rather than regulating them as IEG should not require prior protein *de novo* synthesis for their regulation [119]. I therefore postulate that silencing a combination of these factors would lead to a greater reduction in collagenase gene expression than silencing them individually. Although this may prove difficult, using siRNA, the group is currently developing shRNA-mediated gene knock-

down which, with sustained silencing, may be better able to tease apart these regulatory events.

I would also vary the model of inflammation-dependent collagenase expression by substituting IL-1 and/or OSM with other pro-inflammatory cytokines that are elevated in disease and exhibit synergy in collagenase induction [75-77]. By using other relevant inflammatory mediators in this model I would be able to see if different cytokines elicited different transcriptional regulation or that regulation is, in fact, common to the majority of pro-inflammatory cytokines elevated in disease. In addition to the above alterations to the model of inflammation-dependent collagenase expression, I would also assess whether or not IL-1+OSM stimulation of other collagenase-producing cells within the synovial joint were capable of regulating collagenase in the same way as chondrocytes.

As previously discussed, studies indicate that *RUNX2* may be regulated by ATF3 [297] and that Runx-2 regulates *MMP13* expression but not *MMP1* [113]. This not only accounts for the differential regulation shown by ATF3 but also the fact that the present study still does not fully capitulate the temporal lag between AP-1 peak and initiation of collagenase expression. As such, I would assess genome-wide gene expression changes in HAC stimulated with slightly longer durations (e.g. 2 hours) of IL-1+OSM thereby elucidating genes which were beginning to be expressed after/as a result of contributing factors highlighted in this study. This would involve new DNA microarray studies and the assessment of *RUNX2* temporal gene expression.

Although data from the DNA microarray could be further utilised in order to look at how factors other than those which demonstrated trans-activating capacity could affect collagenase gene regulation, I am convinced that in the case of transcription factor elucidation, further study using ChIP-seq would prove highly informative. This technique allows the user to elucidate the regions at which certain factors are associated with DNA within the entire genome [361]. This involves ChIP using an antibody against a factor of interest to pull-down fragments of DNA which are then sequenced in order to identify specific factor binding sites throughout the genome. With this technique I would be able to ascertain all the sites enriched with c-Fos in the genome following IL-1+OSM stimulation in chondrocytes. This would allow me to

determine whether the genes of factors that are contributing to collagenase expression identified in this study are controlled directly by AP-1. Moreover, I would be able to identify other genes that require c-Fos recruitment to their promoters following different durations of IL-1+OSM stimulation, thereby providing greater insight into which other c-Fos/c-Jun-dependent factors may be contributing to the regulation of collagenase genes. This technique could be further utilised with antibodies for other factors, such as ATF3, in order to assess their binding to collagenase gene promoters but also other genes which themselves may contribute to collagenase transcription.

Not only does ChIP-seq allow for detailed assessment of factor binding at a genome-wide scale but it also gives insight into how regions that are relatively close to the gene are populated with certain factors. By knowing where c-Fos or ATF3 cis-binding occurs in the genome it may be possible to identify possible enhancer or repressor elements that could be playing a role in their collagenase gene regulation as demonstrated in Schmucker *et al* [115]. If candidate regions were identified, chromatin conformation capture could be used to confirm the association of these with more proximal regions and gain insight into the dynamic chromatin remodelling required to regulate these genes.

In order to bring about the initiation of transcription a complex of many factors controlling various levels of gene regulation must assemble around the TSS of genes. As such elucidating the components of this complex at the TSS of collagenases would reveal proteins necessary for transcription and therefore the regulatory mechanisms utilised. It would also provide a basis for screening these components and identifying which are the most crucial in gene regulation of collagenases and can therefore be targeted for further study. This could be achieved by IP with an antibody for a 'target protein' (potentially ATF3) which is a crucial component of this complex. Proteins could then be purified following cell lysis and subject to mass spectrometry analysis which identifies the proteins that co-precipitated with the 'target protein'.

6.2 Summary

This study into the transcriptional regulation of the collagenases, *MMP1* and *MMP13*, in chondrocytes following stimulation with IL-1+OSM has resulted in a greater comprehension of the complexity with which this process is orchestrated. Following a cascade of intracellular signalling events in the chondrocyte following stimulation culminating in STAT activation/dimerisation and promoter binding [82, 97], the IEG *FOS* is rapidly and robustly induced (Figure 46a and Figure 47a) at both mRNA and protein level. It is then found enriched on the proximal promoter of collagenase genes *MMP1* and *MMP13* (section 3.1.4), where it could be potentially acting as an initial anchor for the assembly of other co-factors essential for transcription. In parallel, c-Fos/c-Jun is also enhancing the increase in expression of other factors such as *ATF3*, *EGR2* (Figure 46b) and *AXUD1* (Figure 47b), by potentially binding to promoter regions. These genes are then induced and increase in expression, peaking at a point after c-Fos/c-Jun factors. These factors themselves go on to contribute to the transcriptional regulation of collagenases following the loss of c-Fos enrichment on the collagenase promoters following 1 hour IL-1+OSM stimulation. For *MMP13* expression (Figure 46) my findings combined with previous studies have revealed a number of ways in which the gene may be regulated: ATF3 may be recapitulating the AP-1 dimer bound to *MMP13* promoter with a member of the Jun family, potentially JunD, following the loss of c-Fos enrichment (Figure 46d); EGR2 may be binding a distal consensus binding site upstream of *MMP13* TSS in concert with ATF3 binding at the proximal promoter (Figure 46e); ATF3 may also be regulating factors such as Runx-2, a factor shown to regulate *MMP13* gene expression (Figure 46f); furthermore, some or all of these mechanisms may well be occurring at either proximal promoter region or distal enhancer element (Figure 46g).
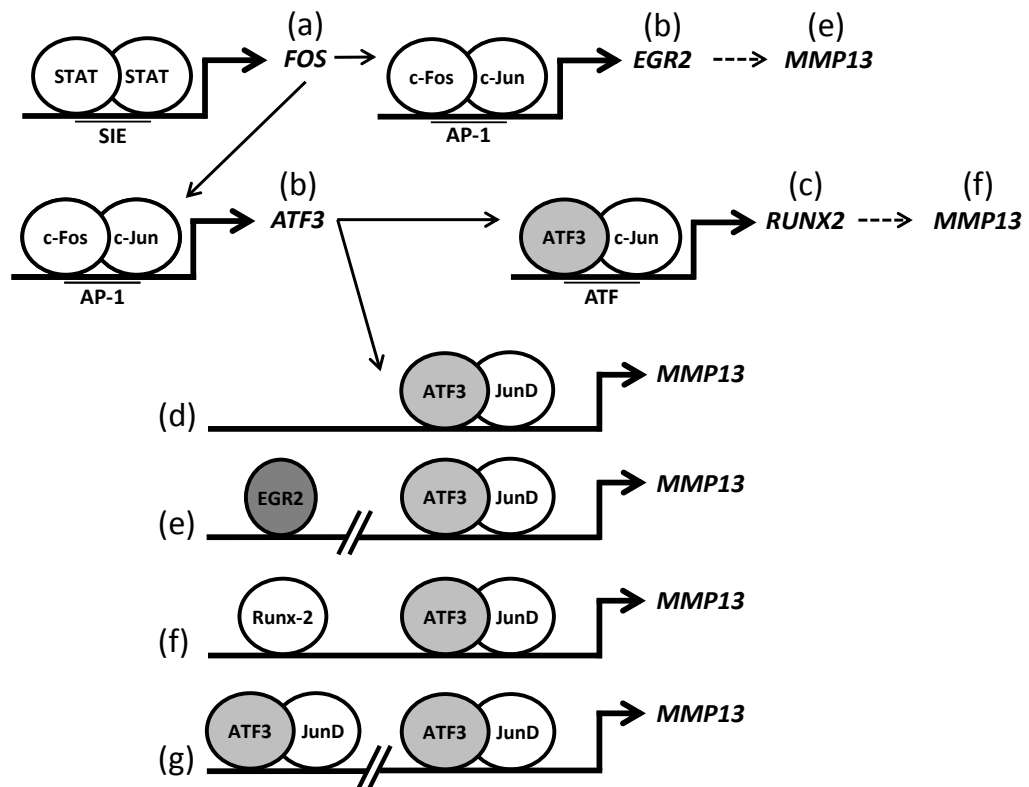
Figure 46: Diagram summarising the potential orchestration of events that lead to *MMP13* gene expression in HAC. Following stimulation of HAC with IL-1+OSM a cascade of intracellular signalling events leads to the increase in c-Fos/c-Jun AP-1 heterodimer in the nucleus via STATs (a). c-Fos/c-Jun heterodimer then regulate *ATF3* and *EGR2* gene expression (b). ATF3 may then also regulate *RUNX2* (c). These factors then contribute to *MMP13* gene expression in a number of potential mechanisms, some of which are highlighted (d-g).

For *MMP1* expression (Figure 47) AXUD1 was shown to contribute to the regulation of this collagenase and like ATF3 for *MMP13*, AXUD1 may well be acting directly (Figure 47c) or in-directly through other factors that regulate *MMP1* (Figure 47d). Again this could well involve a much more complex mechanism comprised of many factors acting in concert at various binding regions.
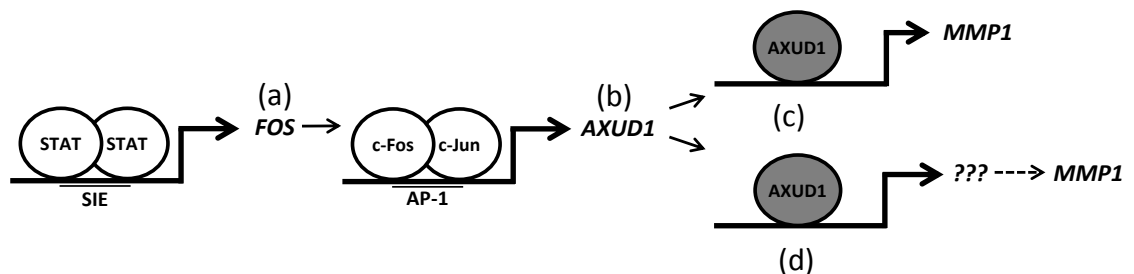
Figure 47: Diagram summarising the potential orchestration of events that lead to *MMP1* gene expression in HAC. Following stimulation of HAC with IL-1+OSM a cascade of intracellular signalling events leads to the increase in c-Fos/c-Jun AP-1 heterodimer in the nucleus via STATs (a). c-Fos/c-Jun heterodimer then regulate *AXUD1* gene expression (b). This factor then contributes to *MMP1* gene expression directly (c) or in-directly the regulation of an unknown gene (d).

143

The majority of therapeutic interventions in arthritis are based on reducing inflammation and/or treating symptoms. Current treatment therefore does little to mitigate the irreversible damage done as a result of proteolytic cartilage degradation. Therapies that have targeted cartilage degradation have proved to be unsuccessful. Broad spectrum inhibition of MMP leads to unwanted side effects that more than offset decreased cartilage destruction [1, 31]. As such the potential of targeted interventions of this pathological process that brings about pain and disability in arthritic patients is considerable. The present study improves our understanding of how collagenases, which facilitate cartilage degradation, are regulated in response to pro-inflammatory stimulus and provides a number of potential targets for therapy. By exploiting the differential regulation of collagenases, it may be possible to specifically abrogate collagenases associated with chondrocytes and cartilage degradation in disease whilst mitigating the effects on other MMPs necessary for normal homeostasis thereby reducing side effects and removing the barrier to successful treatment.

7 Appendix

7.1 Generation of subcellular fractionation western blot figures

In order to elucidate the temporal protein production of c-Fos and the phosphorylation activation of c-Jun, I utilised subcellular fractionation, as outlined in methods section (2.2.8). In order to allow direct comparison of protein level and localisation between different I+O stimulations of HAC, I electrophoresed all 5 subcellular fractions from each I+O duration on a single mini gel. Larger electrophoresis tanks were unreliable and often resulted in irregular migration of proteins and so the samples from the individual stimulation durations were simultaneously electrophoresed on separate mini gels (methods section 2.2.14). Each mini gel was transferred at the same time onto one sheet of PVDF. For ease of use and to minimise antibody wastage, the PVDF membrane was cut into sections which corresponded to each mini gel. All PVDF membrane washes, antibody incubations, exposures to chemiluminescent detection reagents and blot image capture were carried out simultaneously resulting in one single image which contained all exposed membranes as demonstrated in Figure 48. Densitometric scanning was then carried out on these images. In order to best present protein distribution and localisation findings, this single image was cropped using Windows Powerpoint software and arranged into a final figure which was then combined with histograms of relative image density. In Figure 48 the regions highlighted were cropped for the generation of c-Fos western blots in Figure 14Figure 12.
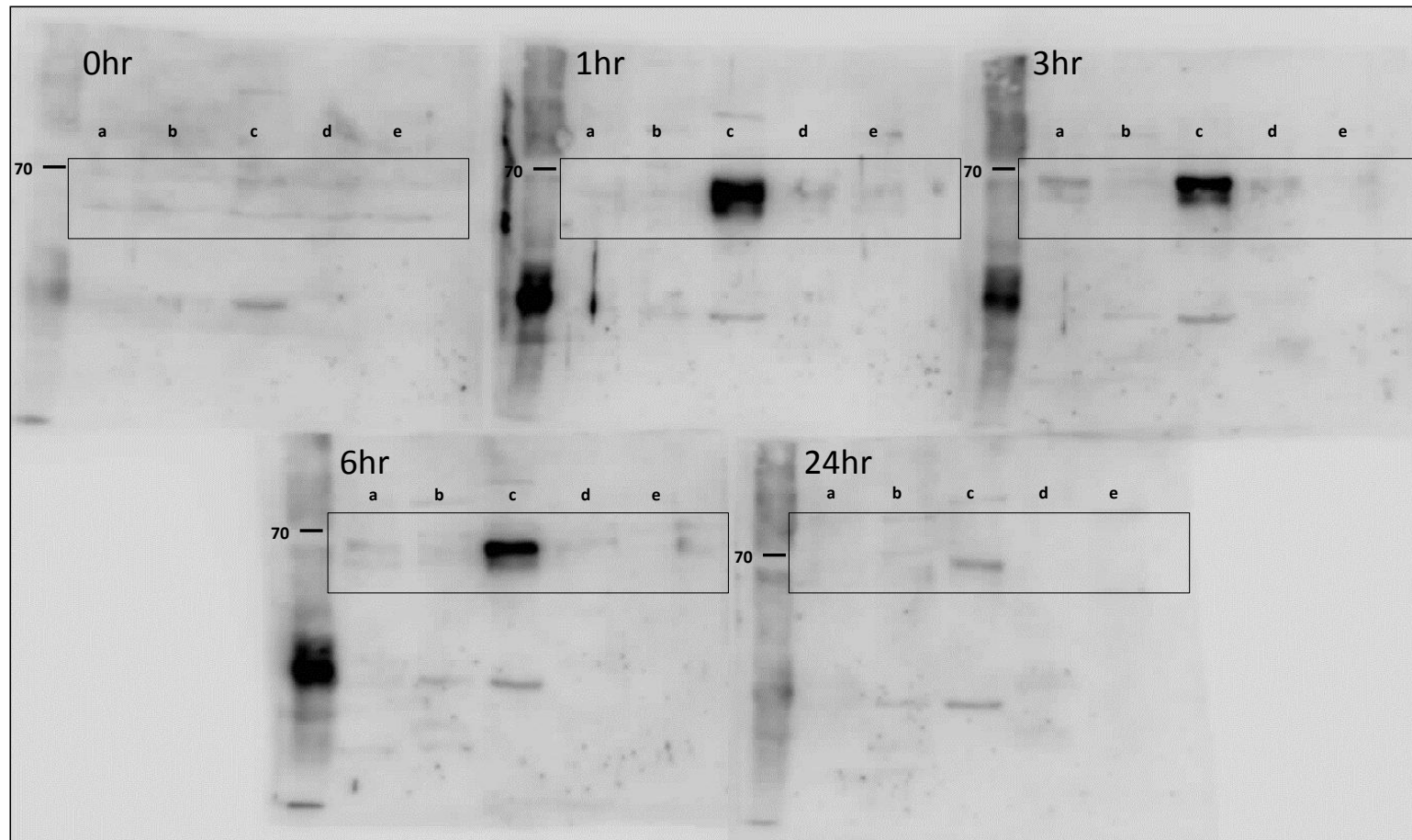
Figure 48: Example of image cropping utilised for generating subcellular fractionation western blot figures HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with c-Fos antibody. Image shows simultaneous exposure of 5 PVDF membranes each corresponding to indicated I+O stimulation. Each rectangular outline corresponds to the representative image used in the results figure 14 (c-Fos). Molecular weight markers (kDa) are annotated adjacent to each I+O duration outline.

7.2 Subcellular fractionation and c-Jun loading controls

A key technique used in my studies was subcellular fractionation and western blotting in order to demonstrate temporal protein distribution and localisation following different durations of I+O stimulation. In Figure 49, only the soluble-nuclear fraction taken from each, individual I+O stimulation duration was loaded on to a single mini-gel. The altered loading confirms the observed temporal protein distribution demonstrated in Figure 14. The same blot was stripped and re-probed for pan-c-Jun antibody. This shows that alterations in levels of p-c-Jun (Ser63) over time were not accounted for by varying c-Jun protein levels. Lamin A/C control confirms equal protein loading.
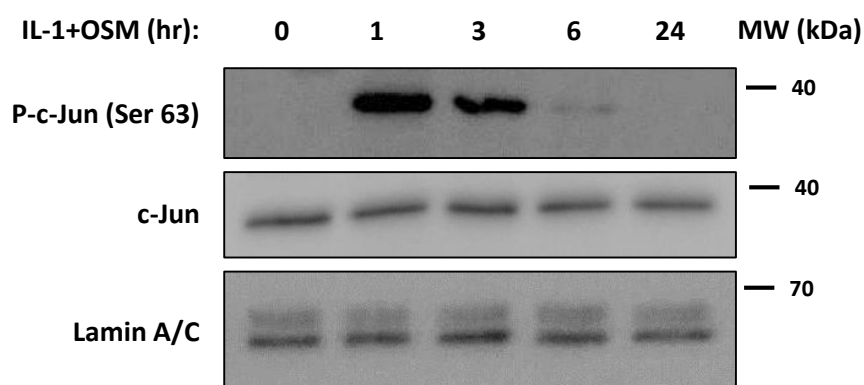
Figure 49: Loading controls for subcellular fractionation P-c-Jun (Ser 63) HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Cells were then subject to subcellular protein fractionation. Proteins from soluble nuclear subcellular fractions were resolved using SDS-PAGE and immunoblotted with the indicated antibodies. Sample volumes loaded for SDS-PAGE were dependent on the relative volume into which the individual fractions were lysed.

7.3 Full length, uncropped gel images of temporal protein distribution and localisation of factors highlighted from siRNA screening

DNA microarray study and functional validation using siRNA highlighted a number of factors that contributed to transcriptional regulation of the *collagenase* genes. The induction and the cellular localisation of these proteins were assessed following IL-1+OSM stimulation time course. Figures 50-53 show the full length, uncropped, gel images used to generate Western blot figures 39-43, respectively, as described in appendix 7.1.
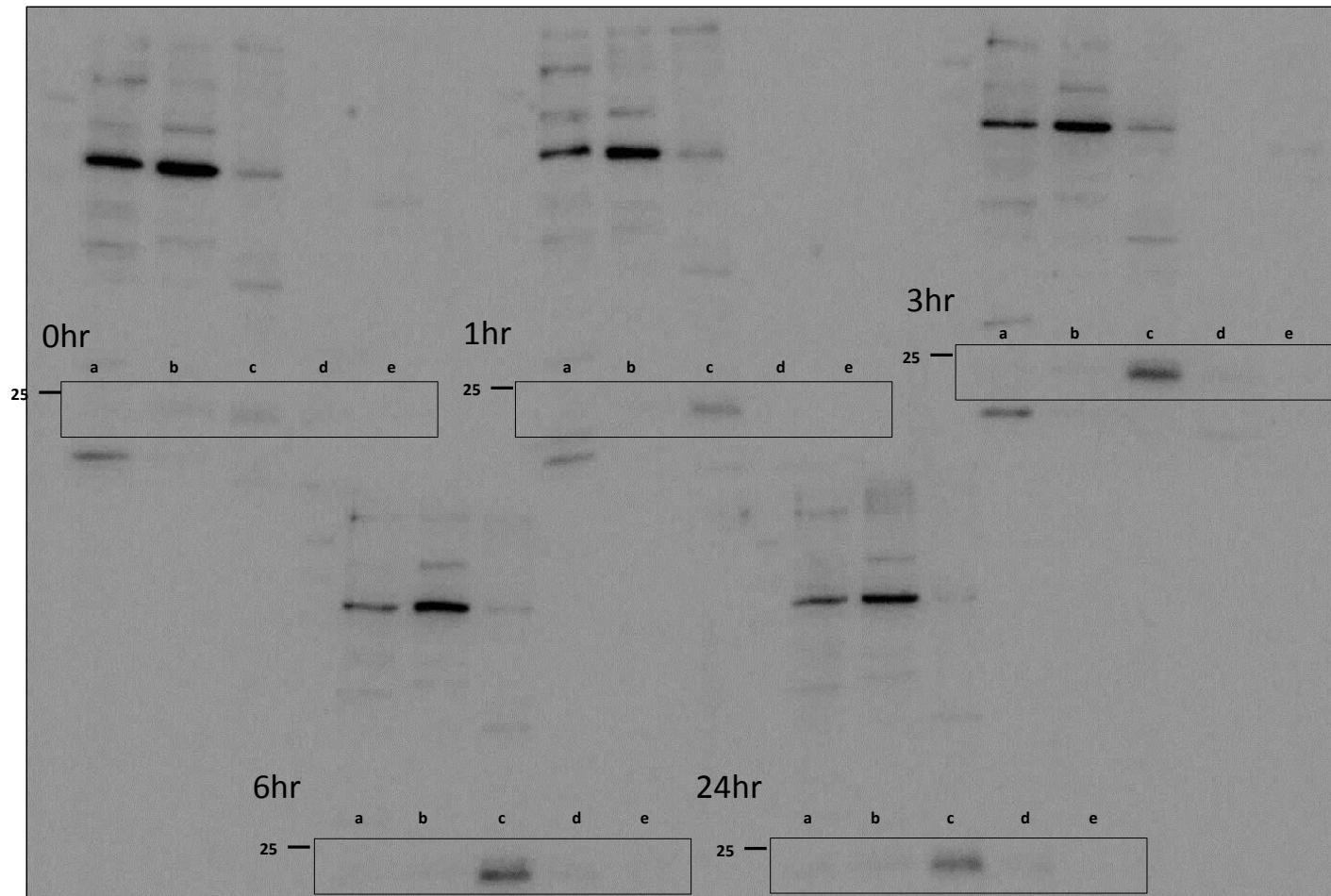
Figure 50: Full length, uncropped, gel image for Western blot for ATF3 in subcellular protein fractionation from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with ATF3 antibody. Image shows simultaneous exposure of 5 PVDF membranes each corresponding to the indicated I+O stimulation. Each rectangular outline corresponds to the representative image used in the results figure 39. Molecular weight markers (kDa) are annotated to the left of each I+O duration outline.

148
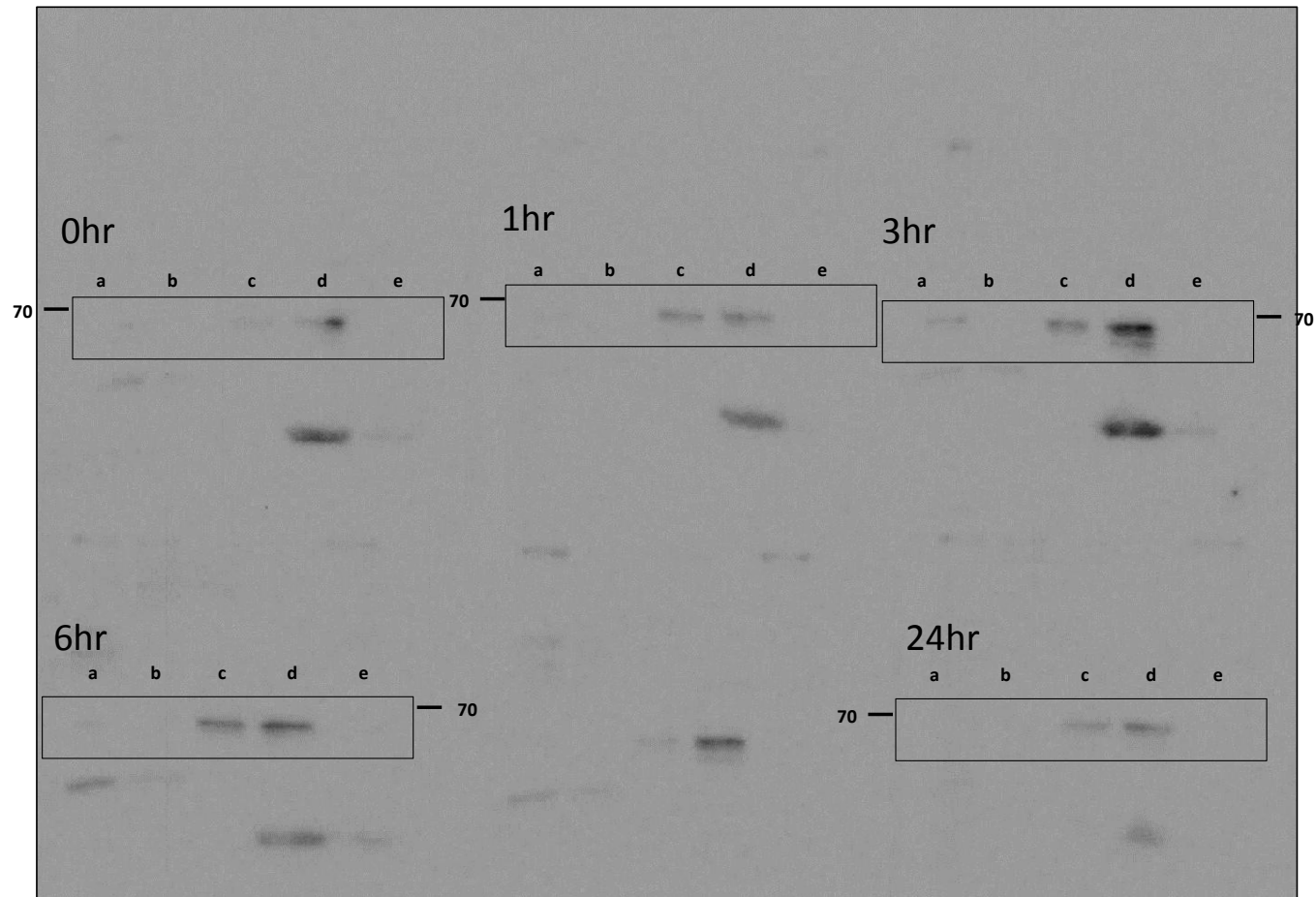
Figure 51: Full length, uncropped, gel image for Western blot for AXUD1 in subcellular protein fractionation from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with AXUD1 antibody. Image shows simultaneous exposure of 5 PVDF membranes each corresponding to the indicated I+O stimulation. Each rectangular outline corresponds to the representative image used in the results figure 40. Molecular weight markers (kDa) are annotated adjacent to each I+O duration outline.

149
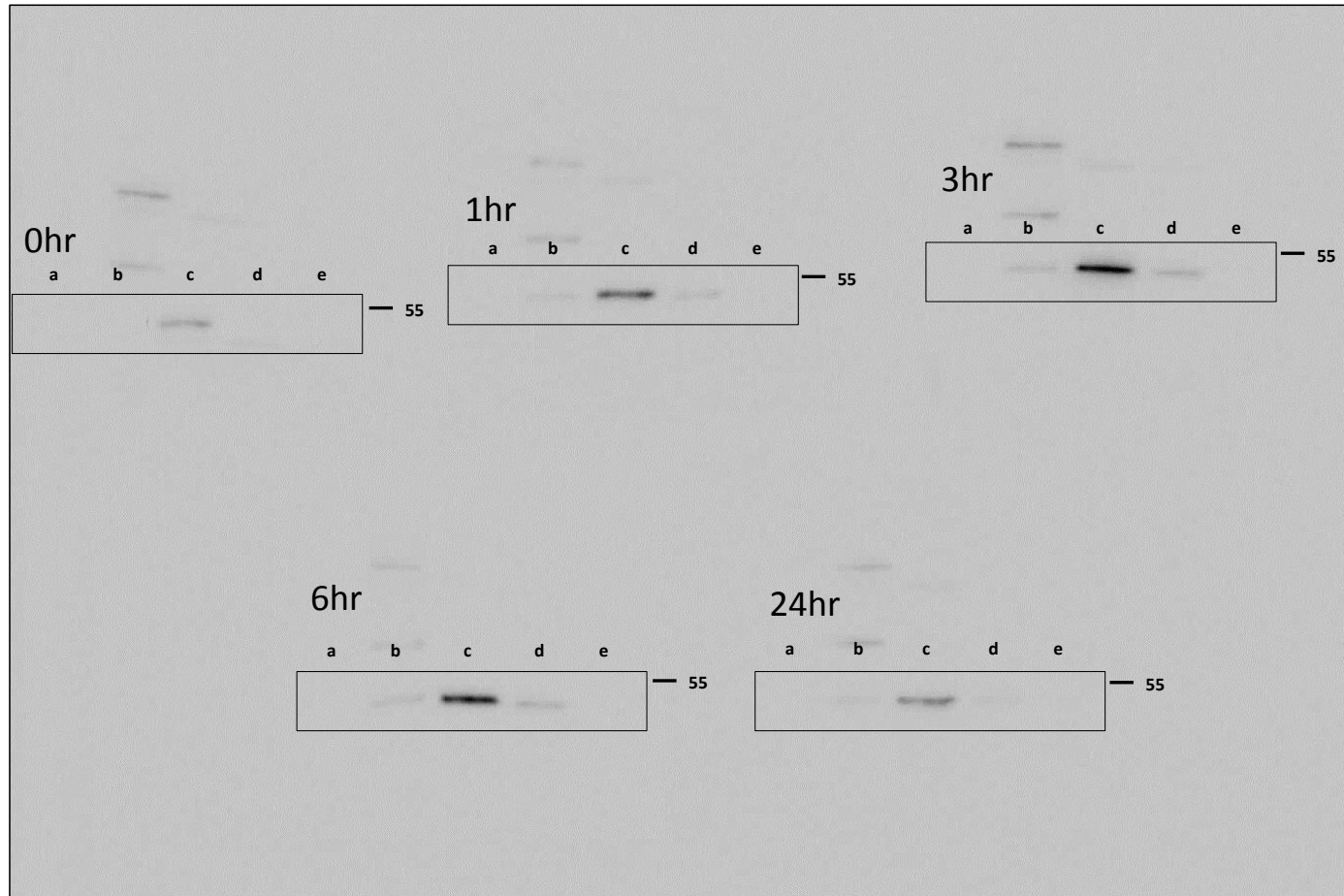
Figure 52: Full length, uncropped, gel image for Western blot for EGR2 in subcellular protein fractionation from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with EGR2 antibody. Image shows simultaneous exposure of 5 PVDF membranes each corresponding to the indicated I+O stimulation. Each rectangular outline corresponds to the representative image used in the results figure 41. Molecular weight markers (kDa) are annotated adjacent to each I+O duration outline.
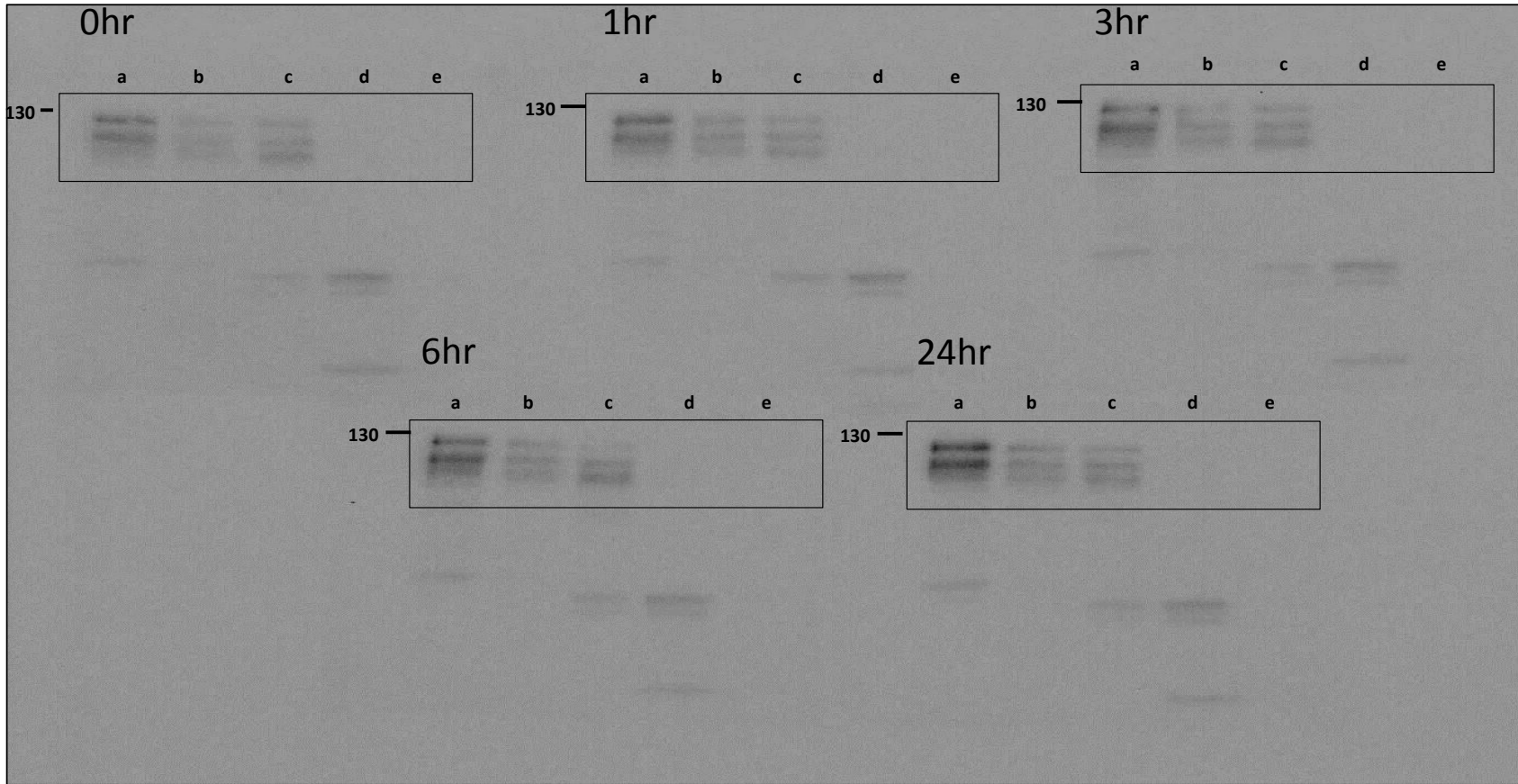
Figure 53: Full length, uncropped, gel image for Western blot for NFATc1 in subcellular protein fractionation from HAC stimulated with IL-1+OSM. HAC were stimulated with IL-1 (0.05ng/ml) in combination with OSM (10ng/ml) for the indicated durations. Proteins from cytosolic (a), membrane-bound (b), soluble nuclear (c), chromatin-bound, (d) and cytoskeletal (e) subcellular fractions were resolved using SDS-PAGE and immunoblotted with NFATc1 antibody. Image shows simultaneous exposure of 5 PVDF membranes each corresponding to the indicated I+O stimulation. Each rectangular outline corresponds to the representative image used in the results figure 42. Molecular weight markers (kDa) are annotated adjacent to each I+O duration outline.

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