The role of novel transmembrane serine proteases: implications for arthritis

Amit Patel BSc(Hons)

Thesis submitted in partial fulfilment of the requirements
of the regulations for the degree of Doctor of Philosophy

Newcastle University

Faculty of Medical Sciences

Institute of Cellular Medicine

October 2010

Declaration

The work reported in this thesis was performed from October 2007 to September 2010. The work in this thesis was carried out in the Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University. Except for commonly held concepts, and where specific reference is made to other work, the content of the thesis is original. All experiments were carried out by myself under the guidance of my supervisors, Prof. Drew Rowan and Dr Jenny Milner.

No part of this thesis has been submitted for the award of any other degree.

Abstract

In the arthritides, the breakdown of articular cartilage and the erosion of subchondral bone lead to loss of efficient joint function. Matrix metalloproteinases (MMPs) can collectively degrade the extracellular matrix of cartilage, and have been strongly implicated in cartilage destruction. MMPs are produced as inactive precursors requiring activation. Addition of interleukin-1 (IL-1) and oncostatin M (OSM) to bovine cartilage in explant culture results in a synergistic loss of the collagen matrix, accompanied by a dramatic increase in pro-MMP synthesis and activation. Increasing evidence implicates serine proteinases in pathologic tissue turnover and the transmembrane serine proteases fibroblast activation protein-α (FAPα), dipeptidyl peptidase IV (DPPIV) and matriptase were recently found to be up-regulated in osteoarthritic (OA) cartilage compared to normal cartilage. The aim of this work was to identify whether these transmembrane serine proteases have roles in cartilage collagen breakdown.

Inhibition of IL-1+OSM-mediated collagen breakdown was not observed using selective FAPα inhibitors, indicating that FAPα enzyme activity *per se* plays no direct role in collagen breakdown. In contrast, DPPIV inhibition showed significant protection of IL-1+OSM-mediated collagen breakdown. However, the mechanism of action of DPPIV remains to be elucidated.

Matriptase was demonstrated to activate pro-MMPs as well as induce MMP expression in OA cartilage via protease-activated receptor 2 activation. This makes matriptase a key protease in the pathology of OA.

Overall, this study has identified matriptase as a key serine protease in OA pathology and confirmed a potential role for DPPIV in cytokine-mediated cartilage degradation. These findings support the need for further research in order that therapeutic interventions targeting these enzymes may be realised.

Table of contents

List of figures

List of tables

Acknowledgements

Firstly, I would like to thank my supervisors, Professor Drew Rowan and Dr Jenny Milner, for their advice over the past 3 years. I would also like to give special thanks to Dr Gary Litherland for his endless help and providing a constant source of amusement.

I would like to thank all of the technical staff for their help and patience, especially for not shooting the messenger when equipment broke down. I must also thank all the Tunnel members, past and present, and everyone in the department, too many to name, for the lab banter and the nights out. Special thanks to Christine Richter for all the support and patience she has shown during this PhD. Thank you to my family for their never ending understanding and support. I thank the Medical Research Council and Ferring Pharmaceuticals for funding this PhD.

Publications

Milner JM, **Patel A**, Rowan AD. (2008). Emerging roles of serine proteinases in tissue turnover in arthritis. Arthritis & Rheumatism 58(12): 3644-3656

Milner JM*, **Patel A*,** Davidson RK, Swingler TE, Désilets A, Young DA, Kelso EB, Donell ST, Cawston TE, Clark IM, Ferrell WR, Plevin R, Lockhart JC, Leduc R, Rowan AD. (2010). Matriptase is a novel initiator of cartilage matrix degradation in osteoarthritis. Arthritis & Rheumatism 62(7): 1955-1966.

* Dr Milner and Mr Patel contributed equally to this work

Abbreviations

^{3}H	Tritium
α_1-AT	α_1-antitrypsin
A	Adenosine
C	Cytosine
G	Guanine
T	Thymidine
ADAM	A disintegrin and metalloprotease
ALK	Activin-like kinase
AMC	7-amino-4-methylcoumarin
ANOVA	One-way analysis of variance
AP-1	Activator protein 1
APMA	4-Aminophenylmercuric acetate
APS	Ammonium persulfate
AT III	Antithrombin III
BNC	Bovine nasal cartilage
Boc	t-Butyloxycarbonyl
bp	Base pairs
BSA	Bovine Serum Albumin
cDNA	Complementary deoxyribonucleic acid
CIA	Collagen induced arthritis
CUB	C1s/C1r, urchin embryonic growth factor and bone morphogenic protein-1
CV	Column volume
Da	Dalton

DAB	p-Dimethylaminobenzaldehyde
ddH_2O	Nanopure water
DEPC	Diethyl pyrocarbonate
DFP	Di-isopropyl phosphorofluoridate
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMM	Destabilisation of the medial meniscus
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DPi	Diphenylphosphonate inhibitor
DPP	Dipeptidyl peptidase
DPPIV	Dipeptidyl peptidase IV
DTT	*(2S,3S)*-1,4-Bis-sulfanylbutane-2,3-diol
ECM	Extracellular matrix
EDTA	Ethylenedinitrilotetraacetic acid
EGR1	Early growth response protein 1
ELISA	Enzyme-linked immunoabsorbant assay
ERK	Extracellular Signal-Regulated Kinase
FACIT	Fibril-associated collagen with interrupted triple helix
FAPα	Fibroblast activation proteinα
FCS	Foetal calf serum
FPLC	Fast protein liquid chromatography
FRZ	Frizzled domain

FSB	Final sample buffer
GAG	Glycosaminoglycan
GLP-1	Glucagon-like peptide-1
GPCR	G-protein coupled receptor
GPI	Glycosylphosphatidylinositol
h	Hour
HA	Hyaluronic acid
HAI-1	Hepatocyte growth factor activator inhibitor-1
HCl	Hydrochloric acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HGF	Hepatocyte growth factor
HOXA4	Homeobox A4
ICE	IL-1β converting enzyme
IGF-1	Insulin-like growth factor-1
IL-1RA	Interluekin-1 receptor antagonist
IL-1RI	Interluekin-1 receptor I
IL-1RII	Interluekin-1 receptor II
IL-1	Interluekin-1α
JNK	c-Jun N-terminal kinase
KO	Knockout
LDLA	Low-density lipoprotein receptor domain class A
LGB	Lower gel buffer
LIF	Leukaemia inhibitory factor
MAM	Merpin, A5 antigen and receptor protein phosphatase μ domain
MAPK	Mitogen-activated protein kinase

min	Minute
MMLV	Moloney murine leukaemia virus
MMP	Matrix metalloprotease
MPa	MegaPascal
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOF	Neck of femur fracture
OA	Osteoarthritis
OPD	o-Phenylenediamine
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease activated receptor
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RCL	Reactive centre loop
ROX	6-Carboxy-X-Rhodamine
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDF-1α	Stromal cell-derived factor-1α
SDS	Sodium dodecyl sulphate
SEA	Single sea urchin sperm protein, enteropeptidase, agrin domain
SEM	Standard error of the mean
shRNA	Small homologous RNA
sIL-6R	Soluble interluekin-6 receptor

siRNA	Small interfering ribonucleic acid
SMAD	Sma and Mad related protein
SR	Scavenger receptor cysteine-rich domain
STAT	Signal transducer and activator of transcription
TEMED	N,N,N'N'-tetramethylenediamine
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteases
TLR	Toll-like receptor
TM	Transmembrane
TNF-α	Tumour necrosis factor-α
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Triton X-100	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
TTSP	Type II transmembrane serine protease
Tween 20	Polyoxyethylenesorbitan monolaurate
U	Units
UBG	Upper gel buffer
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
v/v	Volume by volume
w/v	Weight by volume
Z-	Benzyloxycarbonyl

Amino Acid symbols

Alanine	Ala	A
Arginine	Arg	R

Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z

CHAPTER 1

Introduction

1.1 Cartilage

Cartilage is a highly specialised connective tissue, which falls into the following three distinct categories: elastic; fibro-cartilage and hyaline. The composition of the cartilage matrix thereby determines its classification. Articular cartilage, a specialised form of hyaline cartilage, is found covering the ends of long bones in all synovial joint cavities. Articular cartilage is a unique connective tissue as it is both aneural and avascular (Martel-Pelletier et al., 2008, Kuettner, 1992). It contains only one cell type, the chondrocyte (section 1.1.1), that maintains the large extracellular matrix (ECM) network of the tissue. The ECM is primarily composed of proteoglycans (section 1.1.2) and collagens (section 1.1.3) but other minor matrix components are also present (section 1.1.4) (Bhosale and Richardson, 2008).

Adult articular cartilage displays a zonal architecture (Ikemefuna et al., 2009) (Figure 1.1). The zones are distinguished according to their differing morphologies and comprise:

- The superficial zone;
- The middle (or transitional) zone;
- The deep (or radial) zone;
- The calcified zone.

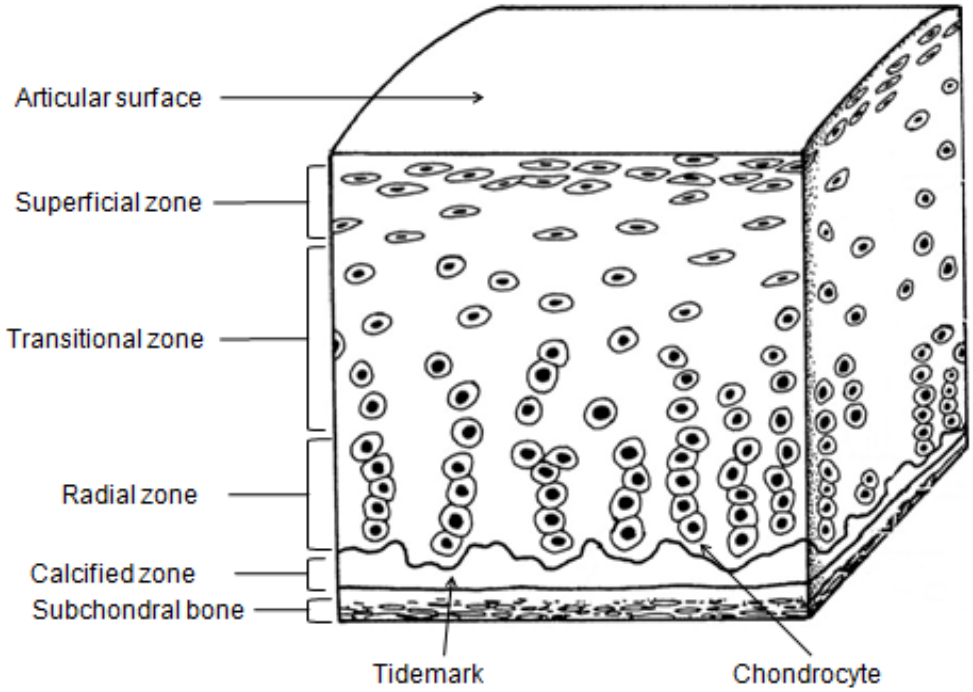


Figure 1.1 Zonal architecture of articular cartilage.

Cross-section of cartilage showing the transition from the articular surface to the subchondral bone. Adapted from Newman (1998).

The superficial zone is exposed to the synovium and represents the thinnest layer of the cartilage. This zone has the highest rate of collagen synthesis in the entire tissue and its chondrocytes are small and flattened surrounded by a matrix composed of thin collagen fibres and little aggrecan. The transitional zone has an extensive ECM composed of aggrecan and large collagen fibres. Here, the chondrocytes are less abundant and exhibit a spherical morphology (Bhosale and Richardson, 2008, Ikemefuna et al., 2009). In the radial zone, the chondrocytes are arranged in parallel to the collagen fibres (Martel-Pelletier et al., 2008) and appear to form columns. The proteoglycan content here is the highest in the whole tissue but its cell density is the lowest. The calcified zone contains a small volume of cells embedded in a calcified matrix and displays low metabolic activity (Bhosale and Richardson, 2008, Ikemefuna et al., 2009). The chondrocytes in this zone display a hypertrophic phenotype, with the synthesis of type X collagen being

the classical marker. The large amount of collagen X in this zone confers structural integrity and transition to the subchondral bone by penetrating into the calcified zone, thereby anchoring the articular cartilage to the subchondral bone (Martel-Pelletier et al., 2008).

This organisation of the macromolecules enables normal, healthy articular cartilage to sustain extremely high pressures of up to 10 MPa (Kerin et al., 2002).

1.1.1 Chondrocytes

Chondrocytes are responsible for the maintenance of the cartilage matrix and have a high rate of metabolism. However, due to the hypocellular nature of cartilage, the overall metabolism of the tissue is quite low (Mankin, 1982), and due to the avascular nature of cartilage, chondrocytes rely on diffusion of oxygen from the synovial tissue (Coimbra et al., 2004, Muir, 1995). Therefore, chondrocytes must rely on anaerobic metabolism. Chondrocytes are known to be encapsulated within a pericellular environment, termed the chondron (Poole, 1997). Within the chondron, the chondrocytes are anchored to the type II collagen network by type VI collagen (Aigner and Stove, 2003). The pericellular environment is composed of two different layers: a glycocalyx, providing attachment for the chondrocyte, and fibrillar capsule, providing attachment for the ECM. The embedding of chondrocytes within this environment is thought to protect them from the high compression strengths in the joint during movement. However, the compressive force does affect cell metabolism (Urban, 1994) and excessive pressures to the cartilage can result in homeostatic imbalance and leads to cartilage degradation.

1.1.2 Proteoglycans

Proteoglycans are composed of a central protein core with numerous covalently attached glycosaminoglycan (GAG) chains. The GAG chains confer the biological

function of proteoglycans as their distinct physio-chemical properties provide hydration and swelling pressure (Gentili and Cancedda, 2009).

The major proteoglycan found in articulating cartilage is aggrecan (Martel-Pelletier et al., 2008). Aggrecan has a large protein core (230 kDa) to which numerous GAGs and N-linked and O-linked oligosaccharide chains are covalently attached. The main GAGs are keratan sulphate and chondroitin sulphate. Keratan sulphate is a multi-repeat dimer of N-acetylglucosamine-galactose and chondroitin sulphate is a multi-repeat dimer of N-acetylgalactosamine-glucuronic acid (Kuettner, 1992). The protein core consists of three distinct globular domains, G1, G2 and G3 (Figure 1.2), which are stabilised by disulphide bridges. The G1 and G2 domains are located near the N-terminus and connected via a short interglobular domain, while the G3 domain is near the C-terminus and separated from the G2 domain by a long GAG attachment region. The G1 domain interacts non-covalently with hyaluronic acid (HA) and link protein (Martel-Pelletier et al., 2008) and the G3 domain is essential for the post-translational modification and secretion of aggrecan. The function of the G2 domain has yet to be elucidated.

The proteoglycan content of the joint can also facilitate signalling in the joint (Gentili and Cancedda, 2009), as heparin sulphate can bind to a number of signalling molecules, including fibroblast growth factors and transforming growth factor-β (TGF-β) 1.

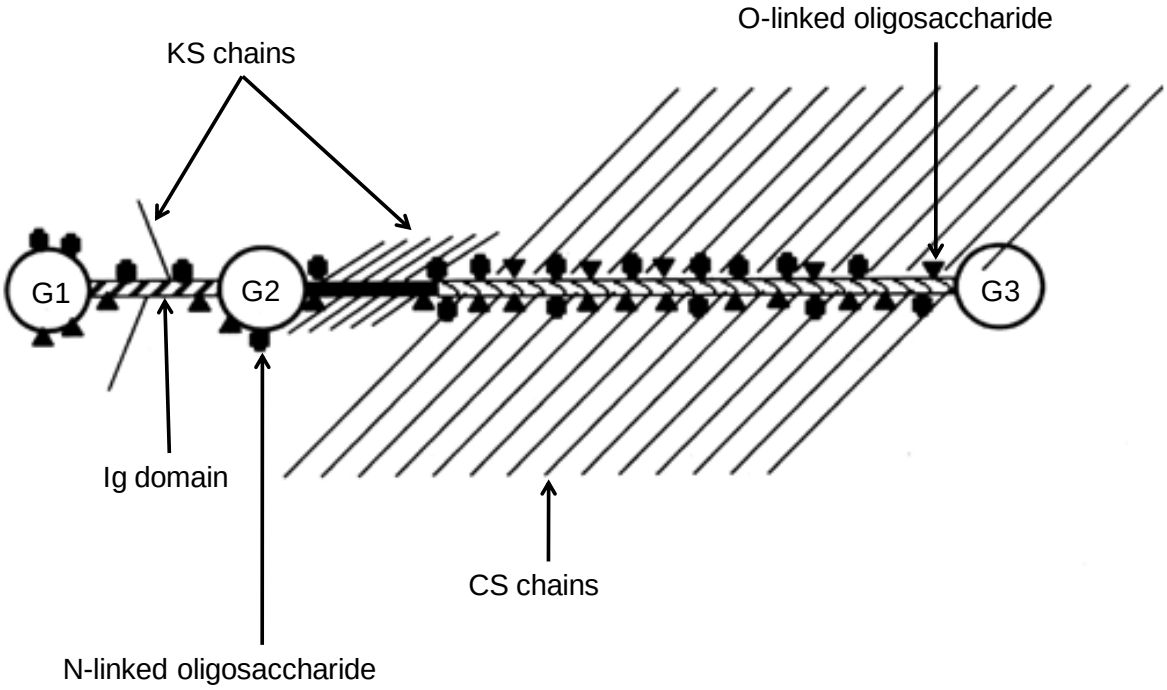


Figure 1.2 Structure of aggrecan.

Protein structure of aggrecan showing a simplified depiction of the domain organization and glycosaminoglycan attachment areas. CS, chondroitin sulphate; KS, keratin sulphate; Ig, immunoglobulin. Adapted from Kiani et al. (2002).

1.1.3 Collagens

Collagens make up a family of closely related but distinct fibrous proteins (Gentili and Cancedda, 2009). There are more than 30 genes encoding collagen proteins and collagen-like proteins. Collagen is the predominant protein found in mammals, constituting the structural components of tissues and organs.

Collagen proteins form a wide array of different structures, such as fibrils (type I, II, III) (Kolacna et al., 2007) and network-like sheets (type IV) (Jamshid et al., 2008). The characteristic amino acid sequence is Gly-Xaa-Yaa, where Xaa is proline every third residue and Yaa is hydroxyproline every seventh residue. The presence of glycine in this tri-peptide repeat helps to stabilise the collagen fibrils as it faces the interior of the helix, as the side chain of glycine is short and allows for tight association between the

fibrils. Hydroxyproline residues help to stabilise the polypeptide chains and are critical to collagen formation (Kolacna et al., 2007).

Type II collagen is the major fibrillar collagen found in articular cartilage. It is synthesised intracellularly as pro-α-chains which contain large pro-peptides at the N- and C-termini. These pro-peptides are separated from the central helix forming region by telopeptides and are essential for trimerisation of the pro-α-chains. Subsequently, this trimer is secreted from the chondrocyte and the pro-peptides are removed by proteolysis. The trimeric α-chains are then assembled into large collagen fibrils by covalent cross-linking in the telopeptide regions (Figure 1.3) (Martel-Pelletier et al., 2008).

Other collagen types, type IX, X and XI, co-exist with type II collagen in the articulating joint. Type IX is a member of the fibril-associated collagen with interrupted triple helix (FACIT) family of collagens and is cartilage specific. It does not form a fibril itself, but is found at the interface of type II/type XI fibrils. Collagen IX fibrils are thought to bridge the collagen network to the glycosaminoglycan network (Martel-Pelletier et al., 2008). Type IX collagen can have chondroitin sulphate attached to the α2 chain, and is sometimes thought of as a proteoglycan.

Type XI collagen is another fibrillar collagen and is also cartilage specific. It is synthesised and secreted in a similar manner as type II collagen, except here only the C-terminal pro-peptide is removed. Type XI collagens do not form their own unique fibrils, but associate with type II collagen molecules to form heterotypic fibrils. Type XI collagen is predominantly found in the thin fibrils of the pericellular network.

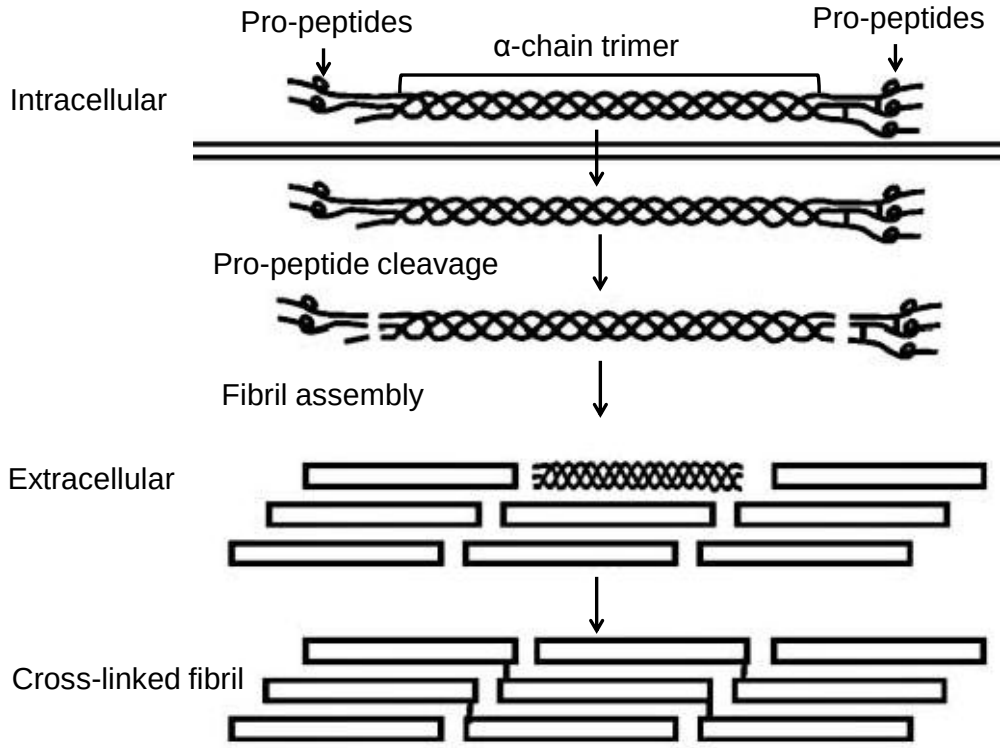


Figure 1.3 Collagen fibril formation.

Collagen pro-α-chains from trimers intracellularly and are then secreted into the extracellular space and proteolytically cleaved. The trimers then assemble into large fibrils which are stabilised by cross-linking. The crosslinking is initiated by the oxidative deamination between lysine and hydroxylysine resides Adapted from Kadler et al. (1996).

Type X collagen is termed a short chain collagen and its fibril is composed of homotrimeric α1(X) chains (Chan et al., 1995, Schmid and Linsenmayer, 1983). As mentioned earlier, the hypertrophic zone of the cartilage is rich in type X collagen, which confers both structural integrity and a transition to the subchondral bone. The expression of type X collagen is closely associated with hypertrophic chondrocytes and is often used as a marker for their presence in this zone (van der Kraan et al., 2001). Furthermore, it is postulated that type X collagen plays a role in cartilage mineralisation (Gibson and Flint, 1985). This may be due to the open lattice structure that type X

collagen adopts that permits vascular invasion, calcification, and remodelling to proceed to the next phase of bone development (Kwan et al., 1991).

1.1.4 Non-aggregating proteins

Biglycan, fibromodulin and decorin are examples of non-aggregating proteoglycans found in articular cartilage (Kuettner, 1992, Gentili and Cancedda, 2009). They share a similar protein structure but differ in their GAG composition. Biglycan possesses two dermetan sulphate side chains, whereas decorin contains one. In contrast, fibromodulin contains multiple keratin sulphate chains attached to the protein core. Biglycan, fibromodulin and decorin are characterised by their ability to interact with collagen proteins. Biglycan is found in the pericellular matrix and binds type VI collagen, while decorin and fibromodulin both bind to the type II collagen fibrils (Kuettner, 1992).

HA is a non-sulphated GAG and the size of the HA molecules in the joint decrease with age (Martin and Buckwalter, 2001). As HA binds aggrecan molecules (section 1.1.2) and this helps to attract water into the joint after the application of a compressive load to the cartilage, a decrease in the size of HA results in decreased water content in the joint.

Link protein has a structure that is analogous to the G1 domain of aggrecan. It plays a number of key roles in cartilage such as the binding of aggrecan to the HA filaments and the protection of HA from degradation. Cleavage of link protein generates the LP3 isoform, which can act as a growth factor to promote matrix production (Martel-Pelletier et al., 2008).

1.2 Arthritis

1.2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a disease characterised by systemic activation of the immune system, which is possibly initiated by the recognition of auto-antigens (de

Vries et al., 2005). Typically, RA affects about 1% of the population at a ratio of 3:1 (female:male) and disease presentation usually occurs at the ages of 30 to 50 years (Majithia and Geraci, 2007).

One of the distinguishing features of RA is the increase in the number of invasive cells into the joint space. There is increased thickening of the synovial lining by peripheral blood T cells, activated macrophages, B cells and synovial fibroblasts (Lundy et al., 2007, Mauri and Ehrenstein, 2007, Lutzky et al., 2007, Muller-Ladner et al., 2007, Kinne et al., 2007). The synovial fibroblast is thought to instigate the major pathways that lead to joint destruction by producing inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6) (Chiu et al., 2008). The chronic activation of the immune system in the joint space leads to the release of inflammatory cytokines, such as IL-1 and oncostatin M (OSM), which have been shown to synergistically increase levels of the matrix metalloproteases (MMPs), such as MMP-1 (Cawston et al., 1998). A further distinct feature of RA is the formation of locally invasive tissue, termed pannus, from synovial tissue. The pannus has a high level of MMP expression and invades the cartilage and subchondral bone, causing permanent tissue impairment (Lee and Weinblatt, 2001).

1.2.2 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis, affecting the majority of individuals over the age of 65 years. However, it is a disease that is not just a disorder of the articular cartilage but affects most of the major tissues of the joint. The subchondral bone shows increased invasion into the tidemark of the cartilage and results in thinning of cartilage. This increased ossification in the subchondral bone is thought to occur due to reactivation of the secondary centre of ossification (Brandt et al., 2006). Cartilage damage in OA is thought to occur due to a number of different reasons such as

ageing, genetic factors as well as excessive loading. The degeneration of cartilage occurs due to an imbalance between the anabolic and catabolic pathways. Excessive loading of cartilage can cause increased collagen degradation and up-regulation of MMPs. This increase in the levels of MMPs leads to an increase in the turnover rate of the cartilage matrix. However, type II collagen gene expression in OA is shown to be up-regulated in the late stages of the disease, indicating that anabolism does occur, in an attempt to repair the damage (Goldring and Goldring, 2007). Nevertheless, the damage to the collagen network is irreversible at this point, as the chondrocyte cannot replicate the complex network as it did during development.

1.3 Cytokines

Cytokines are soluble or cell surface anchored molecules that play an essential role in mediating cell-cell interactions. Cytokines generally act through autocrine, juxtacrine and paracrine signalling by binding their specific receptors. They are important mediators and regulators of inflammation and cartilage degradation in the arthritides (Table 1.1) (Goldring and Goldring, 2004). In RA, the chronic inflammation results in an increased concentration of cytokines in the joint space. The cytokines can then interact with synovial fibroblasts or diffuse through the synovial fluid to the cartilage and cause an increase in the release of catabolic factors.

Catabolic	Anti-inflammatory	Anabolic
IL-1	IL-4	Insulin-like growth factor-1
Tumour necrosis factor-α	IL-10	TGF-β1, 2, 3
IL-17	IL-1 receptor antagonist	Bone morphogenetic protein-2
IL-18		
OSM		
IL-6		

Table 1.1 Classification of cytokines implicated in the modulation of chondrocyte metabolism. Adapted from Goldring and Goldring 2004**.**

1.3.1 Interleukin-1

The IL-1 family currently comprises of 11 structurally similar members with IL-1α and IL-1β being the best characterised members of the family (Kalliolias and C Liossis, 2008). IL-1α and IL-1β are both synthesised as an inactive pro-form in the cytoplasm. IL-1α is then sequestered to the plasma membrane or the nucleus. IL-1β is processed at the plasma membrane by IL-1β converting enzyme (ICE) and secreted as the active protein. Only pro-IL-1α and the mature forms of IL-1α and IL-1β have biological activity.

There are two specific IL-1 receptors, IL-1RI and IL-1RII, and both IL-1α and IL-1β can bind to them. IL-1RI is the biologically active receptor and contains a long cytoplasmic domain. Activation of IL-1RI by IL-1α or IL-1β results in the initiation of a number of pathways, such as the mitogen-activated protein kinase (MAPK) pathways. In contrast, IL-1RII is biologically inert and found on the cell surface as well as a soluble receptor (Arend, 2002). Henceforth all references pertaining to IL-1 will refer to IL-1α.

Numerous different groups have described involvement of IL-1 in arthritis (David et al., 1985, Nobuyuki et al., 1988). IL-1 is secreted by a number of different cells within the joint space in arthritis (Cinzia et al., 1998). In RA, it is the synovial lining which is the major source of IL-1 production and conversely, cartilage is the major source of IL-1 production in OA.

Stimulation of chondrocytes with IL-1 results in the induction of a number of different pro-inflammatory mediators, such as IL-6 and leukaemia inhibitory factor (LIF) (Barksby et al., 2006). These data suggest that IL-1 is an important catabolic mediator in arthritic diseases.

1.3.2 Oncostatin M

OSM is a 28 kDa protein and belongs to the IL-6 family of cytokines (Pelletier and Martel-Pelletier, 2003). It is described to play an important role in the progression of RA. The addition of OSM to IL-1 or tumour necrosis factor-α (TNF-α) treated cartilage causes a reproducible increase in collagen release (Cawston et al., 1998, Hui et al., 2003b). Interestingly, when OSM is added in combination with IL-1 there is a synergistic increase in collagen release and gene induction (Barksby et al., 2006, Morgan et al., 2006). Other members, such as IL-6 and LIF, cannot synergise directly with IL-1 as only the OSM-specific receptor is expressed on the surface of the chondrocyte (Rowan et al., 2001). However, the addition of soluble IL-6 receptor in combination with IL-6 does exhibit the same effect as the addition of OSM to IL-1 treated cartilage. Elevated levels of IL-6 and sIL-6R have been documented in various arthritides, including RA (Houssiau et al., 1988, Robak et al., 1998).

1.3.3 TGF-β

The TGF-β superfamily consists of over 30 members and includes TGF-βs, activins and bone morphogenic proteins. There are three TGF-β isotypes in humans, called β1, β2

and β3, and all three isoforms are produced as inactive dimers (de Caestecker, 2004, Todorovic et al., 2005) and consist of the core TGF-β protein and pro-peptide, also known as latency-associated peptide. The mechanism of physiological activation of TGF-β has yet to be fully elucidated, although proteolytic cleavage and integrin-specific activation have been implicated (Saharinen et al., 1999, Wipff and Hinz, 2008). Active TGF-β can then bind its receptors and activate a number of different signalling pathways, including c-Jun N-terminal kinase (JNK) (van der Kraan et al., 2009).

Historically, TGF-β has an anabolic role in cartilage (Table 1.1) as TGF-β activation stimulates ECM production and can counteract both TNF-α and IL-1 induced gene induction (Hui et al., 2001). However, recent evidence shows that TGF-β can cause significant MMP-13 up-regulation in aging cartilage (Blaney Davidson et al., 2009). This is due to a decrease in activin-like kinase (ALK) 5 receptor levels in aging cartilage, whereas ALK1 levels remain constant. Signalling through ALK1 leads to Smad1/5/8 activation and this leads to MMP-13 gene expression, but ALK5 stimulation leads to increased expression of cartilage matrix genes.

1.4 Proteases

Proteases are enzymes that hydrolyse the peptide bonds linking the amino acids in the polypeptide chain. Proteases are involved in a number of processes such as protein catabolism and specific protein cleavage.

Based upon their catalytic mechanism, proteases can be classified into five distinct categories:

- Serine;
- Threonine;
- Metallo;

- Aspartic;
- Cysteine

These five classes are implicated to be involved in arthritis with cysteine, aspartic and threonine proteases contributing to matrix degradation through intracellular pathways. Recently, intracellular uptake and lysosomal targeting of type II collagen has been characterised and using E64d, a broad spectrum cysteine protease inhibitor, caused collagen accumulation in lysosomes (Kjoller et al., 2004). Furthermore, cathepsin K, a novel cysteine collagenase, has been identified as a protease in the pathology of arthritis (Dejica et al., 2008), although the actual contribution of cathepsin K to cartilage catabolism *in vivo* has yet to be fully elucidated.

Metallo- and serine proteases contribute to matrix degradation in the extracellular space (Cawston and Young, 2010).

1.5 Matrix metalloproteases

The MMPs are members of the metalloprotease family and utilise a divalent zinc cation to catalyse the hydrolysis of peptide bonds (Bode et al., 1993). There are 28 vertebrate MMP genes known to date and all MMPs share common features (Figure 1.4) (Sternlicht and Werb, 2001). Some of these common features include:

- Synthesis as zymogens, with activation requiring removal of the pro-domain;
- Cysteine switch motif PRGC(V/N)PD in the pro-domain (Nagase and Woessner, 1999);
- Zinc binding motif HEXXHXXGXXHS in the catalytic domain.

1.5.1 MMP Structure

The MMP domain structures are shown in Figure 1.4.

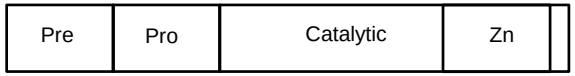
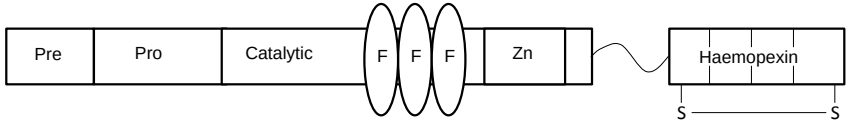
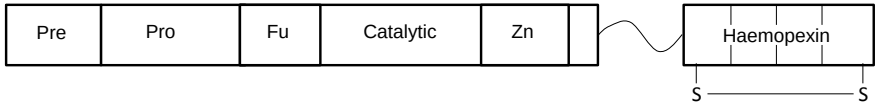
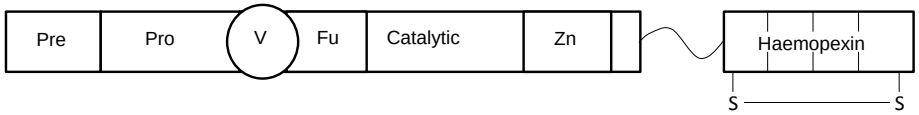
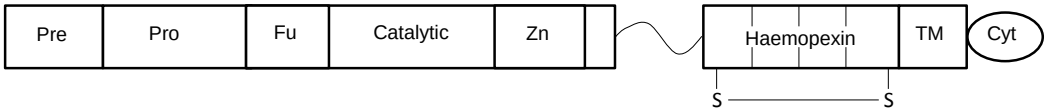
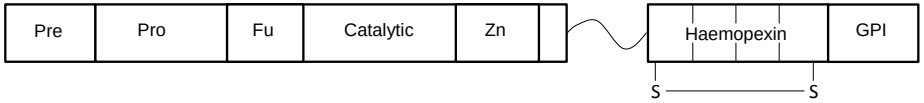
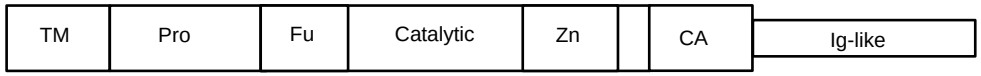
MMP-7, -26	Minimal domain MMPs	
MMP-1, -3, -8, -10, -12, -13, -18, -19, -20, -22, -27	Simple haemopexin domain MMPs	
MMP-2, -9	Gelatin binding MMPs	
MMP-11, -28	Furin activated MMPs	
MMP-21	Vitronectin insert containing MMPs	
MMP-14, -15, -16, -24	Transmembrane MMPs	
MMP-17, -25	GPI-anchored MMPs	
MMP-23	Type II transmembrane MMPs	

Figure 1.4 Domain structures of matrix metalloproteinases (MMPs).
All MMPs have a catalytic domain, which also contains the zinc-binding motif, and pro-peptide that ensures latency. Some contain a furin recognition motif (Fu) that specifically allows for intracellular activation by furin-like proteases . Apart from MMP-7, -23 and -26 all contain a haemopexin domain which determines substrate specificity and is connected by a flexible hinge domain. Other domains include the fibronectin-like domains (F) and the vitronectin-like domain (V). Some MMPs are anchored to the cell surface through either a TM with cytoplasmic tail (Cyt) or a GPI anchor. MMP-23 is structurally unique as it contains a N-terminal TM (actually an N-terminal signal anchor), a cysteine array (CA) and a immunoglobulin-like domain (Ig-like). Modified from Cawston and Wilson (2006).

The pre-domain encodes a signal peptide region that targets the protein for secretion and is removed shortly before the MMPs are secreted (Sternlicht and Werb, 2001).

The pro-domain is up to 90 amino acids in length and contains the conserved PRGC(V/N)PD sequence. This motif is conserved between all MMPs with the exception of MMP-23, which has the sequence ALCLLPA instead (Velasco et al., 1999). The Cys residue in these sequences is termed the cysteine switch and stably interacts with the catalytic zinc ion to maintain latency of pro-MMPs (Nagase and Woessner, 1999, Sternlicht and Werb, 2001). MMP-11, MMP-14, MMP-15, MMP-16, MMP-17, MMP-23, MMP-24, MMP-25, and MMP-28 contain a pro-protein processing motif RXKR in their pro-domain (Milner et al., 2008). This sequence can be cleaved intracellularly by pro-protein convertases resulting in the secretion of active MMPs (Kang et al., 2002).

The catalytic domain contains the zinc binding motif, HEXXHXXGXXHS in MMPs, and a conserved Met residue, which forms a Met-turn structure (Bode et al., 1993). This domain also contains an extra zinc ion and three calcium ions that are essential for stability, structure and secretion of the MMP (Iyer et al., 2007). MMP-2 and -9 are unique as their catalytic domains contain three repeats homologous to the type II domains of fibronectin (Steffensen et al., 1995). These repeats allow for high affinity binding of collagens and help to localise them to the ECM.

With the exception of MMP-7, -26 and -23, all MMPs possess a C-terminal haemopexin domain. This domain is composed of a calcium linked four bladed β-propeller structure in which each blade consists of four anti-parallel β-strands and a single α-helix (Gomis-Rüth et al., 1996). It was demonstrated that without the haemopexin domain, MMP-1 lost its collagenolytic ability but otherwise retained other substrate specificity. Instead

of a haemopexin domain, MMP-23 possesses a cysteine array, but attempts to characterise its substrate specificity have failed so far (Velasco et al., 1999).

1.5.2 Collagenases

The enzymatic degradation of the native collagen triple helix, at neutral pH, was demonstrated in the resorbing tadpole tail (Gross and Lapiere, 1962). The "classical" collagenases, MMP-1, MMP-8 and MMP-13, are mainly described to cleave fibrillar collagen, such as type II collagen, *in vivo*. All three of these collagenase genes are expressed in arthritic cartilage (Boris et al., 1997, Lynne et al., 2001). The key defining feature of the collagenases is their ability to cleave the collagen triple helix at a specific site, about a quarter length in from the C-terminus (Highberger et al., 1979).

MMP-1, also known as collagenase-1, fibroblast collagenase or interstitial collagenase, has been purified from joint tissues (Cawston and Tyler, 1979, Okada et al., 1986) and synovial fluid from arthritic patients (Cawston et al., 1984). As MMP-1 is expressed by a number of cells within the joint, it is thought to be mainly associated with RA (Murphy et al., 2002). MMP-1 preferentially cleaves type I collagen then type III, with type II collagen degradation being the slowest (Welgus et al., 1981).

MMP-8, also known as collagenase-2 and neutrophil collagenase, was originally thought to be only expressed in human neutrophil precursors. However, it has now been shown that a number of cells, such as chondrocytes and plasma cells, produce MMP-8 (Van Lint and Libert, 2006). MMP-8 shows the same preference for collagen cleavage as MMP-1 (Hasty et al., 1987).

MMP-13, also known as collagenase-3, was originally identified from a breast carcinoma cDNA library (Freije et al., 1994). MMP-13 expression is mostly restricted to chondrocytes and is therefore thought to drive disease progression in OA, where cartilage degradation is mainly chondrocyte driven (Murphy et al., 2002). Biochemical

characterisation of MMP-13 activity has shown that it preferentially cleaves type II collagen over type I and type III (Knäuper et al., 1996).

1.5.3 Stromelysins

There are three human stromelysins, MMP-3 (stromelysin-1), MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3) (Sternlicht and Werb, 2001). MMP-3 was first identified as a proteoglycanase from rabbit bone culture. It was later shown that MMP-3 can also degrade other matrix components such as fibronectin and type IV collagen but not fibrillar collagens such as type I collagen (Murphy et al., 1981, Galloway et al., 1983). MMP-3 is able to activate all three collagenases *in vitro* (Ito and Nagase, 1988, Suzuki et al., 1990, Knäuper et al., 1996) to a "superactive" form (Windsor et al., 1993).

MMP-10 was thought to be differentially regulated in regards to MMP-3 (Bord et al., 1998). However, in normal cartilage MMP-3 and -10 are co-expressed and their expression is decreased in OA (Kevorkian et al., 2004). MMP-10 has substrate specificity similar to MMP-3 (Fosang et al., 1991, Busso et al., 1998).

The third member of the stromelysin family, MMP-11, has a structure which is slightly different as it contains a furin recognition site (Figure 1.4). Like other MMPs, it is expressed during wound healing and development (Lefebvre et al., 1995, Okada et al., 1997), but differs from the other stromelysins in its substrate specificity, as it is unable of cleaving the ECM (Noël et al., 1995). MMP-11 can cleave other proteins found in the extracellular compartment, such as serpins (Pei et al., 1994), implicating that it has an indirect role in ECM degradation by increasing the activity of other proteases.

1.5.4 Regulation of MMP activity

The significant role MMPs have in many biological processes, from wound healing through to tumour metastasis and cartilage matrix degradation indicates that their activity needs to be highly regulated. MMP activity is regulated at several levels from

transcription through to their activity in the ECM. Firstly, MMPs are inducible genes, whose expression is tightly regulated by pro- and anti-inflammatory cytokines and growth factors (Rowan, 2001) and ECM interactions, for example type I collagen binding to integrins can induce MMP-1 expression (Vogel et al., 1997). Secondly, the majority of MMPs are then secreted from the cell in their latent form and require activation (Figure 1.5). MMPs can be activated by previously activated MMPs in the ECM as well as a number of serine proteases (Milner et al., 2008). MMP activation can also be achieved *in vitro* by a number of different chemicals, such as organomercurial compounds (Van Wart and Birkedal-Hansen, 1990). Finally, MMP activity can be regulated by their interaction with endogenous inhibitors, tissue inhibitors of metalloproteases (TIMPs), and α2-macroglobulin (Visse and Nagase, 2003, Tchetverikov et al., 2003).

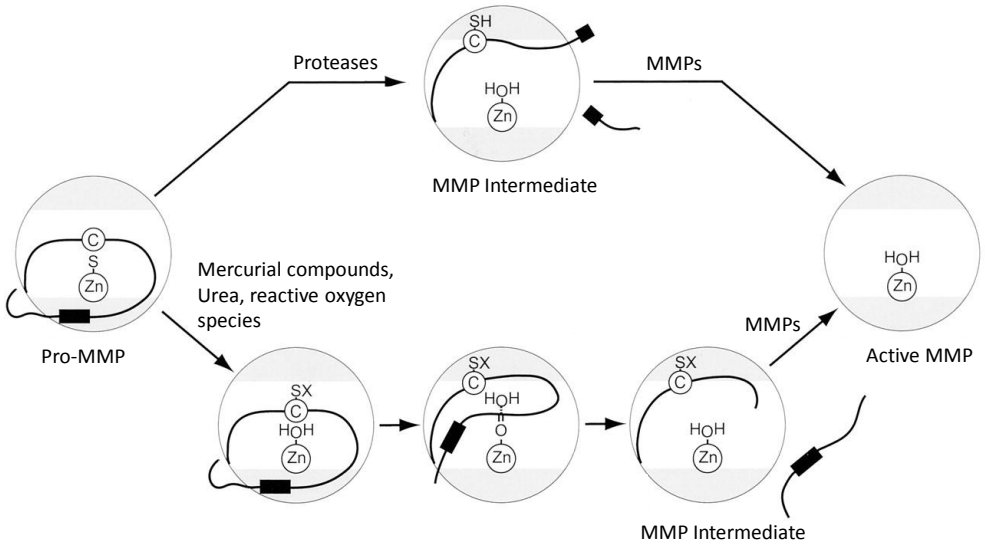


Figure 1.5 Stepwise activation of MMPs.

Pro-MMPs can be activated by proteases (top pathway) or nonproteolytic agents (bottom pathway). Cleavage within the bait region by proteases partially activates the MMP. Chemical activation relies on modification of the cysteine switch sulfhydryl (SX), resulting in partial activation of the MMP and intramolecular cleavage of the propeptide. In either pathway, full activation is attained by complete removal of the pro-domain y intermolecular processing. Adapted from Visse and Nagase (2003).

1.5.5 Tissue inhibitors of metalloproteases

TIMPs are the endogenous inhibitors of MMP enzymes and to date four TIMP homologues (TIMP-1, -2, -3 and -4) have been identified. TIMPs are small proteins (between 21 to 30 kDa) and inhibit MMPs in a 1:1 stoichiometry. All TIMPs inhibit all tested MMPs, with the exception of TIMP-1 that cannot inhibit MMP-14. TIMP-3 is unique in the fact that it can inhibit ADAMs as well as MMPs (Sternlicht and Werb, 2001).

In addition to their MMP inhibitory activity, TIMPs exhibit other biological functions. For example, TIMP-1 caused increased erythroid cell growth (Chesler et al., 1995). TIMP-2 overexpression in smooth muscle cells caused a decrease in proliferation while TIMP-3 overexpression results in apoptosis of these cells (Baker et al., 1998).

1.6 Serine proteases

Serine proteases belong to the protease family that causes peptide bond hydrolysis through a serine residue in their active site. Serine proteases play a role in a number of biological processes such as inflammation, blood clotting and cell signalling as well as digestion. The active site of serine proteases contain the so-called “catalytic triad” (Sigler et al., 1968, Blow et al., 1969, Steitz et al., 1969) which consists of a conserved group of three amino acid residues; serine, histidine and aspartic acid. The mechanism of hydrolysis involves the carboxyl group of the aspartic acid and the nitrogen of the histidine side chain accepting the hydrogen of the active site serine. This increases the electronegativity of the oxygen atom in the hydroxyl group; enabling the nucleophilic attack of the peptide bond. Recently, research has turned to how serine proteases can modulate their local environment through their proteolytic activity.

1.6.1 Classification of serine proteases

The sequencing of the human genome revealed that there are over one hundred and seventy serine proteases (Quesada et al., 2009). Not all of these proteases share a chymotrypsin-like serine protease fold. In fact, the differences can range from slight changes to the amino acids surrounding the active site serine to different catalytic triads (Ekici et al., 2008). For this reason serine proteases have been classified into families or groups of related families (often termed "clans") (Rawlings and Barrett, 1993). This classification is based upon two major criteria:

- Evolutionary divergence in catalytic activity;
- Convergence from separate evolutionary lines.

Previous to this classification standard it was thought that serine endopeptidases belonged to either the chymotrypsin family or the subtilisin family. However, the rigorous standards used in classifying peptidases to these criteria highlighted that there are a large number of distinct serine endopeptidase families.

1.6.2 Serine protease activity in cartilage degradation

Early evidence showed that the MMP enzymes were directly responsible for the breakdown of the collagen network in articular cartilage (Cawston, 1996). At that time, the development of broad-spectrum MMP inhibitors was thought to be a promising therapeutic intervention in the arthritides. However, clinical trials identified musculoskeletal side-effects in patients taking these inhibitors (Nemunaitis et al., 1998) and led to their withdrawal from the clinic.

Bovine nasal cartilage (BNC) has been used as a model of healthy cartilage. When cultured in the presence of IL-1+OSM, there is significant degradation of the collagen matrix (Cawston et al., 1995). In this model there are substantial levels of pro-

collagenases by day 7, although activation rarely happens before day 10 in parallel with collagen release (Milner et al., 2006a). However, Milner et al. (2001) showed that the activation of the pro-collagenases by serine proteases is a critical control point in cartilage collagen degradation and highlighted a novel interaction between the serine and metalloprotease pathways. Primarily, the addition of plasminogen to IL-1+OSM-treated BNC showed a significant increase in collagen release at day 7. This collagen release was found to be metalloprotease dependent since BB94, a general metalloprotease inhibitor, abrogated this release. Conversely, addition of α_1-antitrypsin (α_1-AT) to IL-1+OSM-treated BNC significantly reduced collagen degradation, even if α_1-AT was only added at day 7 of the culture period. In fact, use of another broad-spectrum trypsin-like serine protease inhibitor, ACITIC, displayed a similar trend. While the data do support that plasmin is a key activator of MMPs, other unknown serine proteases are likely to play a greater role since plasminogen is not expressed in this model (Dr J. M. Milner, personal communication).

Furthermore, Milner et al. (2003) show that furin-like enzymes played an important role in cartilage collagen breakdown. However, only addition of the furin inhibitor between days 0 to 7 protected the collagen network. Addition of the inhibitor after day 7 did not have any protective effect on collagen degradation. The data strongly suggest that a second serine protease pathway exists in IL-1+OSM-treated cartilage, directly influencing MMP activity.

Both studies strongly implicate soluble serine proteases in cartilage collagen degradation. On the other hand, there is evidence in the literature that implicates degradation of type II collagen in arthritis originating in the pericellular space, diffusing outwards during disease progression (Hollander et al., 1995). Recently, expression profiling between OA and fracture to the neck of femur (NOF) cartilage showed that 46

serine protease genes were up-regulated in OA (Swingler et al., 2009) and Milner *et al*. (2006b) highlighted that expression of fibroblast activation protein alpha (FAPα), an integral membrane serine proteinase on chondrocyte membranes, is significantly higher in OA compared to normal cartilage. Together these reports highlight that transmembrane serine proteases could play an important role in pathogenesis as their cell surface localisation would support a hypothesis that pericellular degradation is initiated by serine proteases. While many of these proteases have been studied in other pathologies, such as tumour progression, not all of them have been studied in the context of arthritis.

1.7 Type II transmembrane serine protease family

Cloning of the enteropeptidase cDNA revealed that it has a similar sequence to hepsin (Kitamoto et al., 1994) and led to the subsequent discovery of the other 16 members of the type II transmembrane serine protease (TTSP) family. Based on the domain structure and phylogenetic analysis of the serine protease domain, TTSPs can be separated into four subfamilies (Figure 1.6).

Members of the TTSP family have a very unique structure with a N-terminal transmembrane domain followed by a variable "stem region" and finally a C-terminal extracellular serine protease domain of high homology to chymotrypsin (Szabo and Bugge, 2008). Analysis of the amino acid sequence of the TTSPs suggests that they are synthesised as single chain zymogens and need to be proteolytically cleaved after an arginine or lysine residue near the catalytic domain (Szabo and Bugge, 2008). However, there is strong evidence for TTSP auto-activation as they show a high preference for cleavage after an arginine or lysine residue (see section 1.7.2).

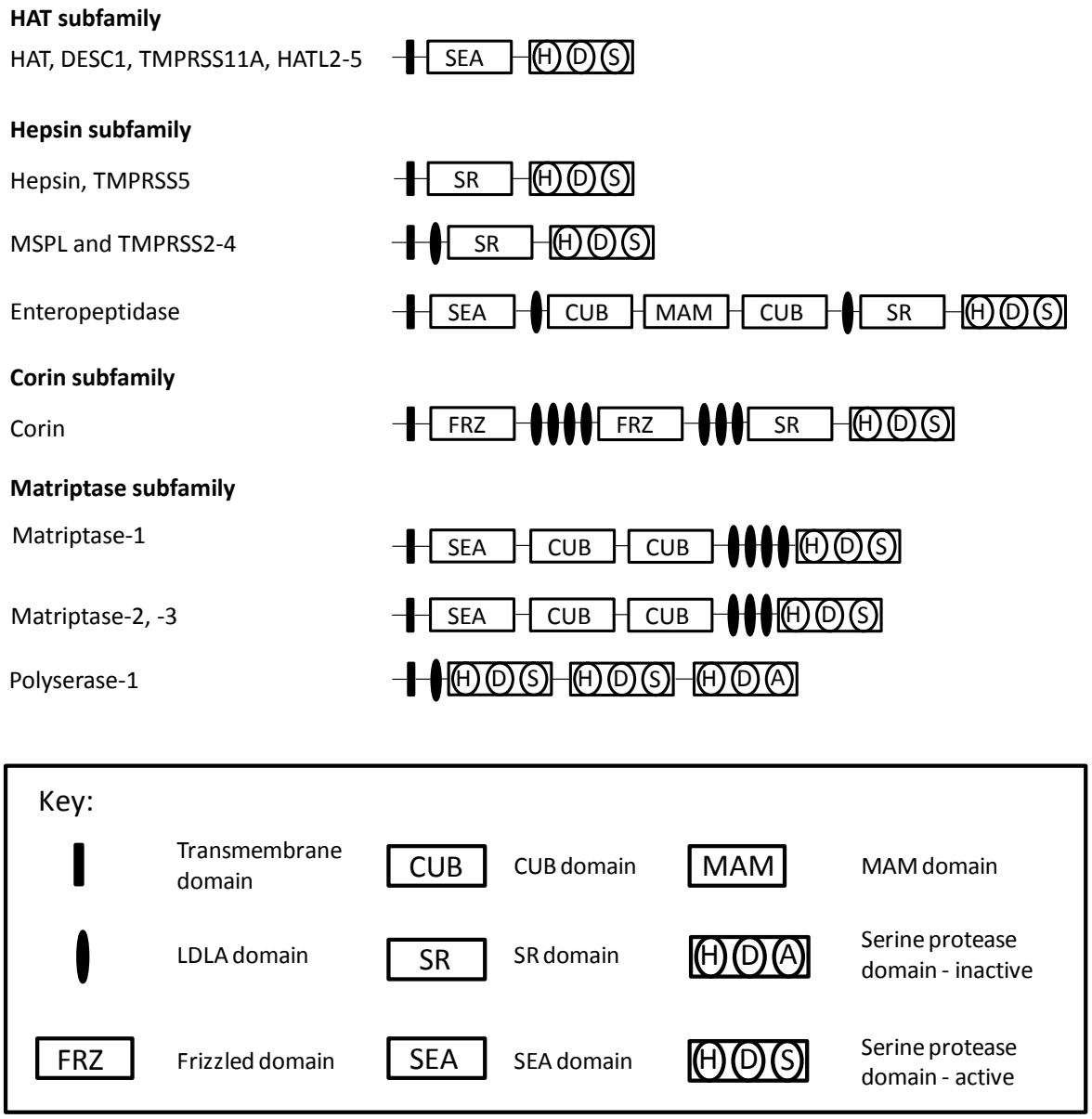


Figure 1.6 Domain structure of the human TTSP family.

TTSPs are grouped into four subfamilies based on domain structure and phylogenetic analysis of the serine protease domains. All members of this family contain a transmembrane domain and serine protease domain. Other domains include CUB, C1s/C1r urchin embryonic growth factor and bone morphogenetic protein-1 domain; FRZ, frizzled domain; LDLA, low-density lipoprotein receptor domain class A; MAM, a merpin, A5 antigen and receptor protein phosphatase μ domain; SEA, a single sea urchin sperm protein, enteropeptidase, agrin domain; SR, scavenger receptor cysteine-rich domain. Adapted from Choi et al. (2009).

1.7.1 Matriptase

Matriptase-1 (matriptase) is a TTSP, first identified as a secreted enzyme from breast cancer cells (Shi et al., 1993). Further characterisation showed that matriptase is a multi-domain transmembrane serine protease that can degrade ECM (Lin et al., 1999, Lin et al., 1997).

1.7.1.1 Matriptase structure

The intracellular region of matriptase is fifty-four amino acids in length and, in conjunction with the transmembrane region, is postulated to associate with the cytoskeleton to help localise matriptase on the cell surface (Lin et al., 1997, Takeuchi et al., 2000). The cytoplasmic region of matriptase also contains consensus phosphorylation sites for protein kinase C and/or casein kinase II (Hooper et al., 2001). However, the role of this phosphorylation has yet to be determined. The extracellular region contains the serine protease domain as well as four low density lipoprotein (LDL) receptor domains, two Cls/Clr, urchin embryonic growth factor and bone morphogenic protein 1 (CUB) domains and a sea urchin sperm protein, enterokinase, agrin (SEA) domain (Figure 1.6) (Hooper et al., 2001). The LDLR and CUB domains are thought to be associated with protein-protein interactions on the cell surface and activation of matriptase (Oberst et al., 2003). The SEA domain is thought to play a role in the non-enzymatic shedding of the protease from the cell surface (Szabo and Bugge, 2008). The serine protease domain has been crystallised (Friedrich et al., 2002) and shows that matriptase has narrow substrate specificity (section 1.7.1.3). Although, matriptase has the same P1 specificity as trypsin, there is a well-defined extended specificity profile for matriptase. This extended specificity is primarily due to the localisation of the benzyl side chains of phenylalanine in the P2 and P4 binding pockets of matriptase, which is uncommon in chymotrypsin-like proteases (Friedrich et al., 2002).

1.7.1.2 Matriptase activation

Similar to most chymotrypsin-like proteases, matriptase is produced as an inactive single chain zymogen (Benaud et al., 2001). Activation of matriptase occurs through cleavage at arginine-614 to produce the canonical active two chain protease; the two chains are linked by a disulphide bridge (Takeuchi et al., 2000).

The non-catalytic domains of matriptase are known to be important for activation (Oberst et al., 2003). Earlier data show that matriptase can be auto-activated by serum; this mechanism has not been elucidated (Benaud et al., 2001). Benaud et al. (2001) postulated that the CUB domain plays an important role in matriptase auto-activation, as C1r has a similar domain and is known to auto-activate as well. These data were corroborated by a later study which went on to show that the LDLR and SEA domains are also required (Oberst et al., 2003). However, the data presented about the role of the LDLR domains in matriptase activation are rather complex. Point mutations in the LDLR domains, that stop the formation of Ca^{2+} cages within the protease, abolish matriptase activation. But, when the LDLR domains are deleted matriptase activation actually increases.

Matriptase can be shed from the cell surface, although the mechanism is not yet known (Benaud et al., 2001). There are reports that matriptase requires hepatocyte growth factor activator inhibitor-1 (HAI-1) to be shed from the membrane (Oberst et al., 2003). However, conflicting data show that HAI-1 is not essential (Miyake et al., 2010) and that only proteolysis within the SEA domain is crucial (Szabo and Bugge, 2008 and references therein). As stated by Miyake et al. (2010), the requirement of HAI-1 is likely to ensure that matripase does not undergo auto-activation in intracellular compartments and self-degrade.

1.7.1.3 Matriptase substrate specificity

The substrate specificity for matriptase has been determined using a positional scanning library and phage substrate library (Takeuchi et al., 2000). Both libraries had their P1 amino acid fixed as arginine. The positional scanning library showed the preferred substrate specificity for matriptase to be either R/K-X-S-R---A or X-R/K-S-R---A, where X is a non-basic amino acid. However, this study showed that glycine or phenylalanine residues are well tolerated in the P2 position, unlike in other canonical serine proteases such as thrombin. Furthermore, this study went on to test the activity of matriptase on protease-activated receptor 2 (PAR-2) and single-chain urokinase-type plasminogen activator (uPA). In fact, matriptase did cause a biological response when incubated with PAR-2 overexpressing *Xenopus* oocytes, as PAR-2 contains the cleavage sequence of S-K-G-R. In contrast, matriptase could not activate PAR-1, -3 and -4 as they do not contain a matriptase consensus cleavage sequence. Furthermore, matriptase was shown to activate pro-uPA *in vitro* as well. The PAR-2 and uPA activation data presented in this study show that matriptase may have an important role in the pathogenesis of arthritis (Takeuchi et al., 2000). PAR-2 has recently been shown to be expressed in the synovial membrane in RA (Kelso et al., 2007) and its activation led to a downstream cytokine release in the synovial membrane. Furthermore, PAR-2 activation in OA cartilage caused the activation of the extracellular signal-regulated kinase1/2 (ERK1/2) and p38 pathways (Boileau et al., 2007) both of which are important in the catabolism of the cartilage matrix. The role of uPA in arthritis has been documented previously (Jin et al., 2003) by the injection of uPA into the joint space of mice which resulted in bone erosion and synovial thickening. uPA causes the induction of MMPs in THP-1 monocytes by activating the ERK1/2 pathway (Menshikov et al., 2006). However, direct evidence for the involvement of matriptase in the activation of PAR-2 and uPA in arthritis is still lacking.

1.7.2 Regulation of matriptase activity

Serine proteases are well known to cleave ECM proteins (Milner et al., 2008) and therefore require tight regulation of their activity. But, with the recent emergence of PARs (section 1.9.1), this regulation is even more important and extends to matriptase. HAI-1 is found to be coexpressed and colocalised with matriptase. HAI-1 is a Kunitz-type serine protease inhibitor (Shimomura et al., 1997), which have a different mechanism of inhibition and different secondary structure compared to the serpins (serine protease inhibitors). The primary loop extends into the active site of the protease and forms stable, reversible interactions with the active site (Creighton et al., 1993, Creighton and Charles, 1987, Kemmink and Creighton, 1993). However, data recently generated show that matriptase activity is not solely regulated by HAI-1. The serpins α_1-AT and antithrombin III have been shown to inhibit matriptase *in vitro* (Janciauskiene et al., 2008).

The serpins also regulate serine protease activity and are the largest superfamily of protease inhibitors, present in all multicellular eukaryotes (Irving et al., 2000). Whilst the name serpin implies that they inhibit serine proteases, there is literature showing that some serpins are able to inhibit cysteine proteases (Schick et al., 1998). The serpins share a common protein fold (Al-Ayyoubi et al., 2004, Loebermann et al., 1984), consisting of three β-sheets, which adopt a twisted conformation, and nine α-helices. The serpins also contain a reactive centre loop (RCL) that constitutes the site of interaction with the target protease. The RCL contains the P1 and P1' site and attracts the protease to the serpin. The RCL is usually found in an exposed conformation above the main body of the serpin (Elliott et al., 1998). The cleavage of the RCL by a serine protease causes a drastic change in conformation and results in the inability of the protease to complete peptide bond hydrolysis, thereby trapping the enzyme (Kaslik et al., 1995).

1.8 Membrane dipeptidyl peptidases

The dipeptidyl peptidase (DPP) class of enzymes comprises non-classical serine proteases that are known to cleave the peptide bond on the carboxyl side of the proline residue in the sequence Pro-Xaa (Xaa≠Pro). The DPP group of enzymes includes intracellular enzymes such as: dipeptidyl peptidase II (also known as quiescent cell proline amino peptidase or dipeptidyl peptidase 7); dipeptidyl peptidase 8 (DPP8) and dipeptidyl peptidase 9 (DPP9). As well, some are active on the cell surface: dipeptidyl peptidase IV (DPPIV, also known as CD26) and FAPα (also known as seprase) (Chen et al., 2003).

A lot of focus has been placed on the membrane DPP enzymes, FAPα and DPPIV, (Gorrell, 2005, Kelly, 2005) as they have been linked to a number of pathologies such as tumour invasiveness (Aoyama and Chen, 1990, Monsky et al., 1994) and type II diabetes (Reimer et al., 2002). These are thought to occur by the modulation of biological processes via Pro-specific N-terminal processing of biologically active peptides by DPPs (Busek et al., 2007, Proost et al., 1998).

Evidence also exists for the modulation of these DPP enzymes in the joint tissues of patients with RA (Ellingsen et al., 2007, Gotoh et al., 1989, Kamori et al., 1991). Recent evidence has shown the expression of FAPα on the surface of chondrocytes from resorbing cartilage (Milner et al., 2006b). Furthermore, the combined inhibition of DPPIV and FAPα increases the invasion of rheumatoid arthritis synovial fibroblasts into articular cartilage (Ospelt et al., 2010). However, the dual inhibitor used in this study does not highlight whether inhibition of either DPPIV or FAPα alone would show a similar result.

1.8.1 Dipeptidyl peptidase IV

DPPIV is the most studied member of the DPP family of enzymes. DPPIV is a 110 kDa glycoprotein that is catalytically active only as either a type 2 integral plasma membrane protein homodimer or a heterodimer with FAPα (Ajami et al., 2003). However, a small proportion appears to circulate in the plasma, cleaved from the membrane by an undetermined mechanism (Gotoh et al., 1989).

The X-ray crystal structure of the DPPIV monomer has been elucidated and shows that it consists of two domains, an α/β-hydrolase domain and an eight bladed β-propeller domain (Aertgeerts et al., 2004). There are limited data showing that DPPIV exhibits endopeptidase activity and can actually cleave gelatin (Bermpohl et al., 1998) although the rate of cleavage for the denatured collagen fibrils is slow. However, the crystal structure highlights that the catalytic pocket is too small to accommodate large proteins. Further structure data show that DPPIV also contains nine N-glycosylation sites (Engel et al., 2003) and this could explain the ability of DPPIV to resist trypsin-induced extracellular cleavage and has been shown to be important for protein localisation to the cell membrane (Ajami et al., 2003).

Adenosine deaminase (ADA) is known to bind to the monomeric and dimeric forms of DPPIV (Ajami et al., 2003) and this association plays an important role in regulating the activity of the cells of the immune system by counteracting the inhibition caused by extracellular adenosine (Wolberg et al., 1975). The inhibition of lymphocytes by extracellular adenosine is thought to occur through A2a receptor signalling which down-regulates the expression of co-stimulatory molecules on the surface of the T cell, such as CD25, and blocks proliferation (Huang et al., 1997). This observation is quite important in diseases such as RA where there is an increase in the amount of ADA isoforms in the synovial fluid (Iwaki-Egawa et al., 2001). Most of the T cells that reside

in synovial tissue are $DPPIV^+$ low and this suggests that $DPPIV^+$ T cells from the periphery actively penetrate into the synovium (Sedo et al., 2005) to continue to potentiate the disease state. Recent evidence suggests that this could be occurring in RA since there is an increase in the expression of DPPIV on the surface of monocytes and lymphocytes (Ellingsen et al., 2007).

The role of ADA binding to DPPIV and the effects this has on the co-stimulatory activation of T cells still remains contradictory. There is a report stating that the co-stimulatory role for ADA is irrespective of its ability to enzymatically degrade adenosine (Martin et al., 1995). Meanwhile, another report states that the activation of $CD4^+$ and $CD8^+$ T cells through DPPIV-mediated signalling was not dependent on activation of the ADA binding site but shown to be through the CD3 pathway (De Meester et al., 1995) which leads to activation and proliferation of T cells. It may be that binding of the ADA protein induces a conformational change in the DPPIV protein and causes the protein to exert a greater co-stimulatory response than DPPIV in the absence of ADA.

The rate of plasminogen activation is shown to be increased by the association of DPPIV-ADA complex with the plasminogen type 2 receptor on the membrane of prostate cancer cells (Gonzalez-Gronow et al., 2004). This finding is interesting in so far as previous data have shown that the use of the protease inhibitor, α_1-AT, possibly blocks a component of the plasminogen/plasmin cascade in a resorbing cartilage model (2003, Milner et al., 2001). Although chondrocytes do not secrete plasminogen themselves, when looking at the disease state of RA the roles of all the cell types in the joint space should be taken into account as there is an increase in the concentration of plasminogen in the synovial fluid (Belcher et al., 1996) and cartilage (Li et al., 2005).

DPPIV activity is known to modulate a number of pro-inflammatory chemokines and cytokines and the processing of the N-terminal ends of these peptides results in changes to both their receptor binding and functional properties (Table 1.2). DPPIV processing of full-length Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) (1-68) to cleaved RANTES (3-68) causes a noteworthy functional change (Proost et al., 1998) such that cleaved RANTES inhibited monocyte chemotaxis in comparison to full-length RANTES. A similar observation is seen in vitro for stromal cell-derived factor-1α (SDF-1α), whereby DPPIV cleavage of SDF-1α causes a reduction in leukocyte chemotaxis (Busso et al., 2005). Therefore, loss of DPPIV in the joint space may lead to unchecked activity of these cytokines and allow for the inflammatory response in the joint to be maintained rather than it cessation.

Substrate	**Effect upon cleavage by DPPIV**	**Reference**
Substance P (SP)	Decreased T cell proliferation	(Covas et al., 1997)
Neuropeptide Y (NPY)	Decreased chemoattractive ability and activation of mononuclear cells	(Mentlein, 1999) (Schwarz et al., 1994)
TNF-α	Decreased IL-1β secretion by macrophages and monocytes	(Bauvois et al., 1992)
RANTES	Decreased monocyte chemotaxis	(Mentlein, 1999, Proost et al., 2001)
Macrophage Inflammatory Protein-1β (MIP-1β)	Decreased T cell and macrophage chemotaxis	(Guan et al., 2004)
Interferon-γ-inducible Protein (IP-10)	Decreased T cell chemotaxis	(Proost et al., 2001)
SDF-1α	Decreased leukocyte chemotaxis	(Mentlein, 1999, Busso et al., 2005, Proost et al., 2001)
Monokine induced by interferon-γ (MIG)	Decreased T cell chemotaxis	(Ludwig et al., 2002, Proost et al., 2001)
Eotaxin	Decreased degranulation of eosinophil and basophils	(Mentlein, 1999, Proost et al., 2001)
Monocyte Derived Chemokine (MDC)	Reduced chemotaxis of monocytes, dendritic cells, activated lymphocytes and NK cells	(Mentlein, 1999, Proost et al., 2001)

Table 1.2 Substrates cleaved by DPPIV

1.8.2 Fibroblast activation protein-α

FAPα was originally identified as an antigen on the surface of reactive stromal fibroblasts (Rettig et al., 1993) and also identified separately as seprase (Monsky et al., 1994, Aoyama and Chen, 1990). Further analysis revealed that both FAPα and seprase encoded the same gene product (Chen and Kelly, 2003).

FAPα is a type II transmembrane protein of 760 amino acids which anchors in the plasma membrane via an uncleaved signal sequence of approximately 20 amino acids and has a short, amino terminal, cytoplasmic domain of six amino acids (Goldstein et al., 1997, Pineiro-Sanchez et al., 1997). There is a small amount of data to indicate that a second cytosolic version of FAPα exists (Goldstein and Chen, 2000) and that this form of FAPα only encodes for the C-terminal region of the enzyme.

The structure of FAPα was modelled on a closely related protease (Cheng et al., 2002) and it was found that the enzyme had a similar domain structure to DPPIV. It possesses an eight bladed β-propeller domain near the N-terminus and an α/β-hydrolase domain at the C-terminus. It is postulated that a pore exists in the β-propeller domain to allow substrate access to the catalytic domain and this was later confirmed by X-ray crystallography (Aertgeerts et al., 2005).

FAPα has gelatinase activity (Aoyama and Chen, 1990, Monsky et al., 1994, Pineiro-Sanchez et al., 1997) as well as dipeptidyl peptidase activity (Park et al., 1999) and both of these functions occur in the same catalytic pocket that is located in the α/β-hydrolase domain. FAPα has similar dipeptidyl peptidase substrate specificity to DPPIV but the rate of hydrolysis is about 100-fold less (Aertgeerts et al., 2005). The dipeptidyl peptidase and endopeptidase specificities of DPPIV and FAPα are due solely to the protonation state of a glutamate residue in the active site pocket. Aspartate-663 of DPPIV causes deprotonation of the glutamate-206 side chain that facilitates binding of

charged amino terminal groups (Aertgeerts et al., 2005). However, the comparable residue in FAPα is alanine-657, and is essential for the endopeptidase activity of FAPα. This is because alanine-657 cannot deprotonate glutamate-204 in FAPα and favours binding of uncharged residues in the P2 pocket of the active site. The gelatinase activity of FAPα is much more pronounced than that for DPPIV but there was some uncertainty in the literature at first as to whether FAPα could cleave native collagen I (Park et al., 1999). It has now been shown that FAPα cannot cleave native collagen fibrils but can cleave substrates which have previously been partially cleaved by MMPs (Christiansen et al., 2007). Another interesting finding is that N-terminal truncation of FAPα can actually increase the gelatinase activity but not the dipeptidyl peptidase activity of the enzyme (Chen et al., 2006a). There is speculation that the use of detergents in this experimental set-up may have caused a conformational change in the membrane bound form of FAPα and therefore activated the proteins ability to cleave gelatin, while *in-vivo* enzymatic truncation may be required before this can occur. Furthermore, it has been shown that FAPα forms a complex with DPPIV on the surface of fibroblasts allowing for their migration on a collagenous matrix (Ghersi et al., 2002). In this environment there will be other proteolytically active enzymes present to cause truncation of FAPα and thus activate its gelatinase activity.

1.9 Cell signalling

Cell signalling is a complex network of communication that governs cellular actions in response to changes in both the local and systemic environments. Undesirable changes in the signalling pathways results in serious pathological consequences such as cancer (Shaw and Cantley, 2006, Vincan and Barker, 2008) or irregular immune responses (Longo et al., 2008).

In basic terms, classical cell signalling is the binding of a signal molecule to a membrane receptor on the cell surface that causes the modulation of cell function. However, recent evidence is starting to shed light on a novel signalling mechanism that involves the direct contribution of proteases to cause a cellular response.

1.9.1 Protease-activated receptors

One mechanism by which serine proteases can act as signalling molecules is through the cleavage of the PARs, which are G-protein coupled receptors (GPCR). There are four PARs known to date; PAR-1, PAR-2, PAR-3 and PAR-4. Most of the data published on the PARs have been generated from endo- and epithelial cells. The modest amount of data on PAR-3 comes from work generated in this manner (McLaughlin et al., 2007) and leads to the possibility that PAR-3 modulates signalling through its ability to bind to PAR-1. However, little is known about the function of PAR-3 on the surface of "normal" cells. Therefore, researchers have transformed cell lines such as HEK-293 to tease out the role of PAR-3 (Ostrowska and Reiser, 2008). Whilst this study seems to suggest that PAR-3 can act independently of PAR-1 to cause IL-8 release, PAR-3 lacks the ability to interact with G-proteins due to the lack of a cytoplasmic tail (Ishihara et al., 1997). Contradictory data have been published showing that PAR-3 is not able to signal independently of PAR-1 (Kaufmann et al., 2005). The latter was shown by the use of PAR-3 and PAR-1 overexpression constructs in KOLF cells (fibroblasts from PAR knockout mice) where PAR-3 alone showed no ability to trigger a signal response after incubation with TFRGAP (the tethered ligand generated by cleavage of PAR-3). However, a definite increase in the amount of phosphorylated p42/44 could be observed when PAR-1 overexpressing cells were incubated with TFRGAP indicating that PAR-1 is important is mediating signalling with PAR-3 acting as a co-stimulatory molecule.

PAR-1 activation is linked to fibroblast proliferation and the release of prostanglandin E_2 and pro-collagen (Blanc-Brude et al., 2005, Sokolova et al., 2005). Furthermore, PAR-1 activation has been linked to the differentiation of fibroblasts to myofibroblasts (Bogatkevich et al., 2001, Materazzi et al., 2007).

PAR-1 is known to be present on the surface of synovial fibroblasts in RA (Morris et al., 1996) and further work has shown that PAR-3 is also expressed on the surface of synovial fibroblasts from patients with rheumatic disease (Hirano et al., 2002). It should be noted that PAR-1 up-regulation is only seen in RA, this phenomenon is not seen in OA or normal synovium (Morris et al., 1996). PAR-1 activation by thrombin on the surface of these fibroblasts causes an up-regulation of RANTES gene expression (Hirano et al., 2002) thus indicating that this pathway could play an important role in the recruitment of inflammatory T cells and NK cells to the joint space thereby propagating the destructive mechanisms. More recent evidence has shown that synovial fibroblasts from the rheumatoid joint can be induced to produce IL-6 by PAR-1 activation (Chiu et al., 2008) through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. This IL-6 production is independent of PAR-3 and PAR-4 as could be demonstrated by the use of small interfering ribonucleic acid (siRNA) to silence these genes as well as the use of specific PAR agonists.

PAR-2 has recently been shown to be expressed in the synovial membrane in RA (Kelso et al., 2007) and its activation was found to lead to downstream cytokine release. One possible mechanism of PAR-2 activation in RA synovium could be its cleavage by mast cell tryptase (Nakano et al., 2007), as a novel form of mast cell tryptase is found at a high concentration in RA and correlates with increased PAR-2 mRNA expression. However, there is the possibility that other trypsin-like serine proteases could activate PAR-2 as well. Mast cells could play an important role as the source of these serine

proteases (Palmer et al., 2007) and could cause auto-activation of the PAR-2 receptors resulting in the perpetuation of the PAR-2 signal. This is supported by findings that mice lacking mast cells were resistant to RA (Lee et al., 2002). Recently, it has been shown that PAR-2 activation in OA cartilage caused the activation of the ERK1/2 and p38 pathways (Boileau et al., 2007) and these two pathways are important in the catabolism of the cartilage matrix. In this study co-stimulation of OA chondrocytes with IL-1β and PAR-2 activating peptide had a synergistic effect on the ERK1/2 pathway.

Little information exists about the role of PAR-4 in the context of connective tissue cells. Nonetheless it has been shown that lipopolysaccharide and TNFα induce expression of PAR-4 on human primary bronchial fibroblasts (Ramachandran et al., 2007) and that activation of PAR-4 counters the PAR-1 mediated proliferation of these cells. This ability of PAR-4 to antagonise the function of PAR-1 is interesting and requires further insight, mainly in the context of connective tissue cells. In endothelial cells PAR-1 and PAR-4 have been reported to counteract each other in the regulation of VEGF and endostatin release from platelets (Ma et al., 2005).

Once the PARs are cleaved they remain constitutively active as the tethered ligand is always available to interact with the PAR. Therefore, after the activation of phospholipase C has occurred, signal attenuation occurs through the recruitment of β-arrestins 1 and 2 (Wang and DeFea, 2006, DeFea et al., 2000) resulting in the desensitisation and endocytosis of the receptors for their degradation.

1.10 Scope of this thesis

Cartilage consists of an extensive extracellular matrix, composed mainly of proteoglycans and collagens, in which the chondrocytes are embedded. Proteolytic degradation of articular cartilage leads to joint dysfunction and is a major hallmark of

arthritis. While the loss of proteoglycan is rapid and reversible, the breakdown of the collagen network is slow and essentially irreversible.

The major extracellular proteases involved in the breakdown of the cartilage matrix are the metallo- and serine proteases. Data from our group have highlighted that both protease families function through interacting cascades (Milner et al., 2001, 2003). MMP expression is increased in arthritis (Kevorkian et al., 2004) and correlates with the excessive degradation of the collagen network (Hollander et al., 1995). The collagenases (MMP-1, MMP-8 and MMP-13) are the key enzymes involved in collagen turnover and they mediate the cleavage of fibrillar collagen into characteristic three- and one-quarter fragments which is the major rate-limiting step in the breakdown of the cartilage ECM. The activation of the pro-collagenases is a key control point with regards to collagen matrix breakdown (Milner et al., 2001) and data have shown that serine proteases are involved (Milner et al., 2001, 2003).

In OA, collagen degradation is initially observed around the chondrocyte before diffusing radially (Hollander et al., 1995). The increased expression of the transmembrane serine proteases DPPIV, FAPα and matriptase could be key mediators of the arthritides. These transmembrane serine proteases could play a vital role via their interaction with either soluble or membrane bound MMPs and localise collagenolytic activity to the cell surface. Therefore, characterising the role of novel transmembrane serine proteases in arthritis is important as it is an area of cartilage biology that has yet to be fully elucidated.

Thus the aims of this thesis are:

- Investigate the roles of FAPα and DPPIV in resorbing IL-1+OSM-treated cartilage.

- Study the role of matriptase in cartilage matrix degradation.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Biochemical assay reagents

Papain (from *Carica papaya*), 2-Amino-2-hydroxymethyl-propane-1,3-diol hydrochloride (Tris-HCl), calcium chloride, sodium chloride, glucose, sodium azide, L-cysteine hydrochloride, *p*-dimethylaminobenzaldehyde (DAB), β-mercaptoethanol, o-phenylenediamine (OPD), phosphate-citrate capsules, L-hydroxy-proline, bovine serum albumin (BSA), sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Chloramine T was purchased from BDH (Poole, UK). 2 ml O-ring screw-cap tubes were obtained from Sarstedt (Leicester, UK) and 1.5 ml eppendorfs obtained from Starlabs (Milton Keynes, UK). Maxisorp Nunc-immuno 96-well plates were obtained from Life Technologies Ltd. (Paisley, UK). Flat-bottomed and V-bottomed 96-well plates were obtained from Bibby Sterilin (Staffordshire, UK). Flexible 96-well sample plates and Optiphase "Supermix" scintillation fluid were obtained from PerkinElmer (Beaconsfield, UK). GP-7- amino-4-methoxy coumarin (AMC), Z-GP-AMC and Boc-QAR-AMC were purchased from Bachem (Essex, UK). The general MMP substrate, FS-6, was obtained from Merck (Nottingham, UK). Pro-MMP-3ΔC was a gift from Dr R. Visse (Kennedy Institute, London, UK). Mono S FPLC column was obtained from GE Healthcare (Chalfont St. Giles, UK). FAPα was purchased from R&D systems (Abingdon, UK). Recombinant DPPIV was a gift from Dr H. Fan (Charité - Universitätsmedizin Berlin, Germany). Matriptase was a gift from Prof. Richard Leduc (Université de Sherbrooke, Canada).

2.1.1.1 Enzyme inhibitors

Ferring pharmaceuticals (Saint-Prex, Switzerland) kindly donated the FAPα-specific inhibitor FE999044 and the DPPIV-specific inhibitor FE999011. Dr B. Gilmore and Prof. B. Walker (Queen's University, Belfast, UK) kindly donated the FAPα inhibitor Z-GP-diphenylphosphonates (DPi) and the DPPIV inhibitors YP-DPi and GP-DPi. The FAPα inhibitors UAMC-583, UAMC-584 and UAMC-442 and the DPPIV inhibitors UAMC-374, Vildagliptin and AB192 were a gift from Prof. I. De Meester (University of Antwerp). The DPPIV inhibitors DPPI 1c and K579 were purchased from Tocris Bioscience (Bristol, UK). The cathepsin K inhibitor, L-873724, was a generous gift from Dr C. Black (Merck). The general metalloprotease inhibitor, GM-6001, and its negative control were purchased from Calbiochem (Nottingham, UK). All of the above inhibitors were dissolved in DMSO such that the highest final concentration of DMSO in the cartilage cultures was 0.1%. Before use they were diluted in culture medium and filter-sterilised through a 0.2 µm filter before addition to cartilage explants. SAM-11 antibody was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). ENMD-1068 was purchased from Enzo Life Sciences Ltd (Exeter, UK) and dissolved in PBS. Before use ENMD-1068 was diluted in culture medium and filter-sterilised through a 0.2 µm filter before addition to cartilage explants.

2.1.2 Cell and tissue culture reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco BRL (Paisley, UK), and L-glutamine, amphotericin, penicillin-streptomycin, collagenase (type I from *Clostridium histolyticum*), hyaluronidase (type I-S from bovine testes) and trypsin (type III, from bovine pancreas) were obtained from Sigma-Aldrich Company Ltd. 96-well, 24-well and 6-well tissue culture plates were obtained from Corning/Costar UK Ltd. (High Wycombe, UK). Sterile 100 mm square

petri dishes and universals (20 and 5 ml) were obtained from Bibby Sterilin Ltd. Syringe filters (0.2 µm) were from Pall Gelman Sciences (Northampton, UK). Sterile disposable scalpel blades were from Swann-Morton (Sheffield, UK). Dulbecco's phosphate buffered saline (DPBS) was obtained from Lonza (Wokingham, UK) and sterile 100 µm cell strainers from BD Falcon (Oxford, UK). IL-1 was a gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, UK) and recombinant OSM was produced in-house. IL-1 at 1 µg/ml in DMEM (with 0.1% BSA) was stored at -20°C. OSM at 60 µg/ml in DPBS (with 0.1% BSA) was stored at -80°C. Immediately prior to use IL-1 and OSM were diluted in culture medium and sterile filtered through a 0.2 µm filter.

2.1.3 Molecular Biology reagents

RNeasy Mini Kit and deoxyribonuclease (DNase) I were from Qiagen (Crawley, UK). Agarose (electrophoretic grade), MMLV and Superscript III were obtained from Life Technologies Ltd. Dialysis membrane was purchased from Medicell International Ltd. (London, UK).

All reagents required for RNA work were molecular biology grade. All RNA work was performed under RNase-free conditions using RNase-free reagents and materials.

2.1.4 Protein electrophoresis

Ammonium persulfate (APS) was obtained from BDH (Poole, UK). β-mercaptoethanol, and N,N,N'N'-tetramethylenediamine (TEMED) were obtained from Sigma-Aldrich Company Ltd. 40% (w/v) acrylamide/bis-acrylamide (37.5:1) solution was obtained from Anachem (Luton, UK). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were obtained from Fermentas (York, UK). Silver staining kit was obtained from GE Healthcare (Chalfont St. Giles, UK).

2.1.5 Other reagents

All other chemicals and biochemicals, unless otherwise stated, were commercially available analytical grade reagents obtained from Sigma-Aldrich Company Ltd., Fisher Scientific, Life Technologies or BDH.

2.2 Cartilage explant culture

Principle: Bovine nasal and human articular cartilage explants cultures are commonly used as a model for cartilage matrix degradation. Cartilage samples are stimulated with combinations of pro-inflammatory cytokines, inhibitors or proteases to influence cartilage homeostasis. Serum was excluded from cartilage explants as it contains chondroprotective agents such as IGF-1 (Luyten et al., 1988, Tyler, 1989, Hui et al., 2001). The absence of serum does not affect tissue viability, with cartilage remaining responsive to fresh serum and exogenous growth factors (Hascall et al., 1983).

2.2.1 Bovine nasal cartilage degradation assay

Culture medium

DMEM containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin.

Dulbecco's phosphate buffered saline (DPBS+)

DPBS containing 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin.

Phosphate buffer

137 ml of 0.1 M NaH_2PO_4 and 63 ml 0.1 M $NaHPO_4$, pH 6.5.

Bovine nasal septum was obtained from a local abattoir after slaughter and used the same day or after overnight storage at 4°C. The connective tissue sheath was removed from the cartilage and cut into 2 mm strips. Discs, 2 mm in diameter were punched out

of the centre of the cartilage strips using a sterilised hole punch; care was taken to avoid cartilage with obvious vasculature. Cartilage discs were washed twice in DPBS. In a 24-well tissue culture plate, 3 discs per well were added in 600 µl of culture medium. In a 96-well tissue culture plate, 1 disc per well was added in 200 µl of culture medium. The plates were then incubated overnight at 37°C in 5% CO_2/humidified air.

Medium was removed from each well and replenished with 600 µl fresh culture medium containing the appropriate cytokine(s) and test reagent(s) in a 24-well tissue culture plate. In a 96-well tissue culture plate each well was replenished with 200 µl of fresh culture medium containing the appropriate cytokine(s) and test reagents(s). For each condition, 4 wells were used in the 24-well tissue culture plate format. Whilst, 6 wells per condition in the 96-well format. Plates were then incubated at 37°C for 7 days. At day 7, supernatants were collected and cartilage discs replenished with identical test reagents to day 0. The experiment was continued for a further 7 days and at day 14, supernatants were removed and stored at -20°C. At day 14, the cartilage explants were placed in capped Sarstedt tubes with phosphate buffer containing 4.5 mg/ml papain, 5mM cysteine-HCl and 5mM ethylenedinitrilotetraacetic acid (EDTA). Following overnight (16-20 h) digestion at 65°C, 450 µl phosphate buffer was added.

2.2.2 Human articular cartilage degradation assay

Reagents

As described in section 2.2.1

Human articular cartilage was obtained from patients undergoing total joint replacement surgery at the Freeman hospital provided under ethical approval and informed consent. The joints were stored at 4°C in DPBS+. Tissue was used up to 24 h after removal from the patient. Macroscopically normal cartilage was dissected and rinsed in DPBS+. Three

pieces of cartilage (approximately 2 mm x 2 mm x 2 mm in dimension) were treated as described in section 2.2.1.

2.2.3 Hydroxyproline assay

Principle: The hydroxyproline imino acid is found in few proteins other than collagen as the sequence glycine-proline-hydroxyproline occurs frequently in collagen. Therefore, collagen levels can be measured by assessing hydroxyproline levels. Proteins were acid hydrolysed into their constituent amino acids. Hydroxyproline was then oxidised by chloramine T to a pyrrole related compound, which was subsequently reacted with DAB to produce a red product. The absorbance was then measured at A_{560}. Hydroxyproline was assayed using a microtitre modification of the assay described by Bergman and Loxley (1963).

Acetate-citrate buffer

420 mM sodium acetate, 130 mM tri-sodium citrate, 26 mM citric acid and 38.5% (v/v) propan-2-ol, pH 6.

DAB

4.5 M stock in 70% (v/v) perchloric acid, stored 4°C.

Chloramine T

250 mM in dH_2O, made fresh.

Method

Supernatants or cartilage digests (200 µl) were mixed with 200 µl of 12 M HCl in 2 ml o-ring screw-cap tubes. Samples were then hydrolysed in a hot-block for 20 h at 105°C. The hydrolysates were dried in a centrifugal evaporator using an acid resistant integrated Savant Speed Vac (Life Sciences International, Basingstoke, UK) or Genevac

EZ-2 speedvac (Genevac Ltd, Ipswich, UK) for 2-3 h. Once dry, the residue was resuspended in 200 µl dH_2O and stored at room temperature until assayed. A 1 mg/ml stock of hydroxyproline was diluted in dH_2O to give a series of standards (0-30 µg/ml). On the day of use, 4.5 mM DAB was diluted 1:3 in propan-2-ol and 250 mM chloramine T was diluted 1:4 in acetate-citrate buffer. 40 µl of sample or standard (neat or diluted in dH_2O) was added in duplicate to a 96-well microtitre plate. 25 µl of 65.5 mM chloramine T was added. After 4 min, 150 µl of 1.5 M DAB was added. The plate was then sealed with a plastic plate sealer and incubated for 35 min at 65°C. The plate was then allowed to cool and the absorbance read at 560 nm (Sunrise microplate reader, Tecan). The hydroxyproline content of the samples was calculated from the standard curve. The release of hydroxyproline from the cartilage was then calculated using the following equation:

% hydroxyproline release =

[hydroxyproline in supernatants (µg)]/[hydroxyproline in supernatants + hydroxyproline in cartilage digests (µg)] x 100

The % release of hydroxyproline was considered to be representative of the % release of collagen.

2.3 mRNA extraction

2.3.1 Bovine nasal chondrocyte isolation

Principle: Bovine nasal septum was used as a source of primary chondrocytes. The cartilage was subjected to a three-step enzymatic digest to isolate the chondrocytes.

DPBS+

DPBS + 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin.

Culture medium + 10% (v/v) FCS

DMEM containing 25 mM HEPES, 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin.

Hyaluronidase

1 mg/ml in DPBS+.

Trypsin

2.5 mg/ml in DPBS+.

Collagenase

2.5 mg/ml in culture medium + 10% (v/v) FCS.

Method

Bovine nasal septum was obtained as described in section 3.2.1. The strips cut into small cubes and washed three times in DPBS. Before use, enzyme solutions were filter-sterilised through a 0.2 μm filter. The cartilage was incubated at 37°C for 15 min on a spiramixer, with hyaluronidase (3 ml/g cartilage). The supernatant was removed and the cartilage washed three times in DPBS. Cartilage was then incubated for 30 min at 37°C with trypsin (3 ml/g cartilage) on a spiramixer. The supernatant was removed and the cartilage washed twice in culture medium + FCS. The cartilage pieces were then incubated overnight at 37°C in a bacterial collagenase solution (3 ml/g cartilage) on a spiramixer. Tubes were allowed to stand for 15 min to permit the debris to settle. The supernatant was then passed through a sterile 100 μm cell strainer and chondrocytes pelleted by centrifugation (217 g for 5 min). Chondrocytes were resuspended in culture medium + 10% (v/v) FCS and $5x10^3$ cells plated into each well of 96-well tissue culture plates (200 μl culture medium + 10% (v/v) FCS/well). The bovine cells took 3-5 days to reach 70-80% confluence. Before cytokine stimulation, cells were washed twice in

DPBS to remove traces of serum and left for 24 h in 100 µl culture medium (serum free)/well.

2.3.2 Cultivation of SW1353 cells

Culture medium + 10% (v/v) FCS

DMEM containing 25 mM HEPES, 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin.

SW1353 were maintained in culture medium + 10% (v/v) FCS and $5x10^3$ cells plated into each well of 96-well tissue culture plates (200 µl culture medium + 10% (v/v) FCS/well). The SW1353 cells took 3-5 days to reach 70-80% confluence. Before cytokine stimulation, cells were washed twice in DPBS to remove traces of serum and left for 24 h in 100 µl culture medium (serum free)/well.

2.3.3 Extraction of RNA

Method

This was performed as per the manufacturer's instructions. Briefly, after cytokine stimulation the plates were washed in ice-cold PBS and then 10 µl of ice-cold lysis buffer added per well. The lysates were then agitated for 2 min at medium speed on a flatbed mixer. The lysates were then transferred to V-bottomed 96-well plates and stored at -80°C.

2.3.4 cDNA production from sidestep RNA extraction

Method

Lysates (5 µl), from section 2.3.3, were transferred to a new 96-well plate and diluted with 15 µl of PCR grade water. Diluted lysate (4 µl) was then transferred to a 96-well PCR plate and then 0.325 mM dNTPs and 0.2 µg random hexamers ($Pd(N)_6$) added and

the mixture then incubated at 70°C for 5 min. The plate was then rapidly cooled on ice for two min. Then DTT (10 μM final concentration), RNaseOUT (5 U), MMLV (100 U) and first strand buffer (50 mM Tris pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) added to the desired volume. The plate was then incubated for 50 min at 37°C and then for 15 min at 70°C. The cDNA was then diluted with water before use for target gene detection and a further 1/100 fold dilution for housekeeping gene detection.

All incubations were performed using the using the TP 600 PCR thermal cycler DICE (Takara).

2.3.5 Human articular cartilage mRNA assay

Principle: Cartilage is a highly specialised and unique tissue (section 1.1) with only a handful of cells embedded in a large ECM network. Interactions between chondrocytes and their matrix are significant and remodelling of the ECM by proteases can release cryptic information and bioactive molecules (Mott and Werb, 2004). A number of studies have studied the gene expression of chondrocytes grown in monolayer (Koshy et al., 2002). However, only few studies have been performed on gene expression in human cartilage that is actively resorbing.

Culture medium

DMEM containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin.

DPBS+

DPBS containing 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin.

Method

Human articular cartilage was obtained as described in section 2.2.2. The cartilage cut into small cubes and washed three times in DPBS. Cartilage (approximately 0.5 g) was then placed into a 12 well dish with 3 ml of culture medium. The plates were then incubated overnight at 37°C in 5% CO_2/humidified air to allow equilibration of the explants.

Medium was removed from each well and replenished with 3 ml fresh culture medium containing the appropriate cytokine(s) and test reagent(s). For each condition and time point, 4 wells were used. At day 7, media samples were collected and stored at -20°C whilst cartilage chips were collected and stored at -80°C.

2.3.6 RNA extraction from cartilage explants

Principle: Trizol is a mixture of guanidinium thiocyanate and water-saturated phenol and is used to solubilise the cells and protein components in cartilage as well as nucleic acids. The guanidinium denatures proteins including RNases thereby protecting nucleic acid during extraction. The addition of chloroform during extraction and subsequent centrifugation separates the components into an upper aqueous phase, where the RNA locates to and the lower and intermediate phase where the DNA and protein mainly locate to (Chomczynski and Sacchi, 1987). RNA was then isolated using Qiagen mini columns that selectively binds to nucleic acids larger than 200 bp, while allowing protein to be excluded, DNase I is added to degrade contaminating genomic DNA. Finally, RNA is eluted with RNase-free water.

Method

Human cartilage was removed from the -80°C freezer and placed in stainless steel grinding vials (Retsch, Germany). The cartilage was then ground under the following

conditions: 5 cycles of cooling in liquid nitrogen for 3 min and then ground for 90 seconds at 20Hz in a Retsch MM200 (Retsch, Germany).

The powered cartilage was then placed in 5 ml of Trizol reagent (Life Technologies), vigorously shaken for 5 min and left at room temperature for 10 min.

The suspension was then placed into 1.5 ml eppendorfs (Starlabs) and centrifuged at 20,000 x *g* for 10 min at 4°C to remove any insoluble material. The supernatant was transferred to fresh eppendorfs and 450 µl chloroform added, then vortexed and left to incubate at room temperature for 10 min. The tubes were then centrifuged for 12,000 x *g* for 15 min at 4°C, the upper aqueous phase then transferred to a new tube and 100% ethanol (0.5 volume) added. This was then loaded onto a Qiagen mini column and the RNA purified according to the manufacturer's instructions. Briefly, 700 µl of sample were applied to the RNeasy mini-spin column and centrifuged for 15 seconds at 8,000 x *g*. The flow-through was discarded and this step repeated until all the sample was applied to the column. The column was then washed to remove contaminants and then DNase I (3 U) added to the column for 15 min at room temperature. The column was then washed three times according to the manufacturer's instructions. The RNA was eluted in diethyl pyrocarbonate (DEPC) water (30 µl). The purified RNA was immediately stored at -80°C. The RNA was then quantified by measuring the absorbance at 260 nm and 280 nm using a ND1000 spectrophotometer (Nanodrop, USA).

2.3.7 Reverse transcription of cartilage explant mRNA

Method

Total RNA (1 µg) was diluted to a final volume of 11 µl with water and then 50 ng random hexamers and 0.5 mM (final concentration) dNTPs were added. This mixture was then incubated at 65°C for 5 min, then chilled on ice for 2 min and DTT (50 µM

final concentration), RNaseOUT (40 U), superscript III reverse transcriptase (200U), and first strand buffer (50 mM Tris pH 8.3, 75 mM KCl and 3 mM $MgCl_2$) added to the desired volume. The reaction mixture was incubated at 25°C for 5 min then 50°C for 1 h. The enzymes were then denatured by incubation at 70°C for 15 min. The cDNA was then diluted 1/100 for target gene determination and a further 1/5 for housekeeping genes.

2.4 Real-time PCR

2.4.1 Primer design

Bovine forward and reverse primers were designed using the Universal Probelibrary software (Roche Applied Science, Burgess Hill, UK). PerfectProbe assay kits were received from PrimerDesign (Southampton, UK). TaqMan assay on demand kits were received from ABI (Warrington, UK).

Primers were designed to span exon/intron boundaries to prevent the amplification of any contaminating genomic DNA. Primers were designed for bovine gene sequences and human gene sequences (Table 2.1).

Gene	Primer (5'-3')	Human Sequence	Bovine Sequence
FAPα	Forward	ATCTATGACCTTAGCAATGGAGAATTTGT	ACCATGAAAAGTGTGAATGCTTCA
	Reverse	GTTTTGATAGACATATGCTAATTTACTCCCAAC	AGTATCTCCAAAGCTTTGAATAATCACTTTCT
DPPIV	Forward	AGCCGTGGCGCCTGTAT	CTGGTCATACGGAGGGTACG
	Reverse	GTCAAGGTTGTCTTCTGGAGTTGG	GGCTATTCCACACTTGAACACA
18S rRNA	Forward	CGAATGGCTCATTAAATCAGTTATGG	AGAAAGGGCTACCACATCCA
	Reverse	TATTAGCTCTAGAATTACCACAGTTATCC	CACCAGACTTGCCCTCCA
	Probe	FAM-TCCTTTGGTCGCTCGCTCCTCTCCC-TAMRA	*Not applicable*
MMP-1	Forward	AAGATGAAAGGTGGACCAAAATT	CAAGCTAACTTTTGATGCCATAAC
	Reverse	CCAAGAGAATGGCCGAGTTC	GCATGTAGAACCGGTCTTTGA
	Probe	FAM-CAGAGAGTACAACTTACATCGTGTTGCGGCTC-TAMRA	*Not applicable*
MMP-13	Forward	AAATTATGGAGGAGATGCCCATT	
	Reverse	TCCTTGGAGTGGTCAAGACCTAA	
	Probe	FAM-CTACAACTTGTTTCTTGTTGCTGCGCATGA-TAMRA	
Matriptase	ABI TaqMan assay on demands		
PAR-2 (F2RL1)			
MMP-3	Forward	TTCCGCCTGTCTCAAGATGATAT	
	Reverse	AAAGGACAAAGCAGGATCACAGTT	
	Probe	FAM-TCAGTCCCTCTATGGACCTCCCCCTGAC-TAMRA	
MMP-14	Forward	AGGCCGACATCATGATCTTCTTT	
	Reverse	AAGTGGGTGTCTCCTCCAATGTT	
	Probe	FAM-CCATGGCGACAGCACGCCCTT-TAMRA	

Table 2.1 Primers for real-time PCR.

2.4.2 SYBR green real-time PCR

Principle: SYBR green is a fluorescent dye that binds to the minor groove of the DNA helix. In solution the dye emits very little fluorescence, but upon binding to an exponentially increasing DNA amplicon, the dye emits fluorescence proportional to the amount of reaction product which is readily increased.

Method

SYBR green PCR reactions for bovine 18S rRNA, MMP-1 and MMP-13 genes were carried out in a total volume of 10 µl using 2.5µl of cDNA with 5 µl of SYBR green reaction mix (Clontech, Paris, France) and primers at 50 nM final concentration and 1X ROX reference dye. Thermal cycling conditions for these PCR reactions comprised a holding stage at 95°C for 10 seconds. This was followed by a three-step programme consisting of 95°C for 5 seconds, 55°C for 15 seconds and 72°C for 20 seconds, repeated for 40 cycles.

SYBR green PCR reactions for FAPα and DPPIV genes were carried out in a total volume of 10 µl using 2.5 µl of cDNA with 4.92 µl of SYBR green reaction mix (Clontech, Paris, France) and primers at 100 nM final concentration and 1X ROX reference dye. Thermal cycling conditions for these PCR reactions comprised a holding stage of 95°C for 10 seconds. This was followed by a two-step programme consisting of 95°C for 15 seconds and 60°C for 60 seconds, repeated for 40 cycles.

For all reactions a melt curve analysis was then performed to confirm the amplification of a single specific product. All reactions were performed using the ABI PRISM 7900HT sequence detector.

2.4.2 Probe based real-time PCR

Principle: This method employs the use of a probe that anneals down-stream of a primer site. The probe has a reporter dye at the 5' end and a quencher at the 3' end. When the probe is intact both the quencher and reporter dye are in close proximity and emit little fluorescence. During extension of the primer sequence by *Taq* polymerase, the 5' nuclease activity of *Taq* cleaves the probe from the DNA. The spatial separation of the reporter dye from the quencher increases the reporter signal. Thus with each cycle the fluorescence intensity increases in proportion to the amplicon product.

Method

Probe based PCR reactions were carried out in a total volume of 10 µl using 5 µl of cDNA with 3.3 µl of SIGMA mastermix (Poole, UK) and primers at 900 nM final concentration, probe at 150 nM final concentration and 1X ROX reference dye. Thermal cycling conditions comprised an activation stage at 95°C for 10 min. This was followed by a two-step programme consisting of 95°C for 15 seconds and 60°C for 60 seconds, repeated for 40 cycles.

Human ST14 and F2RL1 assay on demand kits (Applied Biosystems, Warrington, UK) were used as described above, except 0.5 µl of the assay mix was added instead of primers and probe.

All reactions were performed using the ABI PRISM 7900HT sequence detector.

2.5 Purification and characterisation of type II collagen

2.5.1 Type II collagen purification

Principle: Type II collagen is isolated using the method described by Miller and Rhodes (1982). After tissue homogenisation, proteoglycans need to be removed as they can amalgamate with collagen molecules and form insoluble aggregates at acid pH. Selective degradation of cross-links between the collagen molecules allows the collagen to solubilise in dilute acid solvents. Alternative precipitations from acidic to neutral salt solvents allow for purification of the collagen molecules.

Proteoglycan extraction buffer

4 M guanidium chloride, 50 mM Tris, pH 7.5, 0.02% (w/v) NaN_3

Collagen extraction buffer

0.5 M acetic acid, pH 2.06, 0.02% (w/v) NaN_3, 0.0005% (w/v) pepsin

Method

Human articular cartilage was used a source of human type II collagen; cartilage was dissected from joint tissues received from patients undergoing total joint replacement surgery and stored at -80°C. All tissue was collected with ethical approval and patient consent.

Unless otherwise stated, all work was performed at 4°C.

The cartilage was ground to a fine powder using the Specxmill as previously described (section 2.3.6). The powdered cartilage was then resuspended in proteoglycan extraction buffer and left to agitate for 48 h on a rocker (30 rpm).

The suspension was then spun at 21,875 x *g* for 30 min and the pellet resuspended in collagen extraction buffer and left to shake for 24 h at 30 rpm. The solution was then spun at 21,875 x *g* for 1 h and the supernatant collected and stored at 4°C. The pellet

was resuspended in collagen extraction buffer and left to shake for 24 h at 30 rpm again. After this, the solution was centrifuged at 21,875 x *g* for 1 h, the supernatant collected and combined with that from the first spin. The pH of this solution was then brought to pH 7.6 with 50% (w/v) sodium hydroxide. Sodium chloride was then added very slowly to 27% (w/v) and the solution left for 24 h. It was then spun at 18,381 x *g* and the pellet resuspended in 0.5 M acetic acid and then sodium chloride added to a final concentration of 0.84 M. The solution was then spun for 1 h at 21,875 x *g* and the pellet redissolved in 0.5 M acetic acid, lyophilised (Labconco) and stored at -20°C.

2.5.2 Preparation of dialysis tubing

Method

Regenerated cellulose dialysis tubing (Medicell International Ltd) with a molecular weight cut-off of 14kDa was boiled in 2 M Na_2CO_3 buffer to wash away any impurities and then stored in 40% (v/v) butanol at 4°C.

2.5.3 Characterisation of enzyme activity on purified type II collagen

Assay buffer

50 mM Tris-HCl, pH 7.6, 1 M glucose, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) NaN_3

Method

Lyophilised collagen was dissolved in 0.5 M acetic acid to a final concentration of 1 mg/ml and then dialysed against assay buffer. In some reactions the collagen was denatured by incubation at 56°C for 30 min. Collagen (10 µg) was digested over a 24 h period at 37°C with 100 µM of enzyme. Samples were then separated on 6.5% SDS-PAGE gels and visualised by silver staining as described in sections 2.5.4 and 2.5.5, respectively.

2.5.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Principle: SDS-PAGE allows for the size-dependent separation of proteins regardless of their electrochemical properties. Initially, samples are mixed with loading dye and heated to 105°C. All proteins in the sample are then uniformly coated with SDS, therefore having the same charge. The lower pH of the stacking gel results in the concentration of the proteins into a thin band. Upon entering the resolving gel, the pH increases and the proteins are now separated based on their molecular weight.

4x Lower gel buffer (LGB)

1.5 M TrisHCl pH 8.8, 0.4% (w/v) SDS.

4x Upper gel buffer (UGB)

0.5 M Tris HCl pH 6.8, 0.4% (w/v) SDS.

Upper gel

40% (w/v) bis/acrylamide diluted to 4.5% with water and 4x UGB.

5x Final sample buffer (FSB)

0.625 M Tris HCl pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol.

10x Running Buffer

250 mM Tris, 2 M glycine, 10% (w/v) SDS.

Method

Electrophoresis was carried out in a Bio-Rad Mini-Protean II apparatus with 1.0 mm spacers and combs. Polyacrylamide-bis-acrylamide was diluted with water and 3 ml of 4x LGB to the required percentage (Table 2.2). All solutions were allowed to reach room temperature before mixing. Gel mixture (12 ml) was polymerised by the addition

of 20 µl TEMED and 60 µl APS (0.1% w/v) immediately before pouring the gel. The lower gel was overlaid with propan-2-ol to exclude oxygen and allowed to polymerise. Once set the propan-2-ol was washed off and a 4.5% bis/acrylamide stacking gel laid on top, combs were 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 kit was assembled and filled with 1x running buffer. Samples were prepared as follows: 1x sample buffer was added appropriately to samples which were then boiled at 105°C for 5 min. Molecular weight markers were "Pageruler" protein markers (Fermentas). Proteins were electrophoresed at constant 60 V, through the stacking gel, and then 80 V, through the separating gel until the dye front had migrated to the end of the separating gel.

Percentage gel (%)	**40% (w/v) acryl/bis solution (37.5:1) (ml)**	**ddH_2O (ml)**	**4x LGB (ml)**
10	3.0	6.0	3.0
6.5	1.95	7.05	3.0

Table 2.2 Preparation of SDS-PAGE gels**.**

2.5.5 Visualisation of protein bands

Principle: Silver staining (Heukeshoven and Dernick, 1985) employs the use of soluble silver ions to detect proteins after separation by SDS-PAGE. When a reducing agent, such as formaldehyde, is added the silver ions are reduced and this reduction is enhanced in the presence of protein. Addition of sodium carbonate forms insoluble silver carbonate and highlights protein bands in the gel. The reaction is stopped by the sequestering of the silver ions by a chelating agent, such as EDTA.

Fixing solution

40% (v/v) ethanol, 10% (v/v) acetic acid

Sensitising solution

30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 0.125% (v/v) glutaraldehyde

Silver solution

0.25% (w/v) silver nitrate

Developing solution

2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde

Stop solution

1.5% (w/v) EDTA

Method

Proteins were separated by SDS-PAGE (section 2.5.4) and then the separating gel placed in fixing solution for 1 h. The fixing solution was then removed and replaced with sensitising solution for 1 h. Sensitising solution was then removed and the gel then washed with ddH_2O for 15 min and this step was repeated 3 further times. Silver solution was then added and left for 1 h. The gel was then washed twice with ddH_2O for 1 min. Developing solution was then added and the reaction progressing for 4 to 6 min until the protein bands were of sufficient visibility. The reaction was then immediately stopped by the addition of stop solution for 1 h. Images were captured using the ChemiGenius II system and associated software (Syngene, Cambridge, UK).

2.6 Collagenase assay

Principle: Collagen at neutral pH and at temperatures above 25°C forms into fibrils where individual collagen molecules associate to form a gel. [^{3}H]-acetylated collagen was used to measure the collagenolytic activity in supernatant from bovine and human cartilage explants cultures. At the end of the assay, undigested collagen fibrils are spun down in the centrifuge and the amount of [^{3}H] in the supernatant correlates to the amount of collagen digested. Using APMA in the assay, one can measure total collagenase activity in addition to active collagenase activity.

Tris buffer

100 mM Tris-HCl, pH 7.6, 15 mM $CaCl_2$ and 0.02% (w/v) NaN_3

10mM APMA stock solution

35.2 mg APMA was dissolved in 200 µl DMSO and made up to 10 ml with 100 mM Tris-HCl, pH 8.5. Stored in the dark at 4°C for up to 3 months

Tris-APMA buffer

APMA was diluted to 2 mM in Tris buffer

Cacodylate buffer

25 mM sodium cacodylate, pH 7.6, 0.05% (w/v) Brij-35 and 0.02% (w/v) NaN_3

[^{3}H]-acetylated collagen

Type I collagen was extracted and purified from calf skin (Cawston and Barrett, 1979). The purified collagen was freeze-dried and stored at -20°C. When required the collagen was thawed and redissolved in 0.2 M acetic acid at 4°C. Collagen was radiolabelled with [^{3}H]-acetic anhydride (925 MBq; GE Healthcare (Chalfont St. Giles, UK)) as described in Cawston et al. (2001).

Trypsin

100 µg/ml trypsin in 1 mM HCl

Bacterial collagenase

100 µg/ml bacterial collagenase in cacodylate buffer

Method

To a 96-well V-bottomed plate, 50 µl of Tris assay buffer ± APMA was added to each well followed by 10 µl sample in duplicate (neat or duplicated in cacodylate buffer) and 40 µl cacodylate buffer. Three sets of controls were included in the assay:

1. Cacodylate buffer (50 µl);
2. Trypsin (1 µg);
3. Bacterial collagenase (5 µg).

50 µl of [^{3}H]-acetylated collagen was then added to each well. The 96-well plate was incubated at 37°C (16-20 h) then centrifuged at 1056 x *g* at 4°C for 30 min in Sorvall RC5C Plus centrifuge. Supernatant (50 µl) was removed and placed in a flexible 96-well sample plate with 200 µl of Optiphase "Supermix" scintillation fluid. Counts were read in a 1450 Micro-Beta Trilux liquid scintillation and luminescence counter (PerkinElmer). Collagenase activity was measured in units/ml, where one unit can degrade 1 µg of collagen per min at 37°C

Equation for calculating collagenase activity (units/ml):

=50/(total lysis-blank) x 1000/(sample volume (µl)) x 1/(time (min)) x (sample-blank)

2.7 Enzyme activity assays

2.7.1 Dipeptidyl peptidase activity assays

Principle: FAPα is the only member of the DPP family that has gelatinase activity (Aoyama and Chen, 1990, Monsky et al., 1994, Pineiro-Sanchez et al., 1997) as well as

dipeptidyl peptidase activity (Park et al., 1999) and both of these activities occur in the same catalytic pocket that is located in the α/β-hydrolase domain. However, the exopeptidase activity of FAPα is reported to be 100-fold lower compared to DPPIV (Aertgeerts et al., 2005). Therefore, using a fluorescent N-terminally blocked substrate allows one to probe for the endopeptidase activity of FAPα; using a non N-terminally blocked peptide will probe mainly for the exopeptidase activity. Cleavage of the proline-AMC bond in either case will release AMC, which is highly fluorescent.

Exopeptidase activity is probed for using a fluorescent peptide mimetic, GP-AMC, which closely resembles *in vivo* substrates. Cleavage of the proline-AMC bond will release AMC, which is highly fluorescent.

2.7.1.1 Endopeptidase activity assay

Assay buffer

25 mM Tris HCl, pH 7.9, 140 mM NaCl, 10 mM KCl, 0.01% (w/v) Brij-35. This was filtered through a 0.2 µm filter and pre-warmed to 37°C before use.

Z-GP-AMC

25 mM dissolved in methanol. Stored -20°C in the dark.

FAPα

1.2 µM in assay buffer. Stored at -20°C

Z-GP-AMC was diluted to 62.5 µM in pre-warmed assay buffer and kept at 37°C in the dark. FAPα (120 pM final concentration) was mixed with either 10 µl of test reagent or assay buffer in 96-well white walled plates at 37°C for 30 min. 80 µl of 62.5 µM Z-GP-AMC was added to each well (50 µM final concentration) and the plate incubated for 4 h. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm) in a Perkin Elmer LS-50B fluorimeter.

2.7.1.2 Exopeptidase activity assay

Assay buffer

25 mM Tris HCl, pH 7.9, 140 mM NaCl, 10 mM KCl, 0.01% (w/v) Brij-35. This was filtered through a 0.2 µm filter and pre-warmed to 37°C before use.

GP-AMC

25 mM dissolved in methanol. Stored -20°C in the dark.

FAPα

1.2 µM in assay buffer. Stored at -20°C

DPPIV

9.5 µM in 10 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1 mM EDTA and 10% (v/v) glycerol buffer. Stored at -20°C

GP-AMC was diluted to 62.5 µM in pre-warmed assay buffer and kept at 37°C in the dark. FAPα (120 pM final concentration) or DPPIV (50 pM final concentration) was mixed with either 10 µl of test reagent or assay buffer in 96-well white walled plates at 37°C for 30 min. 80 µl of 62.5 µM GP-AMC was added to each well (50 µM final concentration) and the plate incubated for 4 h. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm) in a Perkin Elmer LS-50B fluorimeter.

2.7.2 Matriptase activity assay

Principle: Matriptase activity is probed for using a fluorescent peptide mimetic, Boc-QAR-AMC, which closely resembles *in vivo* substrates. Cleavage of the arginine-AMC bond will release AMC, which is highly fluorescent.

Assay buffer

100 mM Tris HCl, pH 9, 500 µg/ml BSA, 0.01% (w/v) Brij-35. This was filtered through a 0.2 µm filter and pre-warmed to 37°C before use.

Boc-QAR-AMC

25 mM dissolved in methanol. Stored -20°C in the dark.

Matriptase

3.8 µM in 50 mM Tris HCl, pH9, 1 mM β-mercaptoethanol, 400 mM NaCl, 10% glycerol. Stored at -20°C

Boc-QAR-AMC was diluted to 62.5 µM in pre-warmed assay buffer and kept at 37°C in the dark. Matriptase (10 nM final concentration) was mixed with either 10 µl of test reagent or assay buffer in 96-well white walled plates at 37°C for 30 min. 80 µl of 62.5 µM Boc-QAR-AMC was added to each well (50 µM final concentration) the plate incubated for 30 min. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm) in a Perkin Elmer LS-50B fluorimeter.

2.7.3 FS-6 assay

Principle: FS-6 is a general MMP substrate that has increased reaction rates compared to previous peptide analogues (Neumann et al., 2004). Dpa is an internal quencher that limits the auto-fluorescence of the AMC, but MMP cleavage of the Gly-Leu peptide spatially separates the Dpa and AMC, with fluorescence increasing proportionally.

Assay buffer

100 mM Tris HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.1% (w/v) PEG-6000, 0.05% (w/v) Brij-35. This was filtered through a 0.2 µm filter and pre-warmed to 37°C before use.

FS-6 (AMC-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2)

5 mM dissolved in DMSO. Stored -20°C in the dark

Pro-MMP-1

1.4 µM in 20 mM Tris HCl ,pH7.2, 0.5 M NaCl, 0.05% (w/v) NaN_3, 0.01% (w/v) Brij-35. Stored at -80°C

10 mM APMA stock solution

35.2 mg APMA was dissolved in 200 µl DMSO and made up to 10 ml with 100 mM Tris-HCl, pH 8.5. Stored in the dark at 4°C for up to 3 months

Method

Pro-MMP-1 was activated by incubating with APMA (0.67 mM final concentration) or matriptase (0.28 µM final concentration) for 4 h. FS-6 was diluted to 6.25 µM in pre-warmed assay buffer and kept at 37°C in the dark. MMP-1 (0.14 µM final concentration) was mixed with 10 µl assay buffer in 96-well white walled plates at 37°C for 30 min. 80 µl of 6.25 µM FS-6 was added to each well (5 µM final concentration) and fluorescence read after 5 min (λ_{ex} 325 nm, λ_{em} 400 nm) in a Perkin Elmer LS-50B fluorimeter.

2.7.4 Hide powder azure assay

Principle: Proteins, from bovine hide, are covalently linked to the chromogen Remazol Brilliant Blue R. The substrate was then incubated with active enzyme and upon cleavage of labelled proteins from the hide powder there is an increase in blue product in the supernatant. The absorbance was then measured at A_{595}.

Assay buffer

100 mM Tris HCl, pH 7.5, 0.6 M sucrose, 100 mM NaCl, 10 mM $CaCl_2$, 0.1% (w/v) PEG-6000, 0.05% (w/v) Brij-35. This was filtered through a 0.2 µm filter and pre-warmed to 37°C before use.

Hide azure solution

3% (w/v) dissolved in assay buffer.

Pro-MMP-1

1.4 µM in 20 mM Tris HCl, pH7.2, 0.5 M NaCl, 0.05% (w/v) NaN_3, 0.01% (w/v) Brij-35. Stored at -80°C

10mM APMA stock solution

35.2 mg APMA was dissolved in 200 µl DMSO and made up to 10 ml with 100 mM Tris-HCl, pH 8.5. Stored in the dark at 4°C for up to 3 months

Method

Pro-MMP-1 was activated by incubating with APMA (0.67 mM final concentration) or matriptase (0.28 µM final concentration) for 4 h. MMP-1 (0.14 µM final concentration) was mixed with 1 ml of hide powder azure solution in 1.5 ml eppendorfs and incubated at 37°C for 4 h on an orbital shaker (1000 rpm). Then centrifuged at 1200 x *g* for 5 min and supernatant transferred to 96-well flat bottom plate in duplicate (100 µl/well). The absorbance was read at 595 nm (Sunrise microplate reader, Tecan).

2.8 ELISA

2.8.1 MMP-1 ELISA

Principle: Monoclonal MMP-1 antibody was immobilised on Maxisorb 96-well plates. Samples were added to the plates and any MMP-1 present would bind to the antibody.

A secondary antibody, which is raised against a second distinct epitope, was added. Tertiary antibody, conjugated with HRP, recognising the secondary antibody was then added. A chromogenic substrate was then added and oxidised by HRP producing a coloured product.

Phosphate buffered saline (PBS)

8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 154 mM NaCl, 2.7 mM KCl, pH 7.4

Wash buffer

8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.25 mM thimerosol and 0.1% (v/v) Tween 20, pH 7.0

Blocking buffer

1% (w/v) BSA in PBS

Protein diluents

0.05% (w/v) BSA in wash buffer

Monoclonal antibody to human MMP-1 (RRU-CL1)

2 µg/ml in protein diluent

Rabbit anti-MMP-1 polyclonal antibody (B-anti-CL1)

2 µg/ml in protein diluent

Goat anti-rabbit IgG-HRP

Diluted 1:1000 in protein diluent

Phosphate-citrate buffer

Dissolve one phosphate-citrate capsule per 100 ml of ddH_2O

OPD substrate solution

Prepared immediately before use. 1 tablet (15mg) of OPD dissolved in 12 ml of 0.5 M phosphate-citrate buffer, pH 5.0.

Method

Maxisorb 96-well plates were coated with RRU-CL1 overnight at 4°C (100 µl/well). The plates were washed once with wash buffer (400 µl/well) and blocked with blocking buffer (200 µl/well) overnight at 4°C. The plates were then washed three times in wash buffer. Standards (0-80 ng/ml) were prepared by diluting recombinant human pro-MMP-1 in protein diluent. Samples and standards (100 µl/well) were added to the plates in duplicate and incubated at 4°C overnight. The plates were then washed three times with wash buffer and then incubated at room temperature for 2 h with B-anti-CL1 (100 µl/well). The plates were washed three times in wash buffer and incubated at room temperature for 1 h with goat anti-rabbit IgG-HRP (100 µl/well). The plates were then washed three times in wash buffer and incubated with 100 µl per well of OPD substrate for approximately 5 min at room temperature. The reaction was stopped by the addition of 3 M H_2SO_4 (50 µl/well) and the absorbance read at 490 nm (TECAN Sunrise plate reader, Reading, UK).

2.8.2 MMP-13 ELISA

Principle: Monoclonal MMP-13 antibody was immobilised on Maxisorb 96-well plates. Samples were added to the plate and any MMP-13 present would bind to the antibody. A biotin conjugated secondary antibody, which is raised against a second distinct epitope, is added. Streptavidin conjugated HRP is then added and binds to the biotin labelled secondary antibody. A chromogenic substrate is then added and oxidised by HRP producing a coloured product.

Monoclonal coating antibody to human MMP-13

1.25 µg/ml in protein diluent

Biotinylated detection anti-MMP-13 antibody

125 ng/ml in protein diluent

Streptavidin-HRP

Diluted 1:1000 in protein diluents

Method

Maxisorb 96-well plates were coated with coating antibody overnight at 4°C (100 µl/well). The plates were washed once with wash buffer (400 µl/well) and blocked with blocking buffer (200 µl/well) overnight at 4°C. The plates were then washed three times in wash buffer. Standards (0-20 ng/ml) were prepared by diluting purified recombinant pro-MMP-13 in protein diluent. Samples and standards (100 µl/well) were added to the plates in duplicate and incubated at 4°C overnight. The plates were then washed three times with wash buffer and then incubated at room temperature for 2 h with detection antibody (100 µl/well). The plates were washed three times in wash buffer and incubated at room temperature for 30 min with Streptavidin-HRP (100 µl/well). The plates were then washed three times in wash buffer and incubated with 100 µl per well of OPD substrate for approximately 5 min at room temperature. The reaction was stopped by the addition of 3 M H_2SO_4 (50 µl/well) and the absorbance read at 490 nm (TECAN Sunrise plate reader, Reading, UK).

2.9 N-terminal sequencing

2.9.1 Recombinant pro-MMP-1 isolation by ion exchange chromatography

Principle: Ion exchange chromatography relies on the differences between molecules in relation to their surface charge distribution, overall charge and charge density. MMP-1 at neutral pH displays a net positive charge and binds to the negatively charged methyl sulfonate group in the column. Linear increments in the ionic strength of the buffer

causes elution of the bound proteins, with weakly interacting proteins eluting first at low ionic strength and strongly interacting proteins eluting last at high ionic strength.

Materials

Mono S HR 5/5 column.

Buffer A

20 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 0.05% (w/v) Brij-35, 0.01% (w/v) NaN_3 in ddH_2O

Buffer B

Buffer A including 1 M NaCl

Method

Buffers were filtered through a 0.45 µm filter before use. The Mono S column was used on an ÄKTAexplorer 100 system (GE Healthcare, Chalfont St. Giles, UK) at room temperature. Partially purified recombinant pro-MMP-1 was generously donated by Dr J. Milner. Samples were diluted 10-fold in buffer A. The column was washed with 5 column volumes (CV) of ddH_2O to remove the storage buffer (20% ethanol). 10 CV of buffer A were passed through the column to equilibrate it. The sample was loaded and then washed with 2 CV of buffer A to remove unbound proteins. Next, the column was washed with a salt gradient (0 to 1 M NaCl) using buffer B (33 mM/min). The flow rate throughout the procedure was 1 ml/min. A_{280} was measured to trace the protein peaks. Fractions (0.5 ml) were collected.

2.9.2 N-terminal sequencing of matriptase activated pro-MMPs

Principle: The N-terminal amino acid sequence of proteins can be determined by Edman degradation. Samples are incubated with matriptase and then separated by SDS-

PAGE and transferred onto PVDF membranes. The protein bands were visualised by Coomassie dye staining and excised. Samples were freed from the PVDF by immersion in organic solvents and the protein underwent 5 rounds of Edman degradation. Briefly, phenylisothiocyanate was reacted with the uncharged terminal amino group under alkaline conditions. The derivative is then acid treated to form a thiazolinone derivative and extracted into organic solvent and acid treated again to form phenylthiohydantoin amino acid derivative. The phenylthiohydantoin amino acid derivative is then identified by chromatography.

Buffer

25 mM sodium cacodylate, 10 mM $CaCl_2$, 0.05% Brij, 0.02% azide pH 8 in ddH_2O

Matriptase

3.8 µM in 50mM Tris HCl, pH9, 1mM β-mercaptoethanol, 400 mM NaCl, 10% (v/v) glycerol. Stored at -20°C.

Pro-MMP-3ΔC

22.4 µM in 50 mM TrisHCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 0.05% (w/v) Brij-35, 10% (v/v) glycerol. Stored at -80°C.

Pro-MMP-1

1.4 µM in 20 mM Tris-HCl, pH 7.4, 130 mM NaCl, 5 mM $CaCl_2$, 0.05% (w/v) Brij-35, 0.01% (w/v) NaN_3. Stored at -80°C.

GM6001

50 mM in DMSO. Protected from light and stored at -80°C.

5x Final sample buffer (FSB)

0.625 M Tris HCl pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol.

Transfer buffer

10 mM Caps, pH 11, 10% (v/v) methanol in ddH_2O

Staining solution

0.25% (w/v) Coomassie brilliant blue G250, 40% (v/v) methanol, 10% (v/v) acetic acid

Destaining solution

40% (v/v) methanol, 10% (v/v) acetic acid

Method

1 µM pro-MMP was incubated with 0.28 µM matriptase (± GM6001, 50 µM final concentration) at 37°C for 1 to 4 h. Samples were then run on 10% SDS-polyacrylamide gels (section 2.5.4) and transferred to PVDF membranes by semi-dry electroblotting (Scie-Plas V20-SDB) for 90 min at 1mA/cm^2.

The protein bands were then visualised by placing the membranes in staining solution for 20 min and then destaining for 30 min. The bands were then excised and sent for N-terminal sequencing. N-terminal sequence analysis was performed by Dr Joe Gray (Pinnacle, Newcastle University, UK).

2.10 Statistics

Statistical difference between parametric groups was assessed by one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test. All non-parametric samples were assessed by Mann-Whitney U test. The SPSS 15.0 software package (SPSS UK Ltd, UK) was used in all instances. Significance levels were indicated as * = $p \leq 0.05$, ** $p \leq 0.01$ and *** = $p \leq 0.001$.

CHAPTER 3

Investigating the role of FAPα in cartilage homeostasis

3.1 Introduction

The degradation of the cartilage collagen matrix is irreversible and a major hallmark of the rheumatic diseases. The key enzymes involved in cartilage degradation are the collagenases (section 1.5.2) which belong to the MMP family. All MMPs are synthesised as zymogens that require removal of their pro-domain for activity (section 1.5.4), but the exact *in vivo* mechanisms of MMP activation are not fully understood. However, evidence from within our group shows that serine proteases play an important role in the regulation of MMP activity (Milner et al., 2001, 2003, 2008). The inhibition of either furin-like or trypsin-like cascades in IL-1+OSM-treated cartilage protects the collagen matrix significantly from cytokine-mediated degradation. All these observations clearly implicate serine proteases in pathological collagen turnover.

Data implicate that type II collagen degradation occurs around the chondrocyte before being detected in other zones of the cartilage (Hollander et al., 1995). Recently, novel transmembrane serine proteases have been discovered to be up-regulated in OA cartilage compared to phenotypically normal cartilage (Figure 3.1). Research on newly discovered serine proteases is required as their cell surface localisation of these proteases would support a hypothesis that they have an important role in pericellular degradation of cartilage.

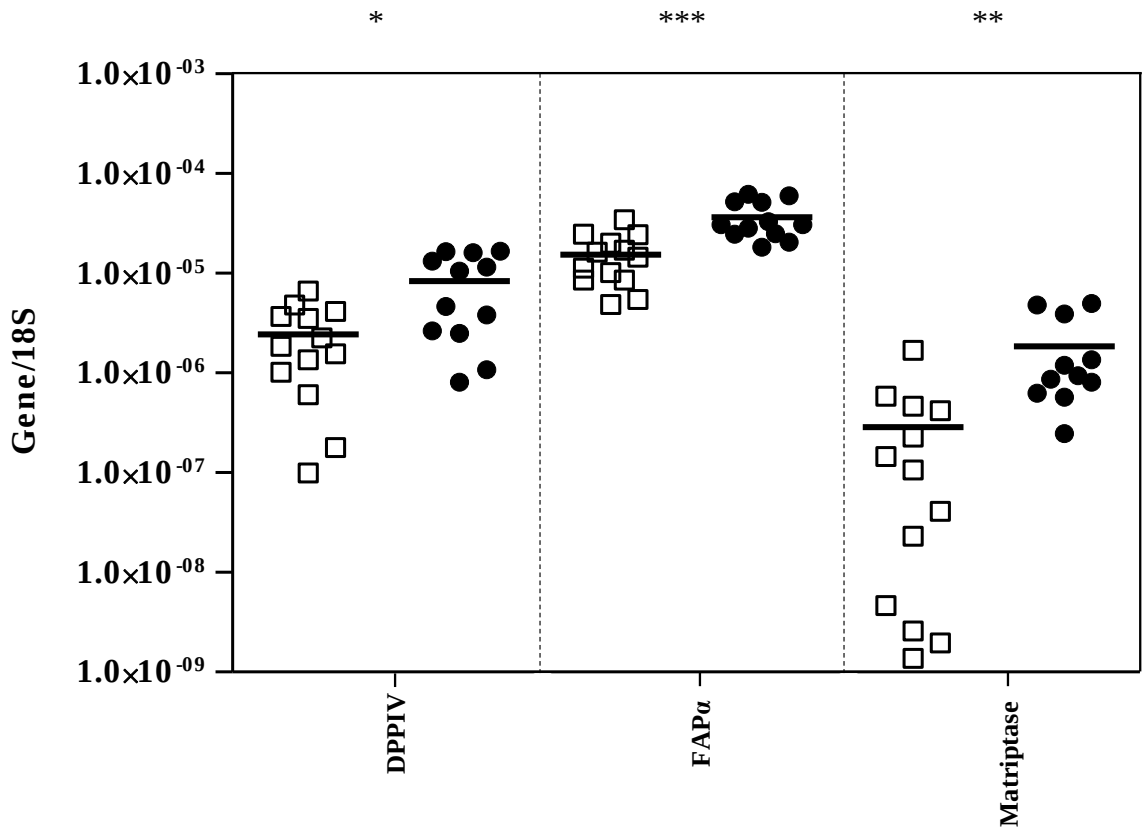


Figure 3.1 Expression of transmembrane serine proteases are elevated in OA cartilage

Gene expression levels in hip cartilage from patients with OA (closed circles; n = 13) or normal controls (open squares; n = 12) of DPPIV, FAPα and matriptase were determined by real-time PCR and normalised to the level of 18S rRNA. Significant differences between the normal and OA groups were determined using a two-sided Mann–Whitney U test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. Data generated by Dr J. M. Milner, Newcastle University, U.K.

FAPα (section 1.8.2) expression was originally thought to be highly restricted to sites of tissue remodelling, on the surface of reactive stromal fibroblasts or synoviocytes (Bauer et al., 2006), at sites of tumour invasiveness and not to be expressed in normal adult tissues (Rettig et al., 1993). However, expression of this enzyme has recently been detected on the cell surface of chondrocytes upon stimulation with IL-1 and OSM (Milner et al., 2006b). Additionally, when BNC was treated with IL-1+OSM, there was a strong correlation between FAPα mRNA expression and increasing collagen degradation (Milner et al., 2006b), suggesting a role for this enzyme in the pathology of inflammatory arthritis.

The β-propeller domain of FAPα plays a role in the interaction of the enzyme with other membrane bound proteins such as α3β1 integrin (Mueller et al., 1999, Zhang et al., 1999) as well as components of the plasminogen activator receptor (Artym et al., 2002). This association with other membrane bound proteins indicates that FAPα has the ability to modulate cellular functions via the recruitment of intracellular signalling molecules such as integrin proteins (Nakahara et al., 1998). Furthermore, there is a substantial body of evidence showing that FAPα associates with a large number of molecules on the cell surface (Kelly, 2005, Monsky et al., 1994, Artym et al., 2002). One study showed that FAPα can associate with uPAR at invadopodia, suggesting a co-operative role in ECM degradation and cellular invasion in cancer. Therefore, the rationale was that modulation of enzymatic FAPα activity in resorbing cartilage would alter collagen matrix degradation in cytokine-treated bovine cartilage.

The aims of this chapter were to:

- determine if FAPα gene expression is regulated by IL-1 and/or OSM in primary chondrocytes;
- examine if FAPα possesses type II collagenolytic or gelatinolytic activity;

- investigate if FAPα enzyme activity has a role in the breakdown of the collagen matrix in IL-1+OSM-treated cartilage, using inhibitors designed to target FAPα;
- assess if addition of exogenous active FAPα would influence IL-1+OSM-induced cartilage breakdown.

3.2 Results

3.2.1 Regulation of FAPα gene expression in primary chondrocytes

FAPα expression has been shown to be elevated in SW1353 cells upon IL-1 and/or OSM stimulation (Milner et al., 2006b). The SW1353 chondrosarcoma cell line has been used routinely in place of primary human chondrocytes since such cells can dedifferentiate when cultured in monolayer (Bonaventure et al., 1994). However, recent data suggest that SW1353 cells have limited potential as a model to study chondrocyte gene expression as long-term culture shows that they exhibit an epithelial phenotype (Gebauer et al., 2005). Therefore, the regulation of FAPα gene expression in primary cells was investigated in bovine nasal chondrocytes.

A modest increase of FAPα expression could be observed in IL-1+OSM-treated SW 1353 cells (Figure 3.2). In contrast to findings by Milner et al. (2006b), however, this increase was not significant. FAPα gene regulation by IL-1 and OSM was then assessed in two different populations of bovine nasal chondrocytes (Figure 3.3). The data were rather variable between the populations with no discernable trend. MMP-1 gene expression was tested in SW1353 and primary cells (data not shown) and synergistic induction upon IL-1+OSM stimulation was observed as previously reported (Barksby et al., 2006). These results highlight the differences between SW1353 and primary cells.

TGF-β1 has been shown to induce FAPα gene expression in a number of cell lines (Chen et al., 2009, Rettig et al., 1994). FAPα expression was also regulated by TGF-β1 in SW1353 cells (Figure 3.2). Whilst TGF-β1 did induce FAPα expression in SW1353 cells even in the presence of IL-1 and/or OSM (Figure 3.2), a similar trend was not observable in primary chondrocytes (Figure 3.3).

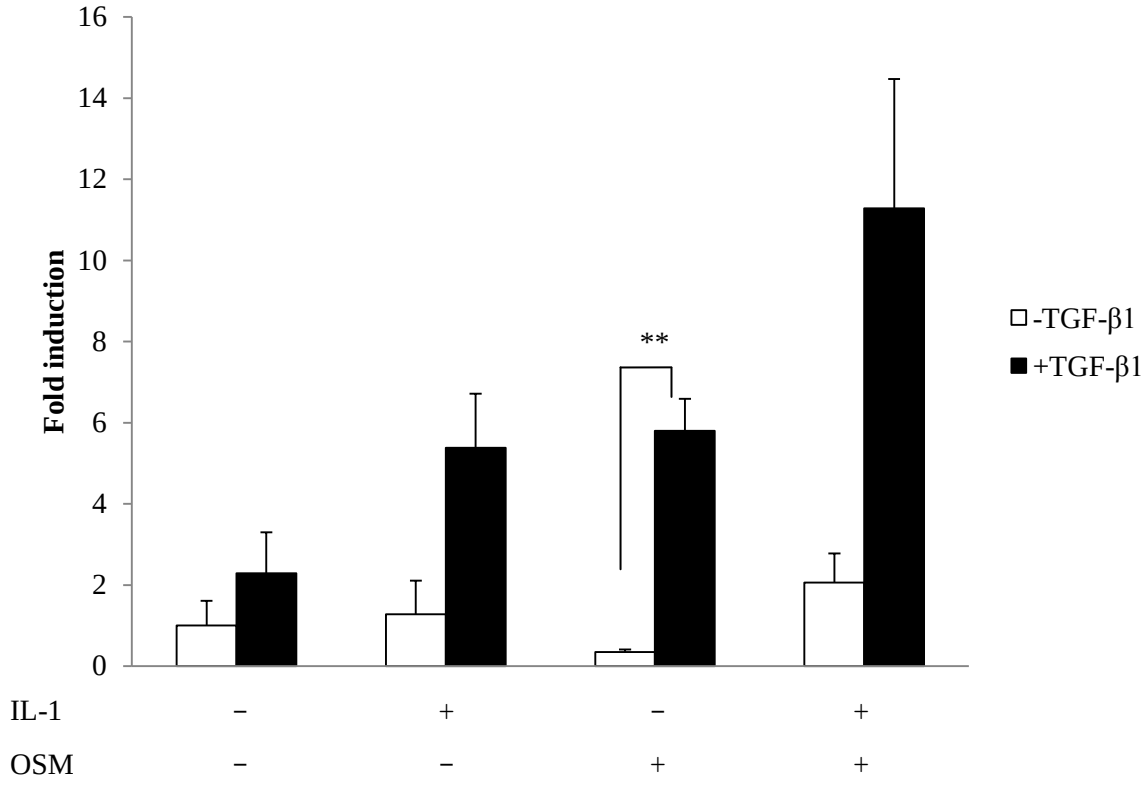


Figure 3.2 The regulation of FAPα gene expression in SW1353 chondrocyte cell line.

SW1353 cells were treated with combinations of IL-1 (1 ng/ml) and OSM (10 ng/ml) ± TGF-β1 (10 ng/ml) for 24 h. Total RNA was extracted and FAPα gene expression determined by real-time PCR and normalised to 18S rRNA levels. The data from two representative experiments are shown and presented as fold induction relative to control. Results are expressed as mean ± SEM (n=4). ** = $p < 0.01$.

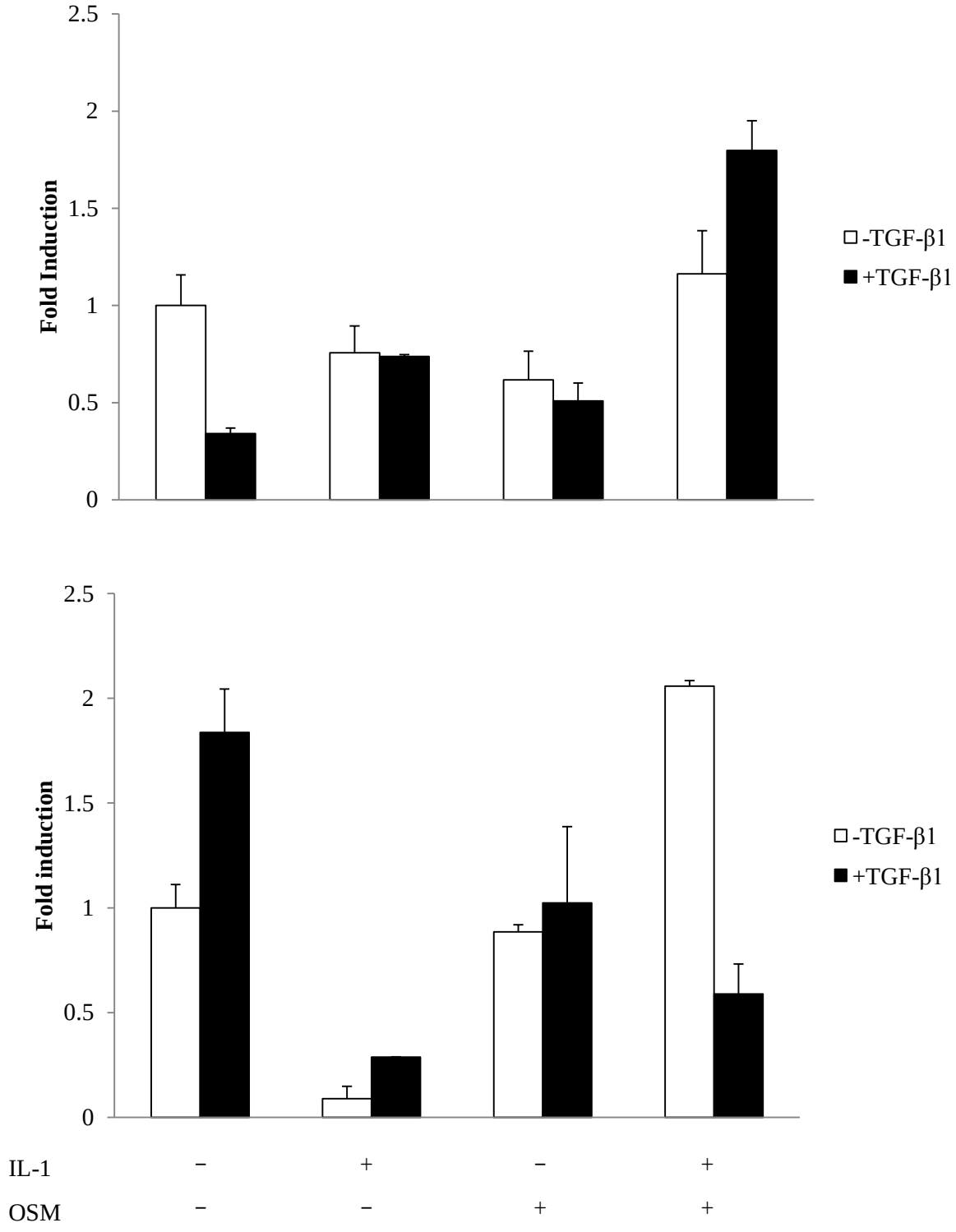


Figure 3.3 The regulation of FAPα gene expression in primary chondrocytes. Bovine nasal chondrocytes were treated with combinations of IL-1 and OSM ± TGF-β1 (10 ng/ml) for 24 h. Total RNA was extracted and FAPα gene expression determined by real-time PCR and normalised to 18S rRNA levels. The data from two experiments are shown and are presented as fold induction relative to control. Results are expressed as mean ± SEM (n=4).

3.2.2 Effect of FAPα enzyme activity on type II collagen

The hypothesis being tested in this experiment was that FAPα should contribute to matrix degradation by hydrolysis of denatured type II collagen. FAPα has been shown to have gelatinolytic activity (Levy et al., 1999) and as proteolysis is thought to occur in the pericellular compartment (Hollander et al., 1995), a transmembrane gelatinase could potentially expedite clearance of matrix proteins and therefore facilitate matrix degradation.

Type II collagen was extracted as described in section 2.5. To ensure that the type II collagen had retained its native triple helical state during the extraction, the collagen was treated with trypsin. The type II collagen extracted from human cartilage still retained the native triple helical conformation as trypsin could not cleave the native collagen band but did hydrolyse the denatured collagen (Figure 3.4, lanes 4 and 5). In the experiment shown, FAPα is only able to cleave denatured collagen and shows for the first time that FAPα can degrade denatured type II collagen (Figure 3.4, lane 7).

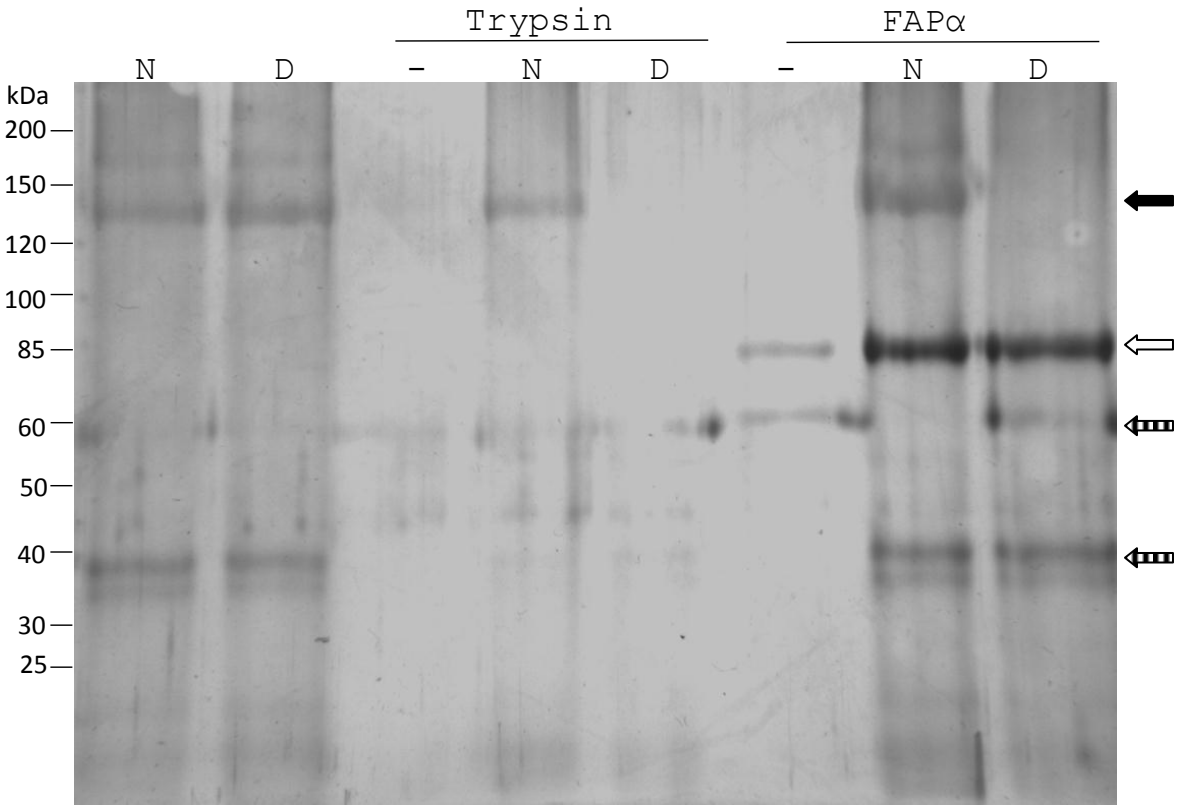


Figure 3.4 FAPα proteolysis of type II collagen.

Type II collagen (10 µg) from human articular cartilage was incubated at 37°C for 24 h in the presence or absence of proteases in 50 mM Tris-HCl, pH 7.6, 1 M glucose, 200 mM NaCl, 5 mM $CaCl_2$ buffer. Lane: (1) Native collagen (N); (2) Denatured collagen (D); (3) 4 µM trypsin; (4) Native collagen, 0.4 µM trypsin; (5) Denatured collagen, 0.4 µM trypsin; (6) 0.1 µM FAPα; (7) Native collagen, 0.4 µM FAPα; (8) Denatured collagen, 0.4 µM FAPα. Digests were reduced and analysed by 6.5 % SDS–PAGE. The closed arrow indicates the position of the intact α(II) chain of type II collagen. The open arrow indicates the position of FAPα. The patterned arrows indicate the presence of unknown low molecular weight contaminants. Results are representative of 2 independent experiments.

3.2.3 The effect of FAPα inhibitors on resorbing cartilage explants

The aim of these experiments was to determine if FAPα enzyme activity contributed to cartilage collagen breakdown in response to cytokine stimulation. Inhibitors specifically targeting FAPα were added to cytokine-treated BNC and collagen release measured.

3.2.3.1 Collagen release upon addition of FE999044 to resorbing nasal cartilages

In the experiment shown (Figure 3.5), cartilage treated with IL-1+OSM yielded 49.84 ± 14.21% collagen release, while the addition of 3 μM FE999044 yielded 50.23 ± 17.12% collagen release and the addition of 0.3 μM FE999044 yielded 60.72 ± 4.92% collagen release. The data show that there was no change in collagen release when resorbing cartilage was treated with FE999044.

Recombinant human FAPα was used to test if FE999044 had potential to inhibit the enzymatic activity of FAPα *in vitro*. While FE999044 showed no inhibitory ability against the endopeptidase activity of FAPα (Figure 3.6a), it showed a strong ability to inhibit its exopeptidase activity (Figure 3.6b). Furthermore, FE999044 showed no cross-reactivity to inhibit the enzymatic activity of recombinant DPPIV *in vitro* (Figure 3.7).

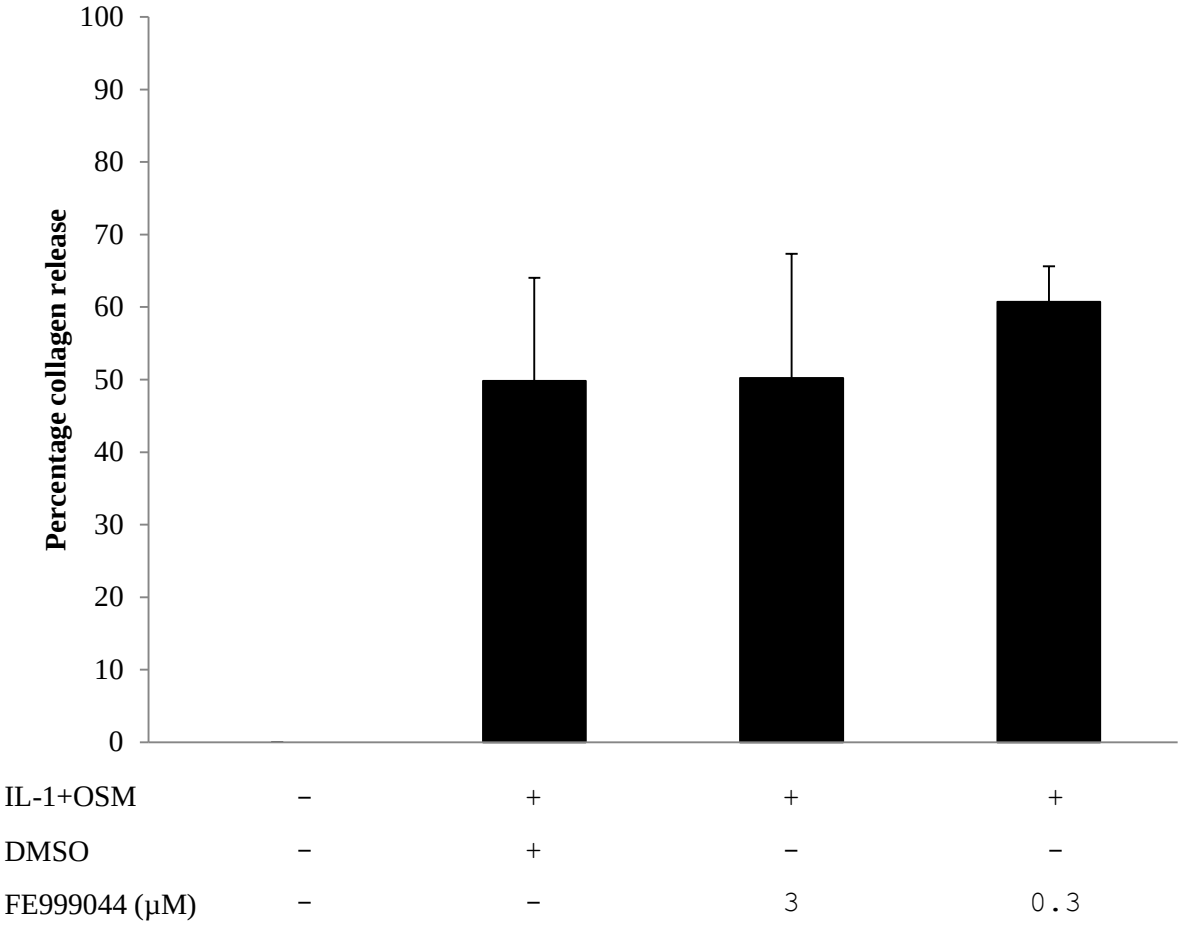


Figure 3.5 Effect of FE999044 on cartilage breakdown.

Bovine nasal cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml respectively) ± FE999044 or DMSO control. Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown are the cumulative collagen release (day 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 independent experiments.

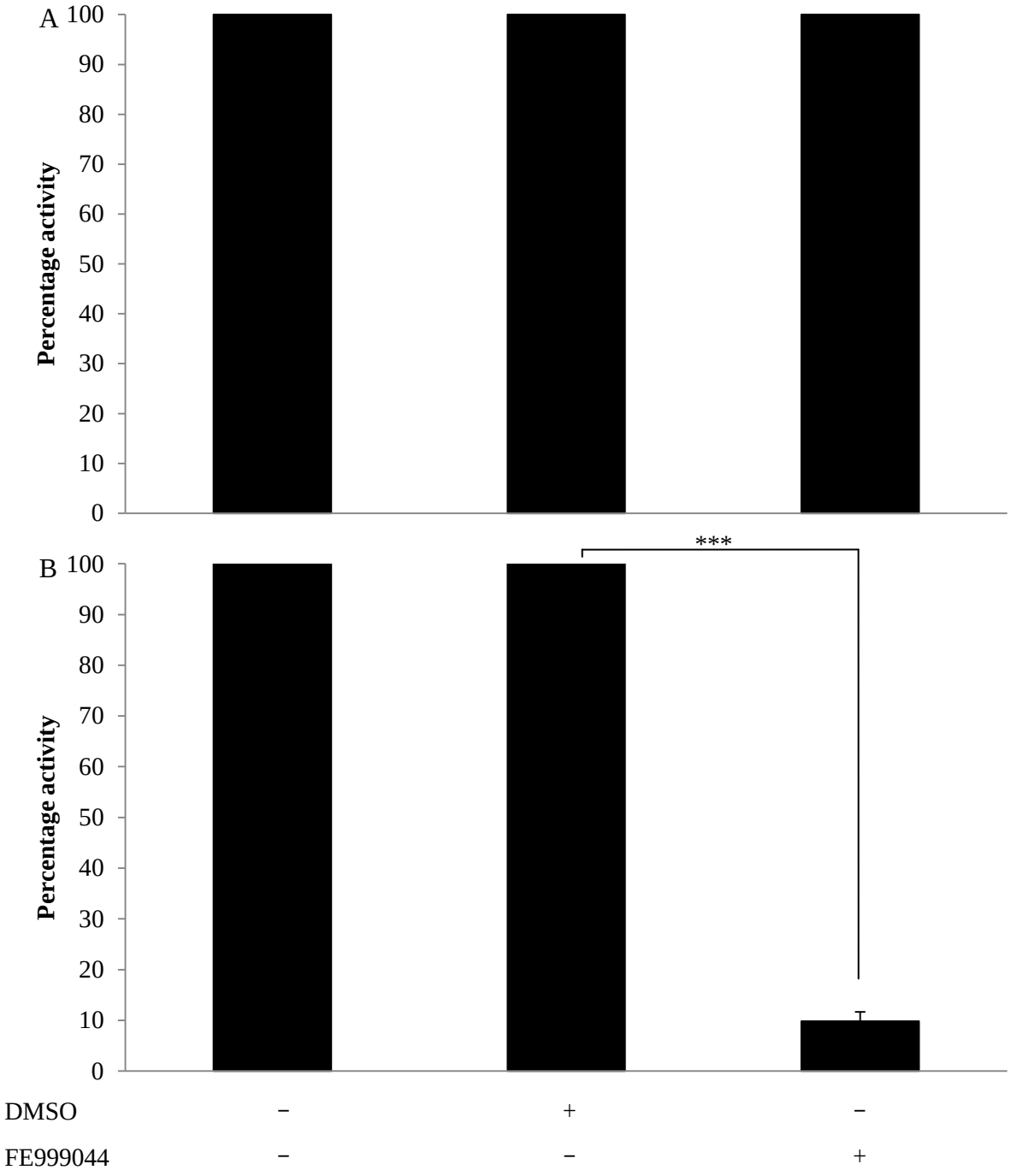


Figure 3.6 Effect of FE999044 on the enzymatic activity of FAPα

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either FE999044 (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 independent experiments. *** = $p < 0.001$ against DMSO.

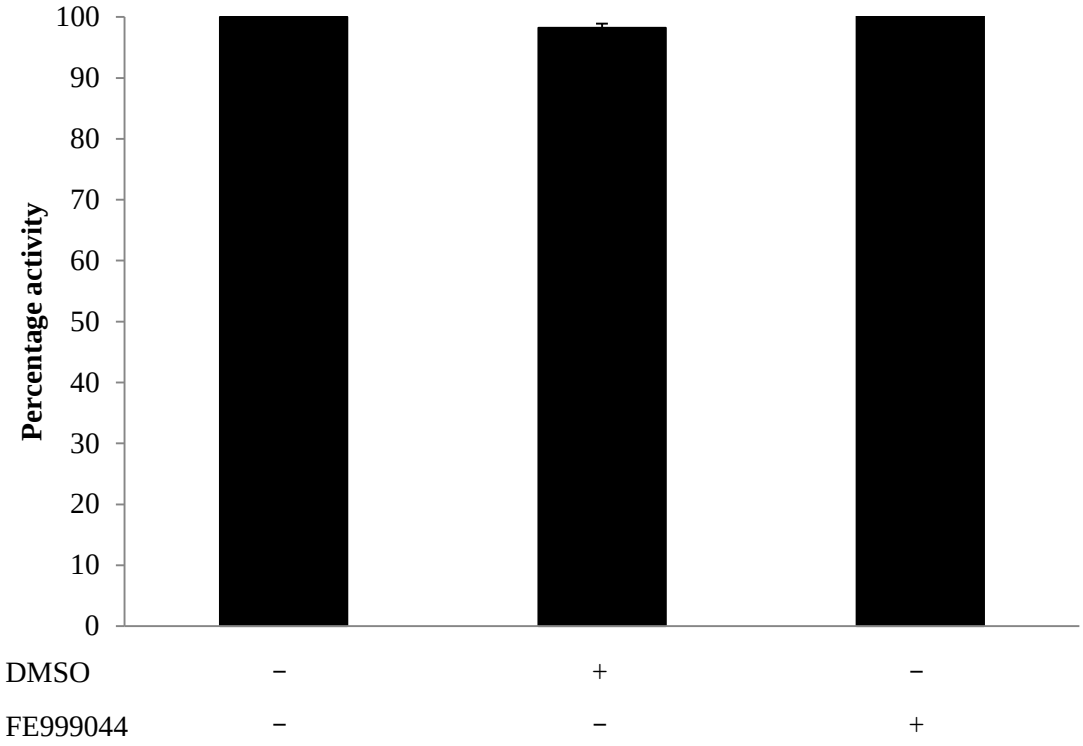


Figure 3.7 Effect of FE999044 on the enzymatic activity of DPPIV

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with either FE999044 (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 mins at 37°C. Substrate (50 µM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

3.2.3.2 Collagen release upon addition of Z-GP-DPi to resorbing cartilage

In the experiment shown (Figure 3.8), cartilage treated with IL-1+OSM and DMSO yielded 69.77 ± 7.73% collagen release. The addition of 150 µM Z-GP-DPi yielded 65.93 ± 5.71% collagen release. No change in collagen release could be observed when resorbing cartilage was treated with Z-GP-DPi.

It has been reported previously that dipeptide diphenylphosphonate inhibitors have short half-lives at physiological salt levels and 37°C (Lambeir et al., 1996). The activation of pro-collagenases in our bovine cartilage explant model is thought to occur around days 10 to 12 (Milner et al., 2006a). However, addition of Z-GP-DPi between days 8 to 12 inclusive showed no change in collagen release (Figure 3.9).

Recombinant human FAPα was used to test if Z-GP-DPi had potential to curtail the enzyme activity of FAPα *in vitro*. Z-GP-DPi showed no inhibitory ability against either the endopeptidase activity of FAPα (Figure 3.10a), or exopeptidase activity (Figure 3.10b). Additionally, Z-GP-DPi did not show any efficacy against recombinant DPPIV activity *in vitro* (Figure 3.11). Consequently, the use of Z-GP-DPi was discontinued as it exhibited no inhibitory potential.

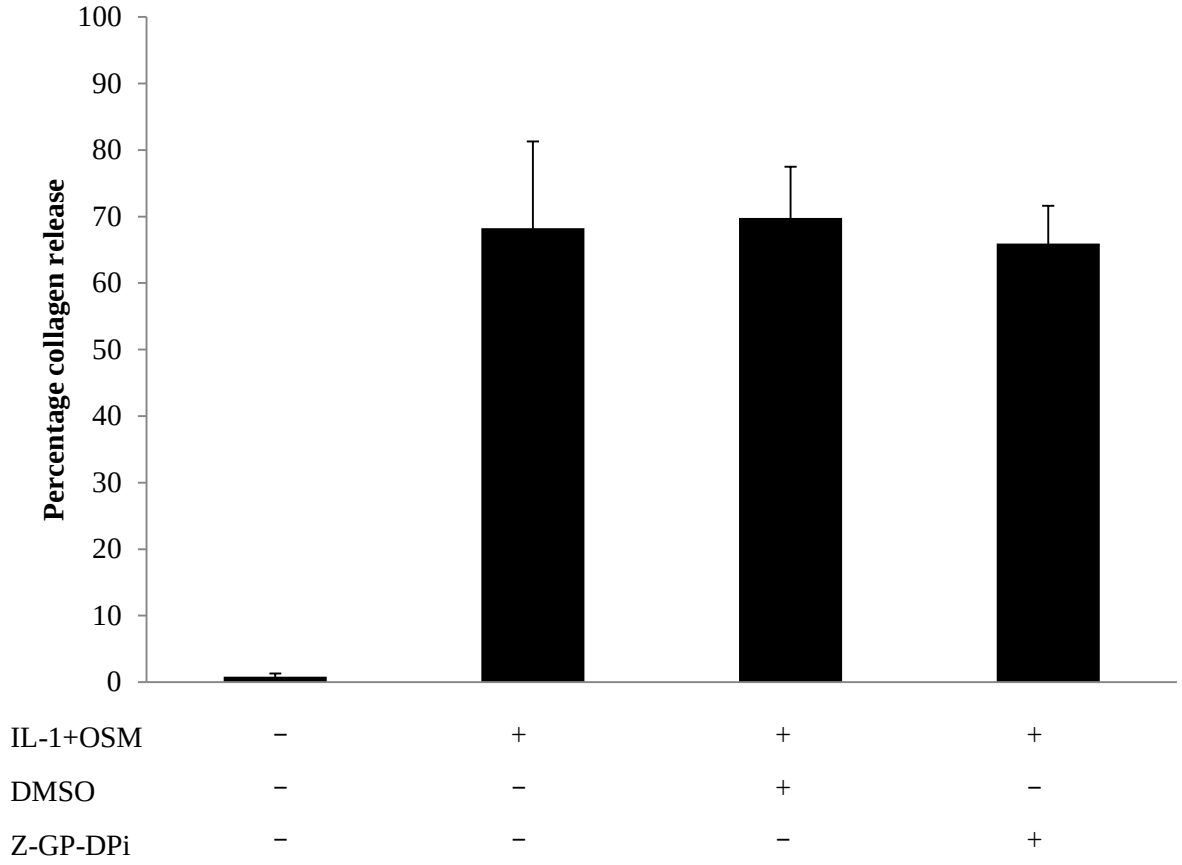


Figure 3.8 Effect of Z-GP-DPi on cartilage breakdown.

Bovine nasal cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively) ± Z-GP-DPi (150 µM) or DMSO control. Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown are the cumulative collagen release (days 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments.

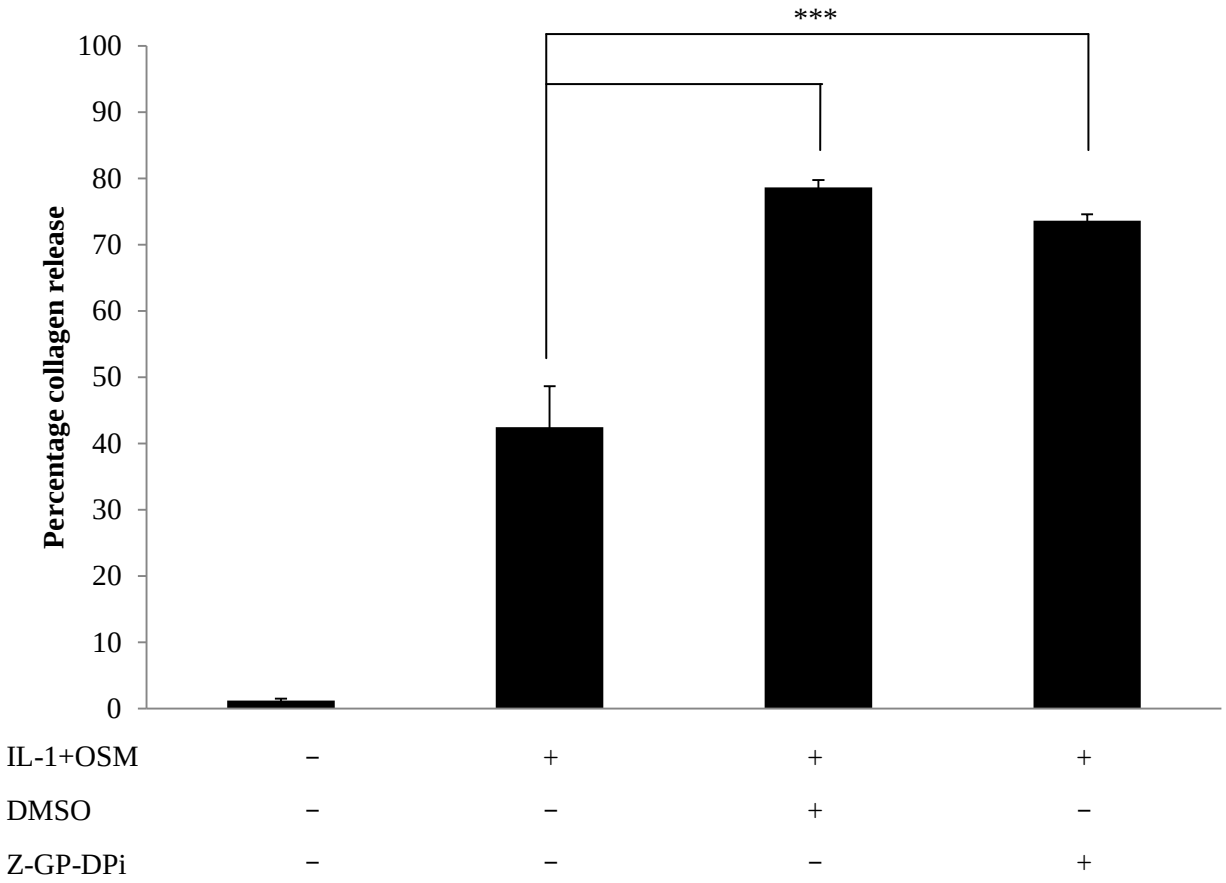


Figure 3.9 Multiple additions of Z-GP-DPi to cartilage show no protective effect.
Bovine nasal cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml respectively) ± Z-GP-DPi or DMSO control. Media were removed on day 7 and fresh reagents added, Z-GP-DPi (150 µM final concentration) was added to the cartilage between days 8 to 12 (inclusive). The experiment was ceased at day 14. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments. *** = $p < 0.001$ against IL-1+OSM alone.

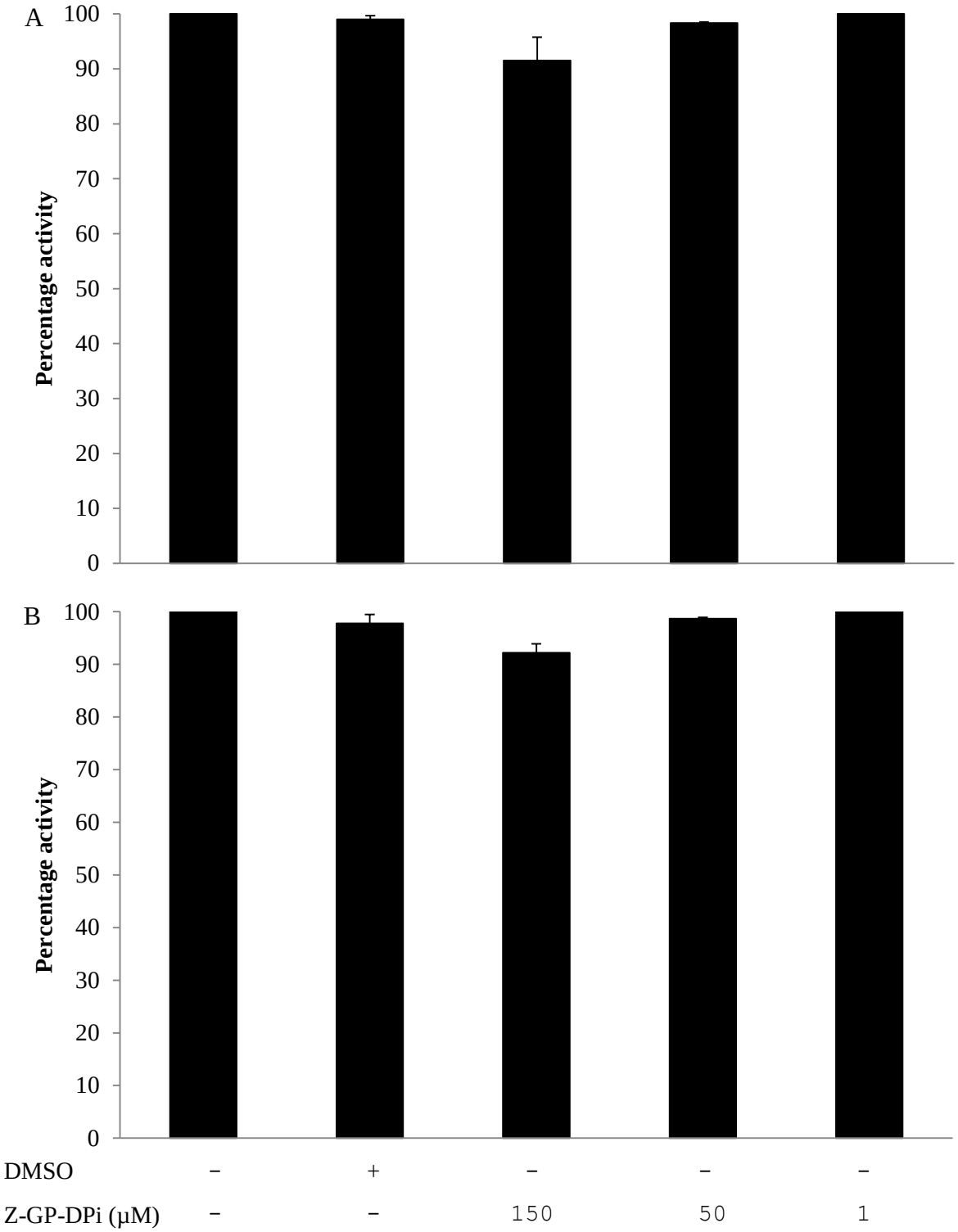


Figure 3.10 Effect of Z-GP-DPi on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either Z-GP-DPi or DMSO (0.1%) as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

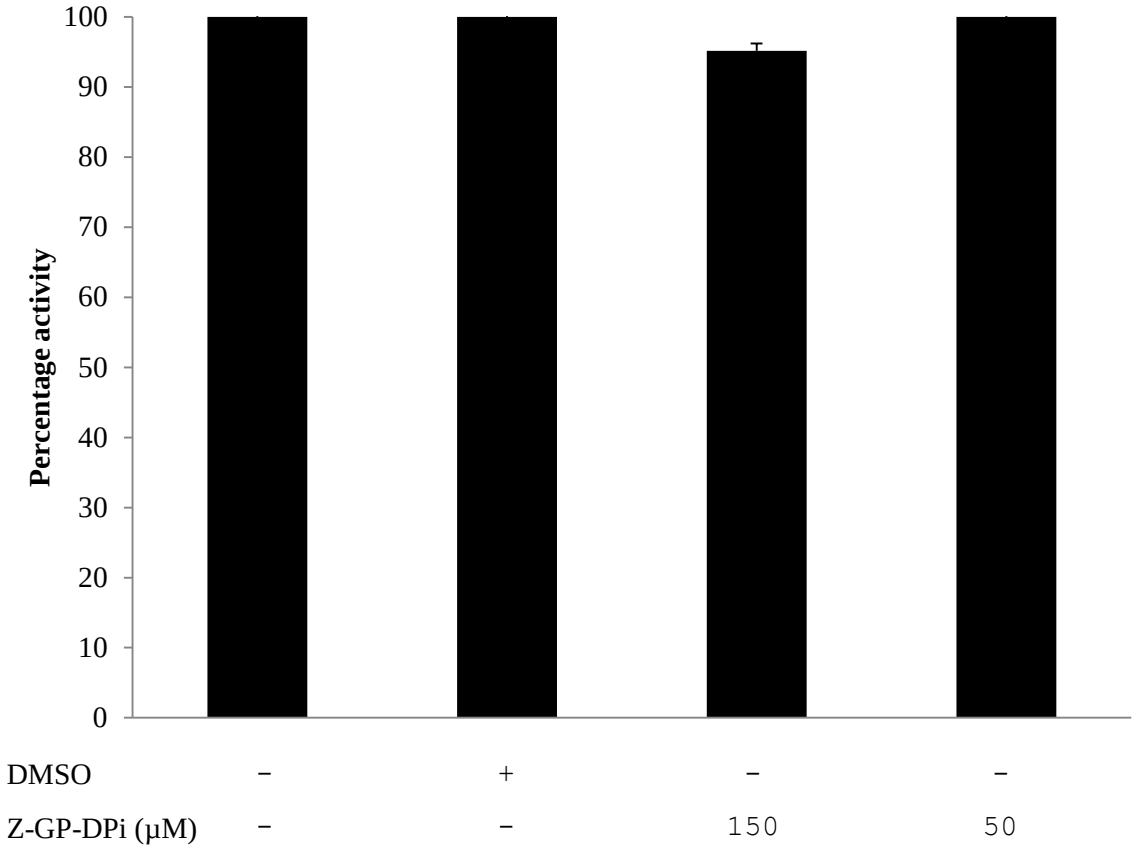


Figure 3.11 Effect of Z-GP-DPi on the enzymatic activity of DPPIV

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with either Z-GP-DPi or DMSO as a solvent control were incubated in assay buffer for 30 mins at 37°C. Substrate (50 µM) was then added to each well and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

3.2.3.3 Collagen release upon addition of UAMC-583 and -584 to resorbing bovine cartilage

In the experiment shown (Figure 3.12), cartilage treated with IL-1+OSM and DMSO yielded 77.55 ± 13.27% collagen release. Addition of 10 µM UAMC-583 and UAMC-584 yielded 78.91 ± 9.87% and 69.47 ± 11.62% collagen release, respectively. No change in collagen release could be detected when resorbing cartilage was treated with any of these FAPα inhibitors.

Recombinant human FAPα was used to test if any of the inhibitors had potential to reduce the enzymatic activity of FAPα *in vitro*. UAMC-583 and -584 showed significant inhibitory ability against the endopeptidase activity of FAPα (Figure 3.13a) but not exopeptidase activity (Figure 3.13b).

None of the FAPα inhibitors tested showed any affect against recombinant DPPIV activity *in vitro* (Figure 3.14).

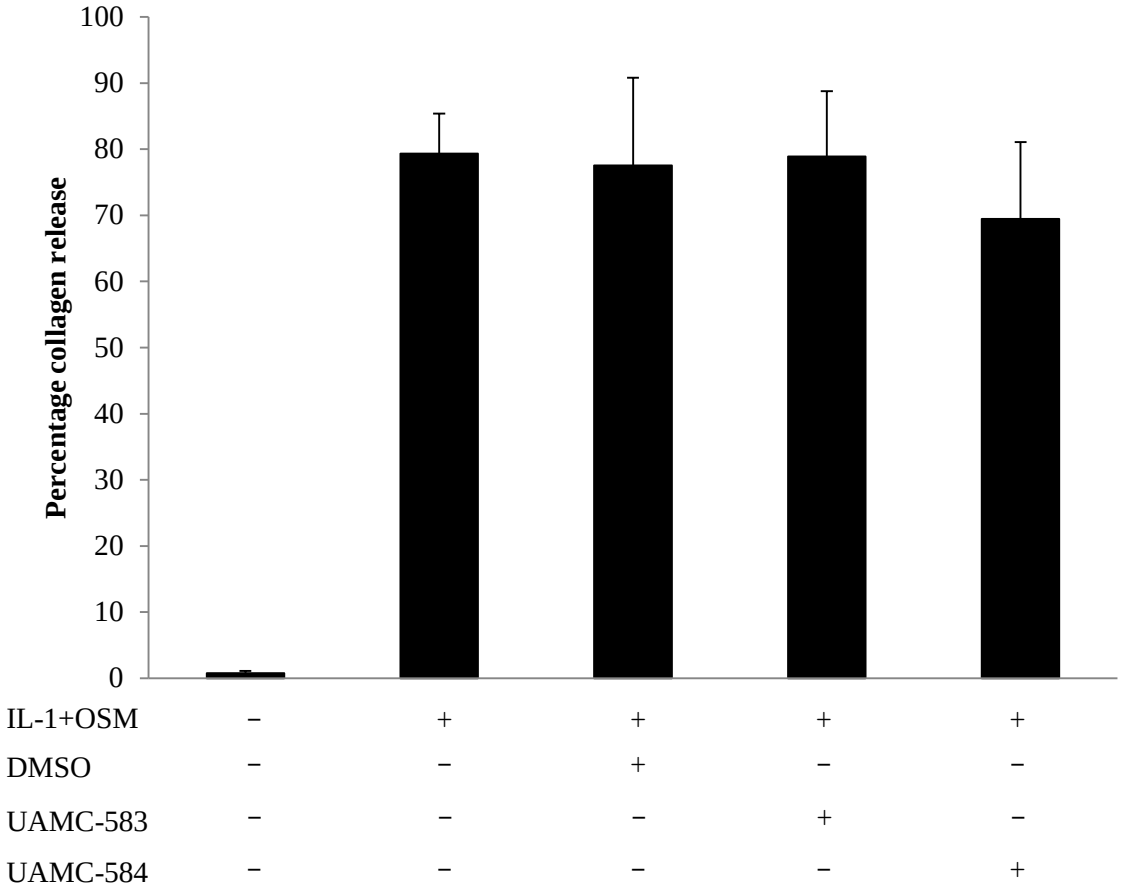


Figure 3.12 Effect of FAP*α* inhibitors on cartilage breakdown.

Bovine nasal cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml respectively). At days 0, 3, 7 and 10, the inhibitors UAMC-583 and -584 (10 µM) or DMSO control were added to cartilage. Media were removed on day 7 and fresh reagents added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments.

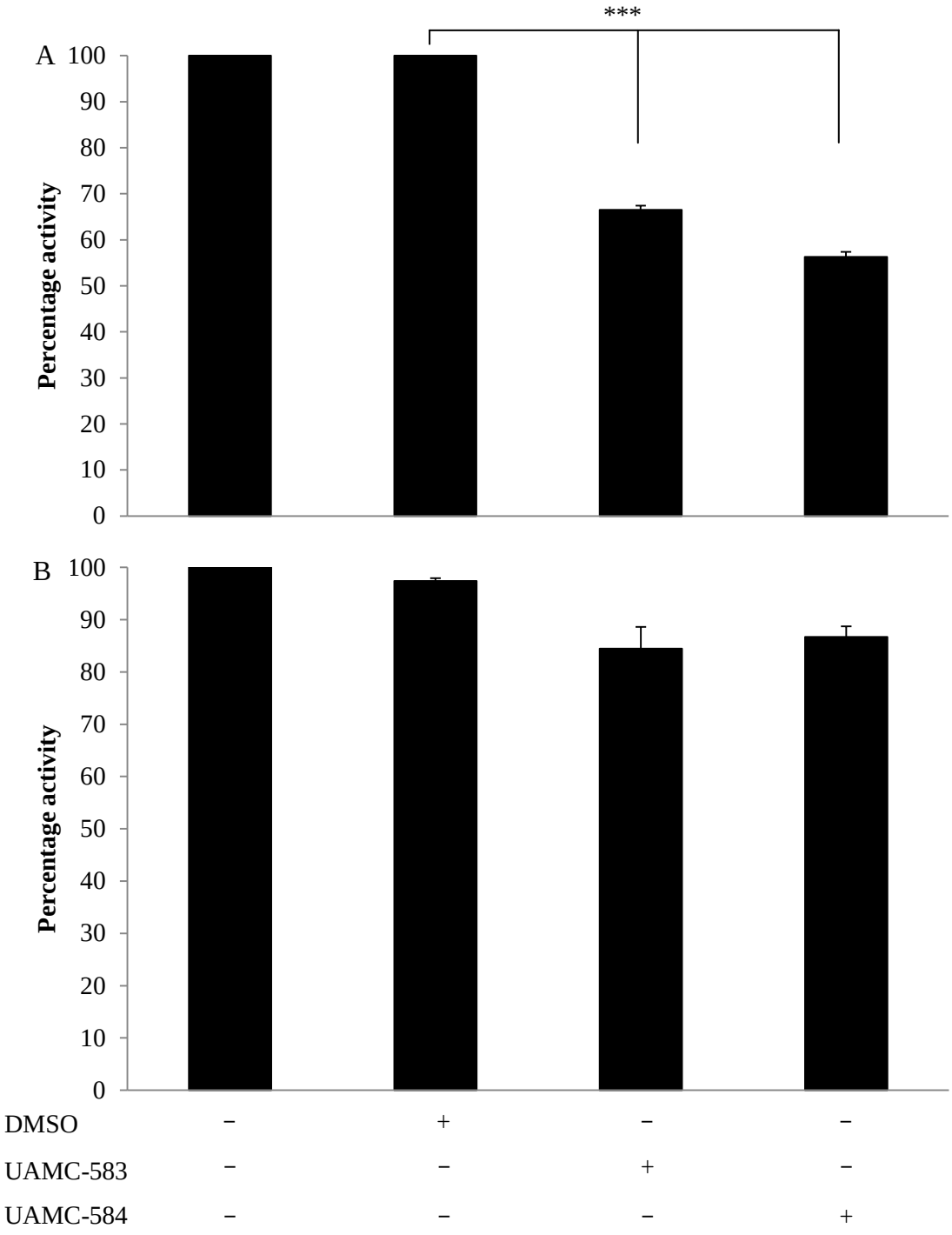


Figure 3.13 Effect of FAPα inhibitors on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either FAPα inhibitors (10 µM) or DMSO (0.1%) as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activities were calculated. Results are expressed as mean ± SD (n=3) and are representative of 2 separate experiments. *** = $p < 0.001$ against DMSO.

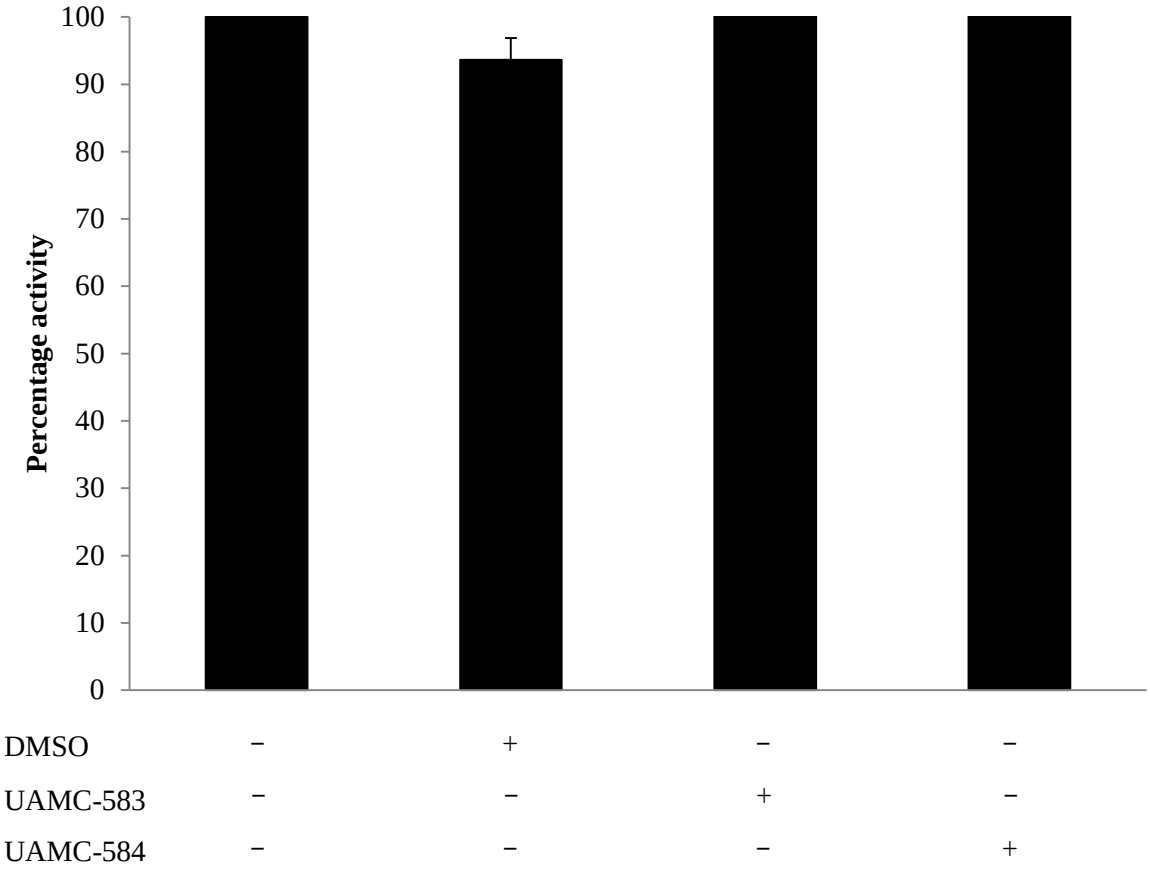


Figure 3.14 Modulation of DPPIV activity by FAPα inhibitors

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with FAPα inhibitors (10 μM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 μM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

3.2.4 Effect of FAPα on IL-1+OSM-treated bovine cartilage explant culture

The hypothesis being tested in this experiment was that, if FAPα has a catabolic role in cartilage breakdown, the addition of active FAPα should lead to higher collagen release compared to IL-1+OSM treated cartilage. Milner et al. (2006a) showed that in IL-1+OSM-treated cartilage the activation of pro-collagenases occurs around days 10 to 12. Data from our group have highlighted that factors that expedite collagen release from this model are best identified at a time point soon after we expect to begin seeing collagen release. Therefore, the effect of exogenous FAPα was assessed at day 12.

A modest decrease in collagen release could be observed with the addition of active FAPα compared to IL-1+OSM-treated bovine cartilage explants by day 12 (Figure 3.15). However, this decrease was not significant in either experiment.

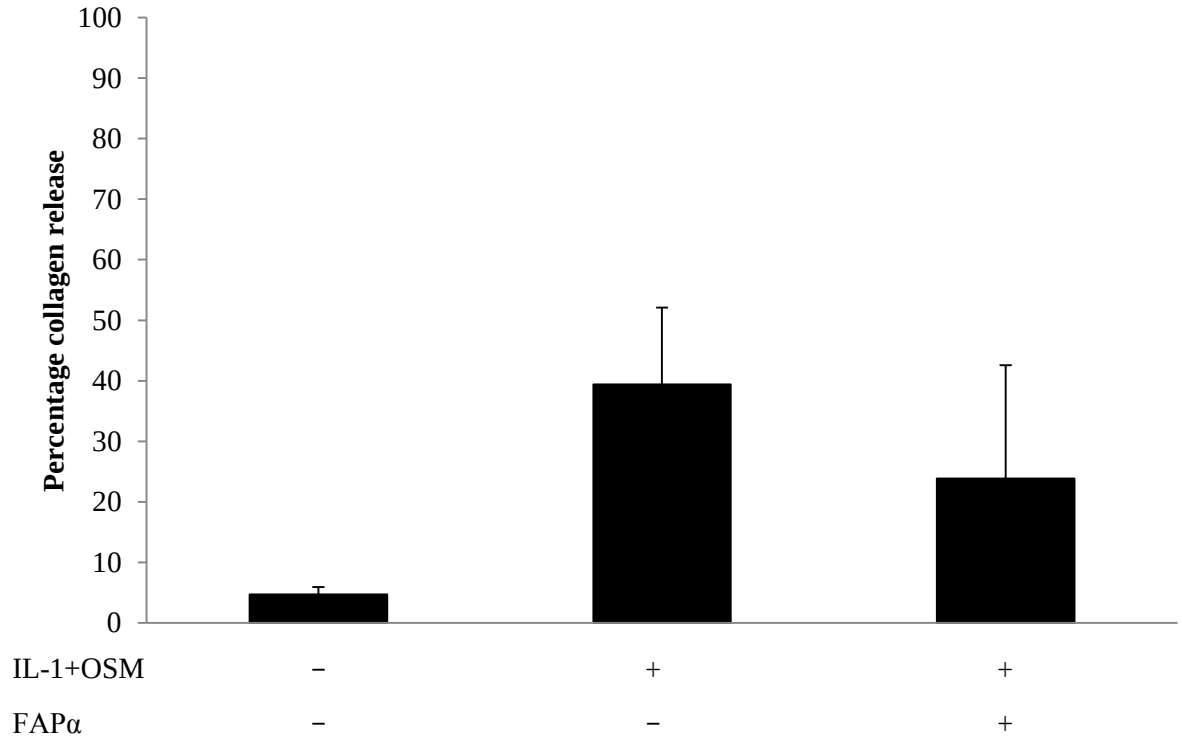


Figure 3.15 Effect of exogenous FAPα on cartilage breakdown.

Bovine nasal cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively). Media removed on day 7 and fresh media ± FAPα (100 nM) were added until day 12 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 12 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 12), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=6) and representative of 2 separate experiments.

3.3 Discussion

3.3.1 IL-1+OSM does not regulate FAPα gene expression in primary chondrocytes

Milner et al. (2006b) previously reported that FAPα gene expression was increased in OA cartilage and correlated with collagen release in IL-1+OSM-treated BNC. In addition, FAPα gene expression was detected in SW1353 chondrosarcoma cells although Gebauer et al. (2005) have described SW1353 chondrosarcoma cells to have limited potential as a model for gene expression in primary chondrocytes. The data presented here show that neither IL-1, OSM nor TGF-β1 regulate FAPα gene expression in primary bovine chondrocytes.

FAPα expression was shown to be up-regulated during inflammation in RA and OA synovial cells (Bauer et al., 2006) and in cirrhotic livers (Levy et al., 1999). However, there is little information on the regulation of FAPα gene expression in these pathologies. Recently, the promoter sequence of FAPα has been analysed and three transcription factor binding sites have been identified; HOXA4 (homeobox A4), E2F1 and EGR1 (early growth response protein 1) (Zhang et al., 2010). In this study, EGR1 knockdown showed a fifty percent decrease in FAPα expression, while HOXA4 and E2F1 knockdowns did not show any effect on gene expression. EGR1 has previously been demonstrated to be up-regulated in OA cartilage compared to phenotypically normal cartilage (Wang et al., 2000). However, the role of EGR1 is arthritis remains unclear as EGR1 has been shown to repress the cartilage matrix genes, such as Col2a1, upon stimulation by IL-1β (Tan et al., 2003) or TNF-α (Rockel et al., 2009) and EGR1 is required for maximal MMP-9 expression after TNF-α stimulation in carcinomas (Shin et al., 2010). Conversely, TGF-β1-stimulated fibroblasts show increased Col1a2 and this effect was dependent on enhanced EGR1 binding to the promoter sequence

(Chen et al., 2006b). The enhanced EGR1 binding upon TGF-β1 stimulation could account for the increased FAPα expression seen in SW1353 cells.

The data presented in this chapter show that FAPα gene expression is not regulated by IL-1 or OSM or TGF-β1 in primary chondrocyte culture. EGR1 was shown to be an early response gene to IL-1 stimulation in SW1353 chondrosarcoma cells (Vincenti and Brinckerhoff, 2001) and this would explain the result seen by Milner et al. (2006b). It is possible that EGR1 regulates FAPα gene expression in primary chondrocytes but EGR1 may not be the major transcription factor regulating FAPα expression as Zhang et al. (2010) showed only fifty percent reduction when EGR1 was knocked down, indicating other transcription factors may be important as well.

3.3.2 FAPα directly contributes to the degradation of denatured collagen

FAPα possesses endopeptidase ability (Aertgeerts et al., 2005, Lee et al., 2006, Levy et al., 1999, Edosada et al., 2006) and can cleave gelatin derived from type I collagen (Aggarwal et al., 2007) as well as other macromolecular proteins such as α_2-antiplasmin (Lee et al., 2004). Recently, Christiansen et al. (2007) showed that FAPα could cleave type I, III and IV collagen after the triple helix had been unwound by the action of MMPs. However, there was no report on the degradation of denatured type II collagen by FAPα until now, and alludes to the prospect that FAPα acts in concert with other proteases to potentiate pericellular cartilage degradation in OA (Hollander et al., 1995) or pannus invasion of the cartilage in RA (Lee and Weinblatt, 2001).

In fact, FAPα is known to associate with a number of proteases and signalling molecules, such as MMP-2, MMP-14, uPAR and integrins, at the invadopodia of malignant cancer cells (Monsky et al., 1994) and DPPIV on lung fibroblasts (Ghersi et al., 2002). It is possible that FAPα acts as a focal point to bring transmembrane proteins together in order to facilitate ECM degradation.

3.3.3 FAPα enzyme activity does not affect cartilage degradation

FAPα expression is usually increased in most malignant carcinomas (Garin-Chesa et al., 1990). FAPα inhibitors are being designed to address this therapeutic need in the field of cancer. A study by Edosada et al. (2006) highlighted that by utilising the differences in substrate specificity between DPPIV and FAPα, specific inhibitors could be produced. The authors showed that N-blocked-boroProline derivatives exhibited high specificity for FAPα over DPPIV (Edosada et al., 2006, Tran et al., 2007). A second class of inhibitors, the dipeptide-derived diphenyl phosphonates have also been designed to target both FAPα and DPPIV (Lambeir et al., 1996). Unlike the boroProline inhibitors, modulating the N-terminal amino acids of these inhibitors did not affect the second order rate constant of inactivation of FAPα (Gilmore et al., 2006).

So far the clinical use of FAPα inhibitors alone to treat carcinomas has shown little promise in clinical trials (Narra et al., 2007). This study was carried out using patients with metastatic colorectal carcinoma and highlighted that FAPα inhibition at earlier stages of the disease may have greater beneficial effects. However, two further studies using the same inhibitor in concert with traditional treatments showed no additional effect over the traditional treatment alone (Eager *et al*. 2009a, b).

Complementary to the inhibitors, FAPα antibodies are being trialled to treat disease progression in advanced FAPα positive cancers (Scott et al., 2003, Tahtis et al., 2003). The FAPα antibodies used in these studies were shown to have minimal uptake into normal tissues after infusion. Furthermore, they showed no adverse side effects in the majority of the patients in the clinical trial. The long-term effects from targeting FAPα in cancer are still not known yet. The role of FAPα in the tumour microenvironment is complicated as FAPα positive stromal fibroblasts in the tumour environment are associated with increased survival (Ariga et al., 2001). However, an *in vivo* mouse

model of human breast cancer showed that FAPα expressing breast carcinoma cells actually increase tumour growth and microvessel density (Huang et al., 2004).

Using FAPα specific inhibitors in IL-1+OSM-treated BNC did not diminish collagen release. All of the inhibitors used here, except Z-GP-DPi, did abrogate either the endopeptidase or exopeptidase activity of FAPα. However, without detailed information about the chemical structures of these FAPα specific inhibitors, no conclusions can be drawn as to why these inhibitors could not inhibit both activities. The active site of FAPα and DPPIV differs by one amino acid and this change affects the preferred activity of the enzyme. Aertgeerts et al. (2005) showed that Ala-657 in the active site of FAPα favours endopeptidase activity over dipeptidyl peptidase activity as uncharged residues are preferred in the P2 pocket. Steric hindrance between the inhibitor and the side chain of Ala-657 could result in a relatively weak binding of the inhibitor to the active site of FAPα. Consequently, if the substrate has a higher binding affinity to the active site then the inhibitor could be displaced from the active site, and explain why the inhibitors had no effect *ex vivo*.

Addition of soluble recombinant FAPα did not influence cartilage degradation suggesting that FAPα enzyme activity does not play a role in the degradation of IL-1+OSM-treated bovine cartilage. There is the possibility that the β-propeller domain of FAPα plays a greater role than the catalytic domain in the context of cartilage degradation. Monsky *et al* (1994) showed that FAPα was located at the invadopodia of melanoma cells and co-localised with a large number of other molecules, such as MMP-2, MMP-14, uPAR and integrins. Artym et al. (2002) went on to show that FAPα and uPAR formed a complex on the surface of melanoma cells and that this was dependent on β_1-chain integrin and the cytoskeleton. Furthermore, the β-propeller domain of FAPα has high homology to the β-propeller region of α_3-chain integrin (Kelly, 2005) and this

domain of the α_3-chain binds uPAR (Zhang et al., 2003). Articular chondrocytes predominantly express α_5 and β_1 integrin chains with α_1 and α_3 chains found at lower levels (Salter et al., 1992). Therefore, there is the distinct possibility that FAPα, in conjunction with integrins, could form large complexes at the chondrocyte membrane (Figure 3.16) and the main focal point of this hypothetical complex would be matrix degradation. However, these complexes could actually influence cell signalling as uPAR has been shown to influence signalling in conjunction with integrins and GPCRs (Binder et al., 2007). uPAR has also been shown to activate the JAK/STAT pathways in a kidney epithelial tumour cell line (Koshelnick et al., 1997) and this is thought to be mediated by the gp130 receptor. Data from our group show that activation of the gp130 receptor, by OSM or IL-6 (in the presence of soluble IL-6 receptor), in cartilage can synergise with IL-1 and promote cartilage matrix degradation (Rowan et al., 2001). This suggests that FAPα may act more as a scaffold rather than a protease in terms of cartilage degradation.

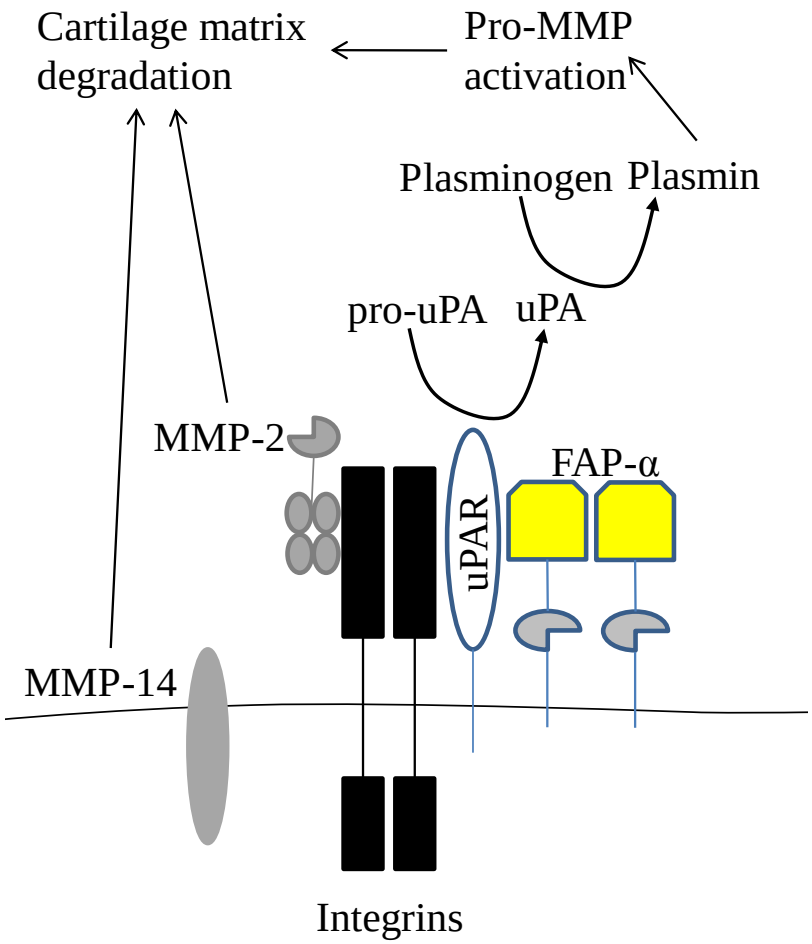


Figure 3.16 Model of a putative FAPα super-complex on the chondrocyte cell surface

FAPα forms complexes with uPAR (Artym et al., 2002) and through this binding comes into close proximity with $\alpha_3\beta_1$ integrin (Zhang et al., 2003). This close association is likely to allow FAPα to associate with ECM proteins (Takada et al., 2007). MMP-2 has been shown to be closely associated with FAPα at the invadopodia of melanoma cells and MMP-14 is also postulated to be located there (Monsky et al., 1994).

3.4 Conclusion

Milner et al. (2006b) previously reported that FAPα gene expression was increased in OA cartilage and correlated with collagen release in IL-1+OSM-treated BNC. The correlation of FAPα expression and collagen release suggested that FAPα had a catabolic role in cartilage matrix homeostasis. However, FAPα specific inhibitors have not corroborated this hypothesis as there is no change in collagen release observed with any of the inhibitors used (section 3.2.3). In fact, all the inhibitors, except Z-GP-DPi, utilised in this chapter were only able to inhibit either the endopeptidase activity or exopeptidase activity of FAPα, but not both.

The cleavage of denatured type II collagen by FAPα does suggest that FAPα has a catabolic role in the degradation of the cartilage matrix (section 3.2.2). However, there is evidence for an anabolic role of FAPα as it has similar dipeptidyl peptidase activity as DPPIV. DPPIV cleavage of chemokines has been shown to decrease the inflammatory response (Table 1.2) and FAPα is likely to cleave the same chemokines, although this has yet to be confirmed. Taken together, the up-regulation of FAPα in OA is likely to have an anabolic effect with the cleavage of chemokines to dampen the inflammatory response and ultimately decrease MMP levels. Whilst the cleavage of denatured type II collagen is a catabolic process, this could be occurring to stimulate remodelling and repair of the surrounding matrix. As β1 integrin signalling has been shown to occur at the invadopodia of LOX cells (Nakahara et al., 1998), and this it has been previously shown that this is where FAPα localises to (Monsky et al., 1994).

3.5 Summary

- FAPα gene expression is not regulated by IL-1, OSM or TGF-β1 in primary chondrocytes.

- FAPα is able to cleave denatured type II collagen.
- FAPα inhibitors targeting enzyme activity do not alter cartilage matrix degradation in the presence of IL-1+OSM.
- Exogenous active FAPα does not expedite collagen breakdown from IL-1+OSM-treated bovine cartilage.

CHAPTER 4

Studying the role of DPPIV in cartilage homeostasis

4.1 Introduction

Recently, DPPIV (section 1.8.1) expression was found to be significantly increased in OA compared to normal cartilage (Figure 3.1) (Swingler et al., 2009). This corroborates an earlier finding that DPPIV is expressed on the surface of OA chondrocytes (Lapadula et al., 1995) and expression is decreased in areas where there is progressive worsening of the osteoarthritic lesions. Collagen-induced arthritis (CIA) in $DPPIV^{-/-}$ mice was found to be more severe than in wild-type $DPPIV^{+/+}$ mice (Busso et al., 2005). This down-regulation of DPPIV expression has important consequences on the joint environment as DPPIV is known to modulate the function of a number of inflammatory cytokines and chemokines (Table 1.2).

Inhibition of DPPIV activity decreases pathogenesis in a CIA model (Tanaka et al., 1997). At first these data appear to be contradictory to substrate data for DPPIV stated above, as DPPIV activity on inflammatory chemokines and neuropeptides modulates their function to cause a decrease in immune cell function and localisation to the joint space. However, data have shown that DPPIV inhibitors play a role in the up-regulation of TGF-β1 secretion (Reinhold et al., 2006) and this would lead to a decrease in T cell activity due to its immunosuppressive and chondroprotective (section 1.3.3) functions.

As stated above DPPIV is involved in a number of immune cell functions and this inhibition could prevent a number of different pathways within the immune system that could cause this anti-arthritic effect.

In RA, DPPIV activity is shown to decrease in both the synovial membrane and fluid of patients (Gotoh et al., 1989, Kamori et al., 1991). Furthermore, there is a decrease in the serum levels of DPPIV that is inversely proportional to the degree of inflammation experienced in the joint (Busso et al., 2005), although recent data also show that DPPIV activity is actually higher in RA synovium compared to OA (Solau-Gervais et al., 2007). However, the authors used homogenised synovium extracts to test for DPPIV activity and as a large number of T cells are shown to infiltrate the tissue in RA (section 1.2.1), this finding is not unexpected as T cells are known to express high levels of DPPIV (Sedo et al., 2005).

While previous data show that DPPIV levels decrease inversely to disease progression, but there are little data as to the role of DPPIV in cartilage. Therefore, the aim of this chapter was to elucidate the function DPPIV enzymatic activity plays in cytokine treated bovine cartilage.

The aims of this chapter were to:

- determine if DPPIV gene expression is regulated by IL-1 and/or OSM in primary chondrocytes;
- investigate if DPPIV enzyme activity has a role in the breakdown of the collagen matrix in IL-1+OSM treated cartilage using inhibitors designed to target DPPIV;
- examine if DPPIV possesses type II collagenolytic or gelatinolytic activity;
- assess if addition of active DPPIV to IL-1+OSM-treated bovine cartilage would affect cartilage breakdown.

4.2 Results

4.2.1 Regulation of DPPIV gene expression in SW1353 cells and primary chondrocytes

DPPIV has been shown to be expressed on OA chondrocytes and expression is decreased in areas where there is progressive worsening of the osteoarthritic lesions (Lapadula et al., 1995). DPPIV knock-out mice with CIA have increased disease severity compared to wild-type mice (Busso et al., 2005).

In SW1353 cells, stimulation with IL-1 and/or OSM caused an increase in DPPIV gene expression, and treatment with TGF-β1 in the presence of these pro-inflammatory cytokines significantly decreased DPPIV gene expression (Figure 4.1). However, SW1353 chondrosarcoma cells have limited potential as a model for chondrocyte gene expression, as mentioned previously (section 3.2.1). Therefore, the regulation of DPPIV gene expression was investigated in primary bovine chondrocytes to see if the result from SW1353 cells could be corroborated.

MMP-1 gene expression was tested in SW1353 and primary cells (data not shown) and synergistic induction upon IL-1+OSM stimulation was observed as previously shown (Barksby et al., 2006). However, DPPIV gene expression in bovine chondrocytes was considerably varied between populations (Figure 4.2). While, one population of bovine chondrocytes showed similar DPPIV gene expression upon pro-inflammatory stimulation (Figure 4.2a), however the other population showed little difference (Figure 4.2b). The effect of TGF-β1 alone on DPPIV gene expression showed a similar variation between the different populations, as one population showed a decrease comparable to SW1353 cells (Figure 4.2b) but the other an increase (Figure 4.2a). Therefore, the results seen with SW1353 cells were not reproducible in primary bovine chondrocyte cultures and highlight the differences between SW1353 and primary cells.

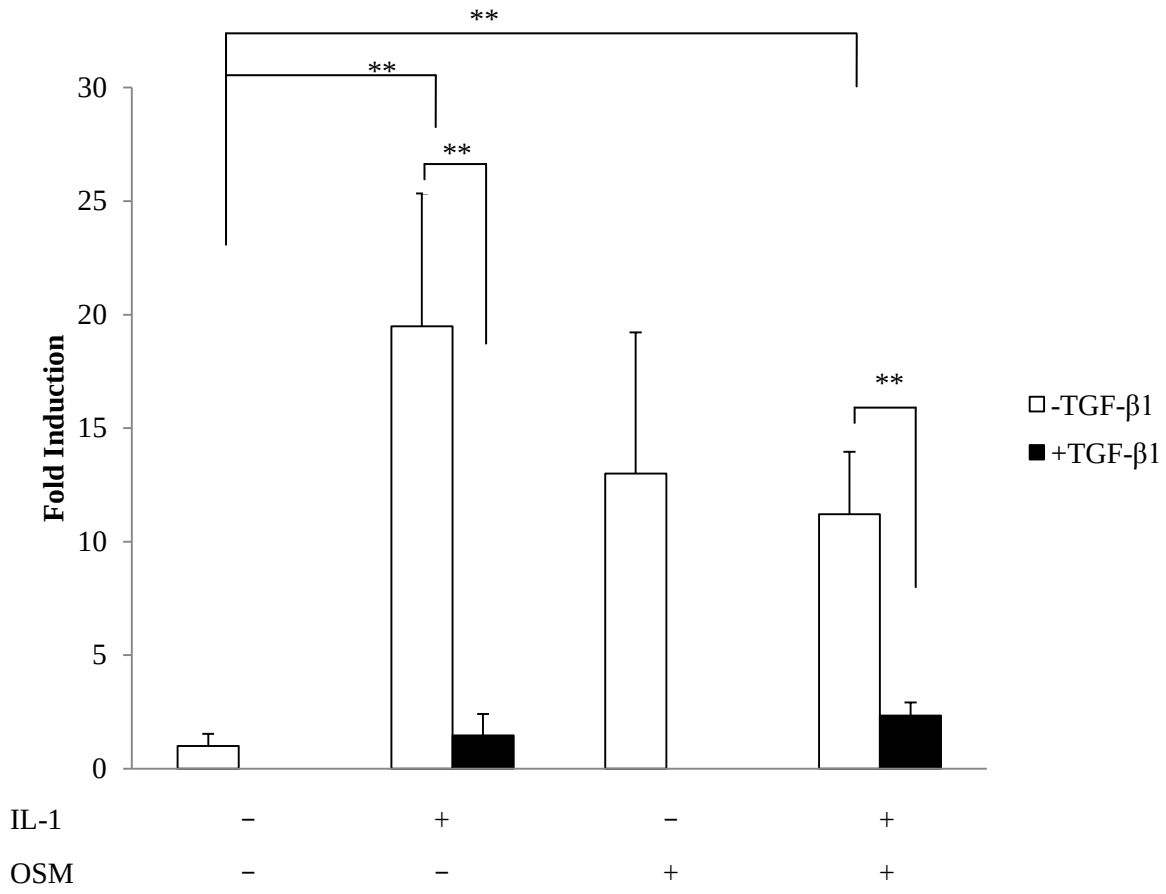


Figure 4.1 The regulation of DPPIV gene expression in SW1353 chondrocyte cell line.

SW1353 cells were treated with combinations of IL-1 (1 ng/ml) and OSM (10 ng/ml) ± TGF-β1 (10 ng/ml) for 24 h. Total RNA was extracted and DPPIV gene expression determined by real-time PCR and normalised to 18S rRNA levels. The data from two representative experiments are shown and presented as fold induction relative to control. Results are expressed as mean ± SEM (n=5). ** = $p < 0.01$

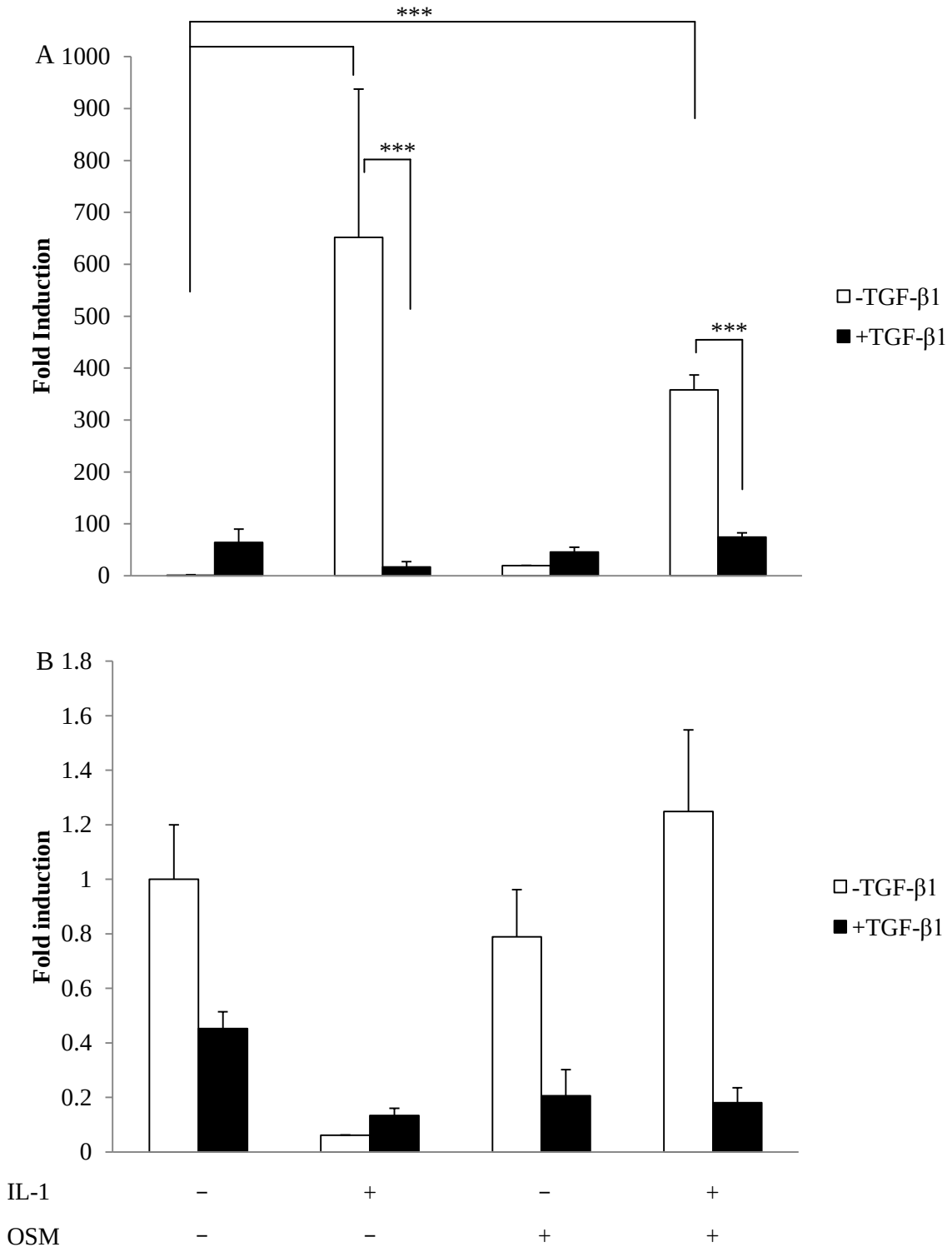


Figure 4.2 The regulation of DPPIV gene expression in primary chondrocytes. Bovine chondrocyte cells were treated with combinations of IL-1 (1 ng/ml) and OSM (10 ng/ml) ± TGF-β1 (10 ng/ml) for 24 h. Total RNA was extracted and DPPIV gene expression determined by real-time PCR and normalised to 18S rRNA levels. The data from two representative experiments are shown and presented as fold induction relative to control. Results are expressed as mean ± SEM (n=5). *** = $p < 0.001$.

4.2.2 The effect of DPPIV inhibitors on resorbing cartilage explants

To determine if DPPIV activity plays a role in cartilage matrix resorption, DPPIV inhibitors were added to IL-1+OSM-treated bovine cartilage and collagen release was measured. DPPIV inhibitors are now widely used in the clinic to treat type II diabetes and indicate that patients show good tolerance to all these compounds (Yazbeck et al., 2009 and references therein). However, there are no data highlighting the impact that DPPIV inhibitors would play in resorbing cartilage.

4.2.2.1 Collagen release upon addition of FE999011 to resorbing cartilage

In the experiment shown (Figure 4.3), cartilage treated with IL-1+OSM yielded 79.16 ± 8.86% collagen release. The addition of DMSO did decrease collagen release in this experiment, but this result is not reproducible between different cartilages (Prof. A. D. Rowan, personal communication). Addition of FE999011 over a range of concentrations resulted in no significant change in collagen release.

Recombinant human DPPIV was used to test if FE999011 had potential to inhibit the enzymatic activity *in vitro*. FE999011 showed a strong ability to inhibit activity (Figure 4.4) and therefore shows that the compound was still active when added to the cartilage explants. Furthermore, FE999011 showed no cross-reactivity to inhibit the enzymatic activity of recombinant FAPα *in vitro* (Figure 4.5).

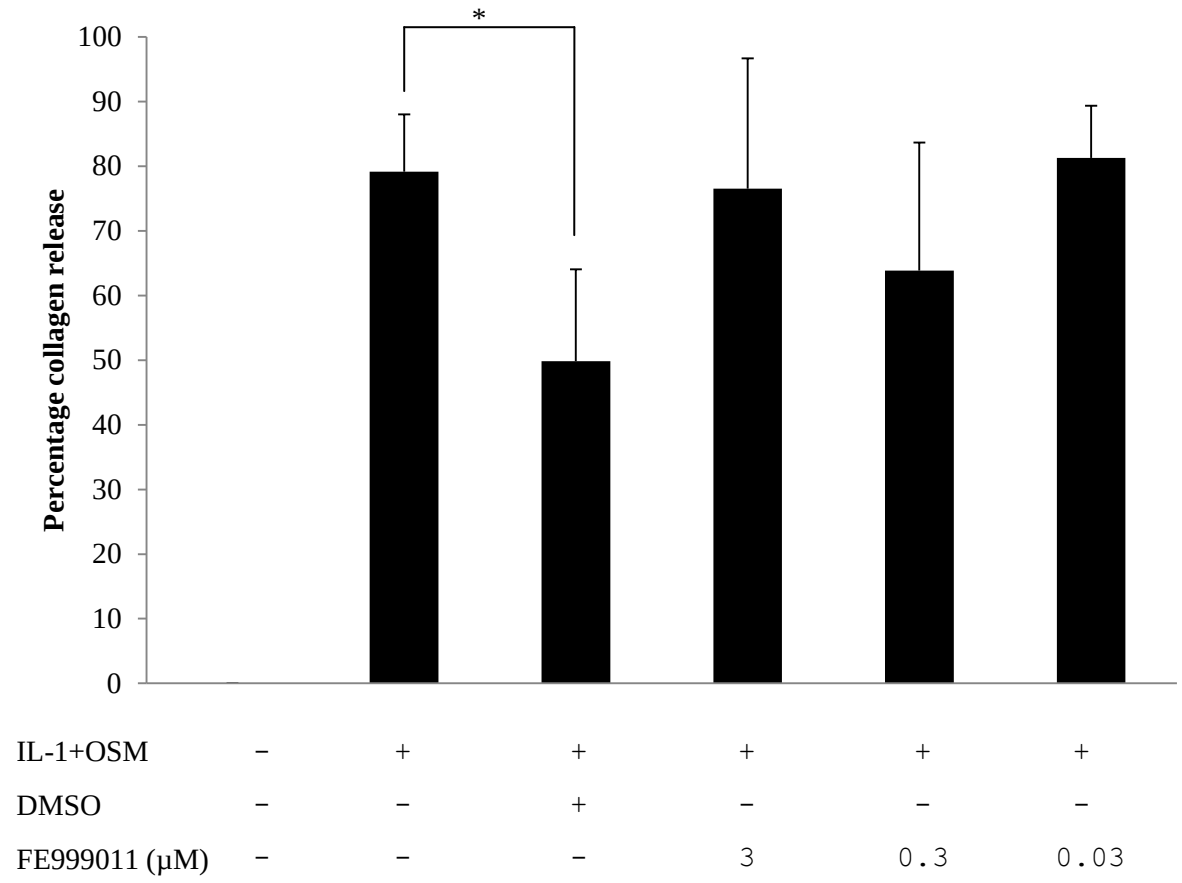


Figure 4.3 Effect of FE999011 on cartilage breakdown.

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively) ± FE999011 or DMSO control. Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release (day 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments. * = $p < 0.05$.

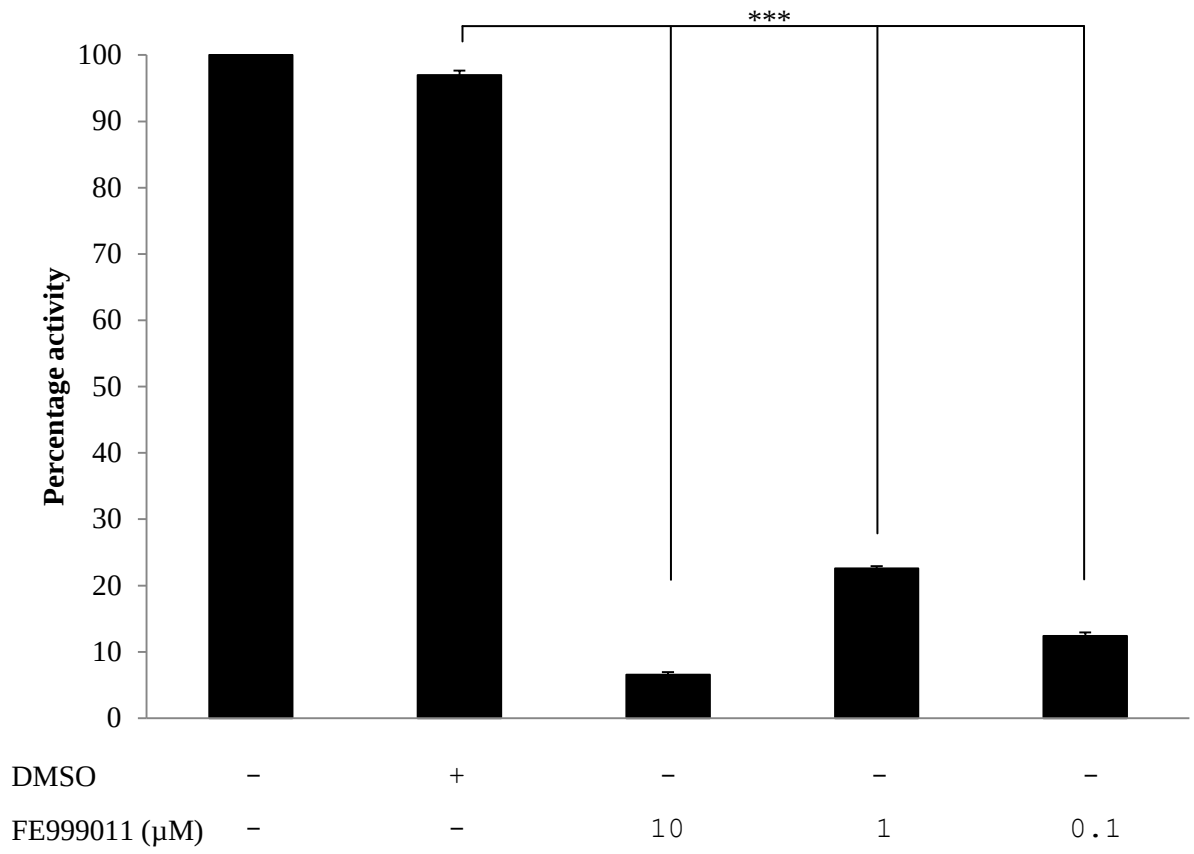


Figure 4.4 Effect of FE999011 on the activity of DPPIV

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with either FE999011 or DMSO as a solvent control were incubated in assay buffer for 30 mins at 37°C. Substrate (50 μM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments. *** = $p < 0.001$ against DMSO.

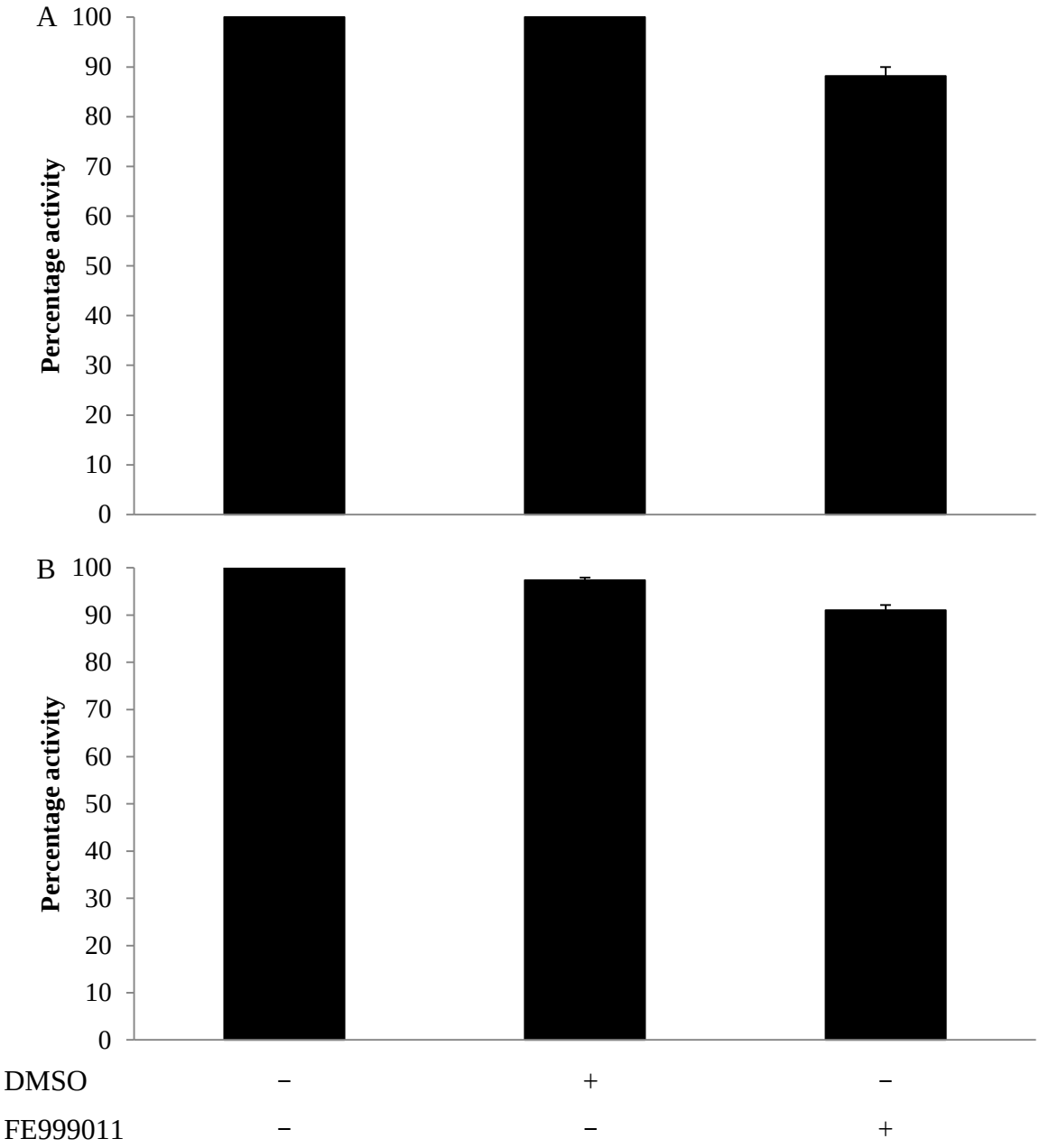


Figure 4.5 Effect of FE999011 on the enzymatic activity of FAPα

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either FE999011 (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

4.2.2.2 Collagen release upon addition of diphenylphosphonate inhibitors to resorbing cartilage

In the experiment shown (Figure 4.6), cartilage treated with IL-1+OSM and DMSO yielded 69.77 ± 7.73% collagen release. The addition of 150 µM YP-DPi yielded 74.18 ± 4.25% collagen release and 150 µM GP-DPi yielded 58.38 ± 10.37% collagen release. The data show that there was no statistically significant change in collagen release when resorbing cartilage was treated with either YP-DPi or GP-DPi. Recombinant DPPIV was used to test whether GP-DPi or YP-DPi displayed any inhibitory action *in vitro* (Figure 4.7 and Figure 4.8, respectively). The data show that both inhibitors displayed a strong ability to inhibit DPPIV *in vitro*.

It has been previously reported that dipeptide diphenylphosphonate inhibitors exhibit short half-lives at 37°C (Lambeir et al., 1996). The results show that both inhibitors lost approximately 20 - 30% of their inhibitory potential by 48 h (Figure 4.9). Therefore, if DPPIV activity is associated with cartilage collagen breakdown then the addition of the inhibitors would need to be added at a time point preceding and during pro-MMP activation. The activation of pro-collagenases in the bovine cartilage explants model is thought to occur around days 10 to 12 (Milner et al., 2006a). Addition of either inhibitor between days 8 to 12 inclusive showed no change in collagen release (Figure 4.10).

Neither GP-DPi nor YP-DPi showed cross-reactivity to inhibit the enzymatic activity of recombinant FAPα *in vitro* (Figure 4.11).

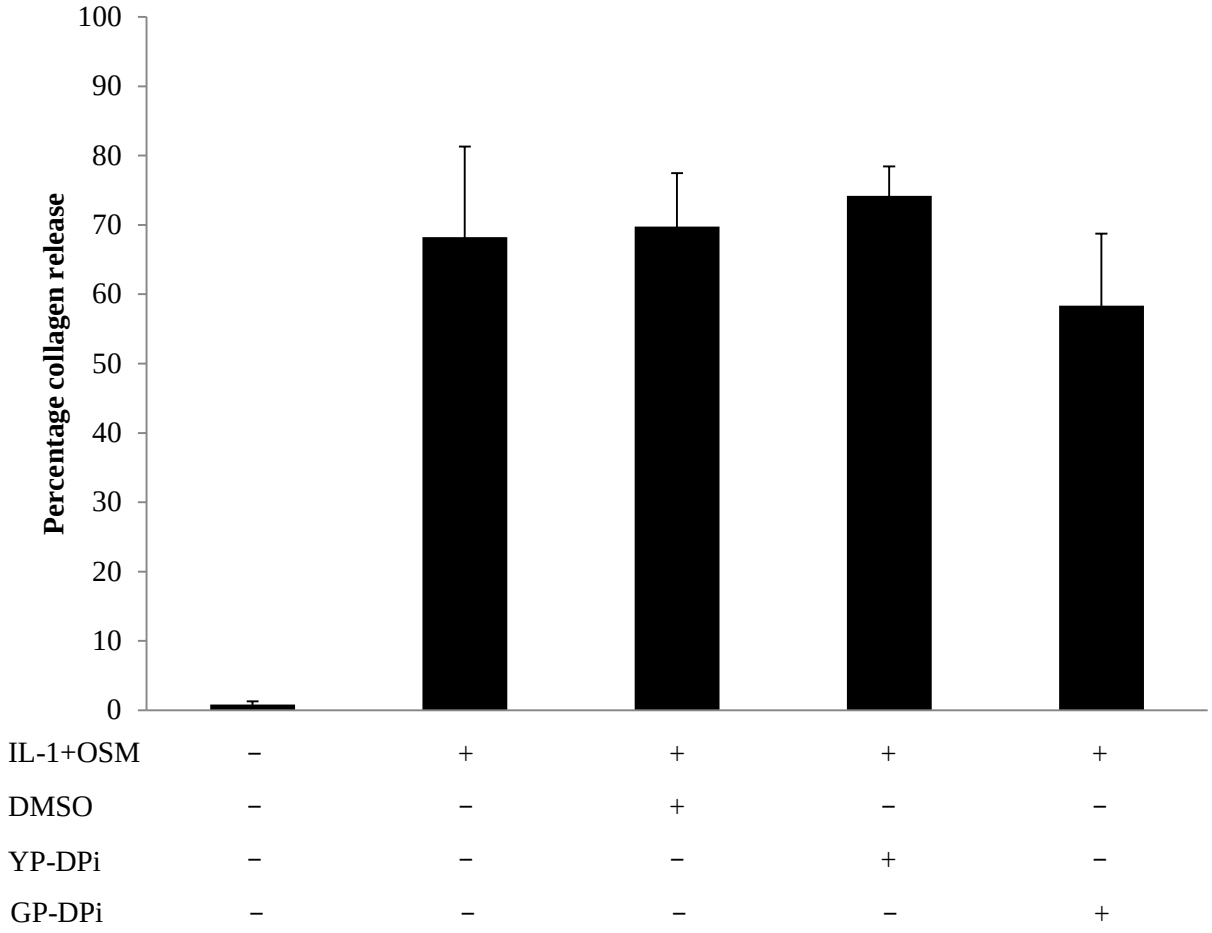


Figure 4.6 Effect of diphenylphosphonate inhibitors on cartilage breakdown.
Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively) ± DPi (150 µM) or DMSO control. Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 14) expressed as percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments.

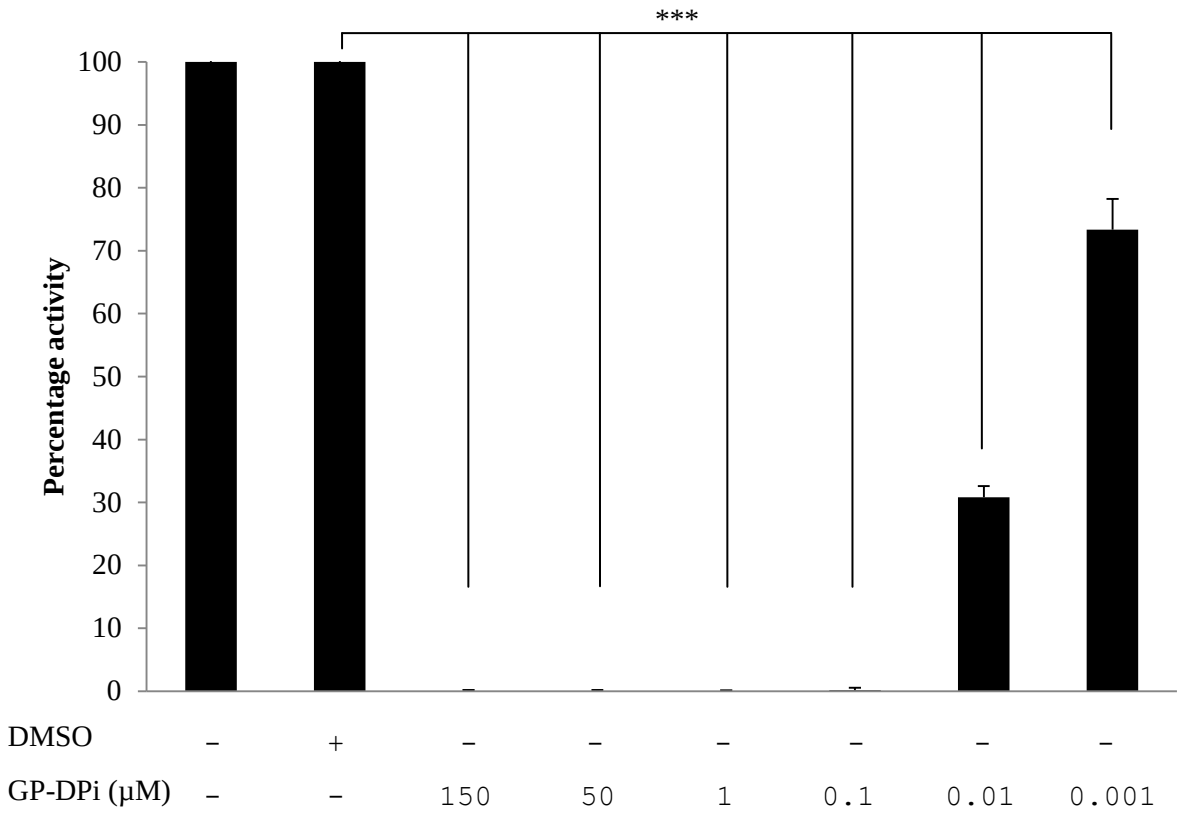


Figure 4.7 Inhibition of DPPIV activity by GP-DPi

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) ± GP-DPi were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments. *** = $p < 0.001$ against DMSO.

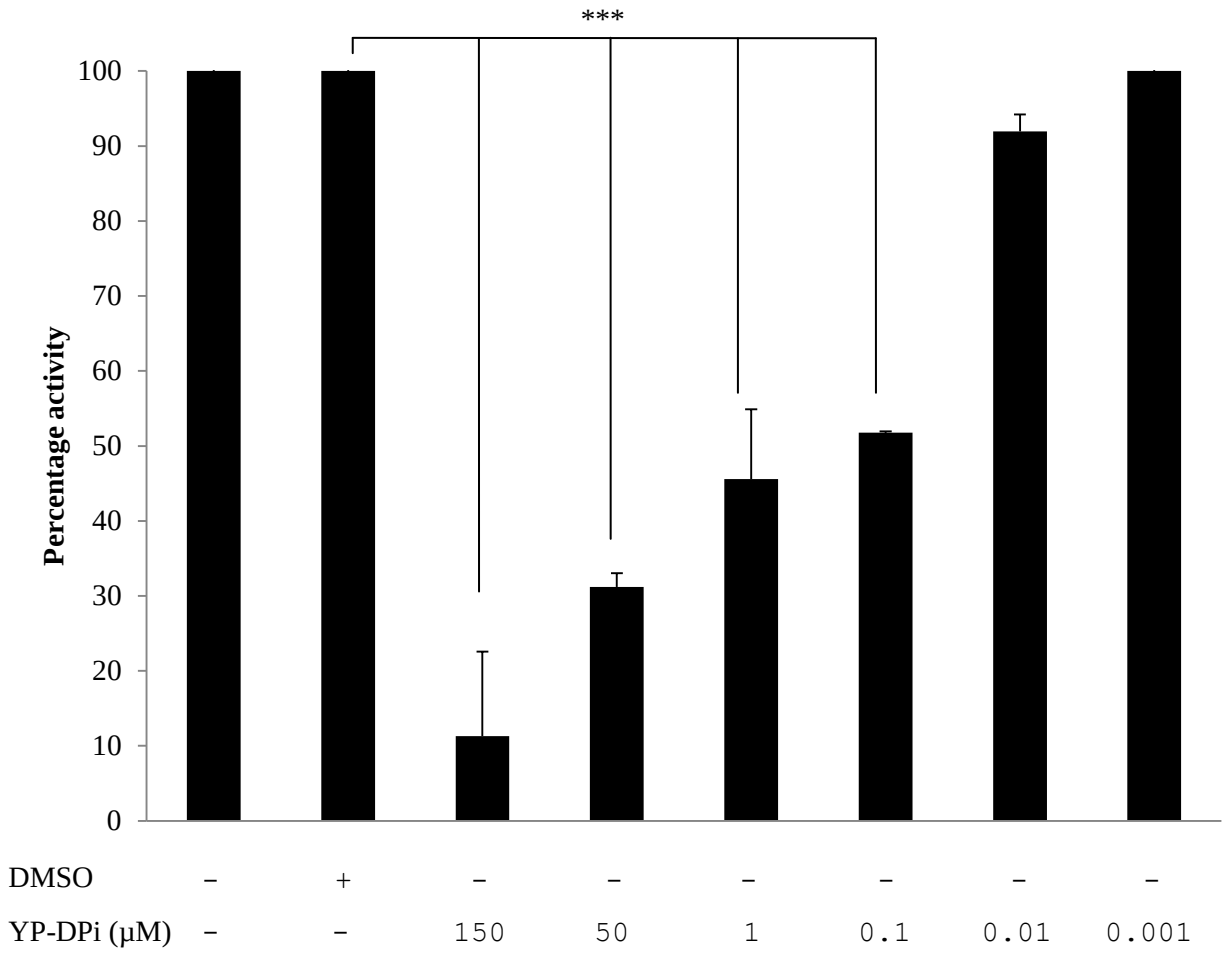


Figure 4.8 Inhibition of DPPIV activity by YP-DPi

DPPIV activity was determined using a quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) ± YP-DPi were incubated in assay buffer for 30 mins at 37°C. Substrate (50 µM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments. *** = $p < 0.001$ against DMSO.

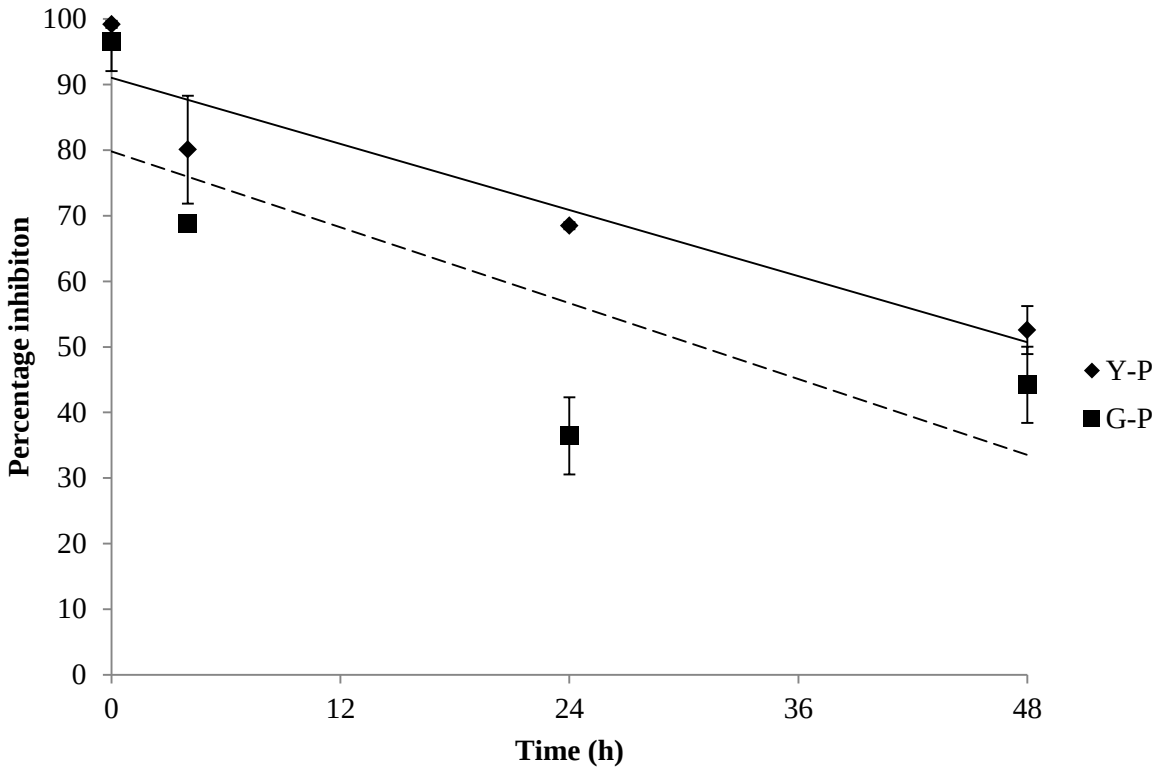


Figure 4.9 Time course to measure the stability of diphenylphosphonates at 37°C

0.1 µM GP-DPi (—) and 1 µM YP-DPi (− −) were incubated in PBS at 37°C for 4, 24 and 48 h time points. The inhibitory activity against DPPIV function was determined using a quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with either GP-DPi or YP-DPi were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added to each well and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3).

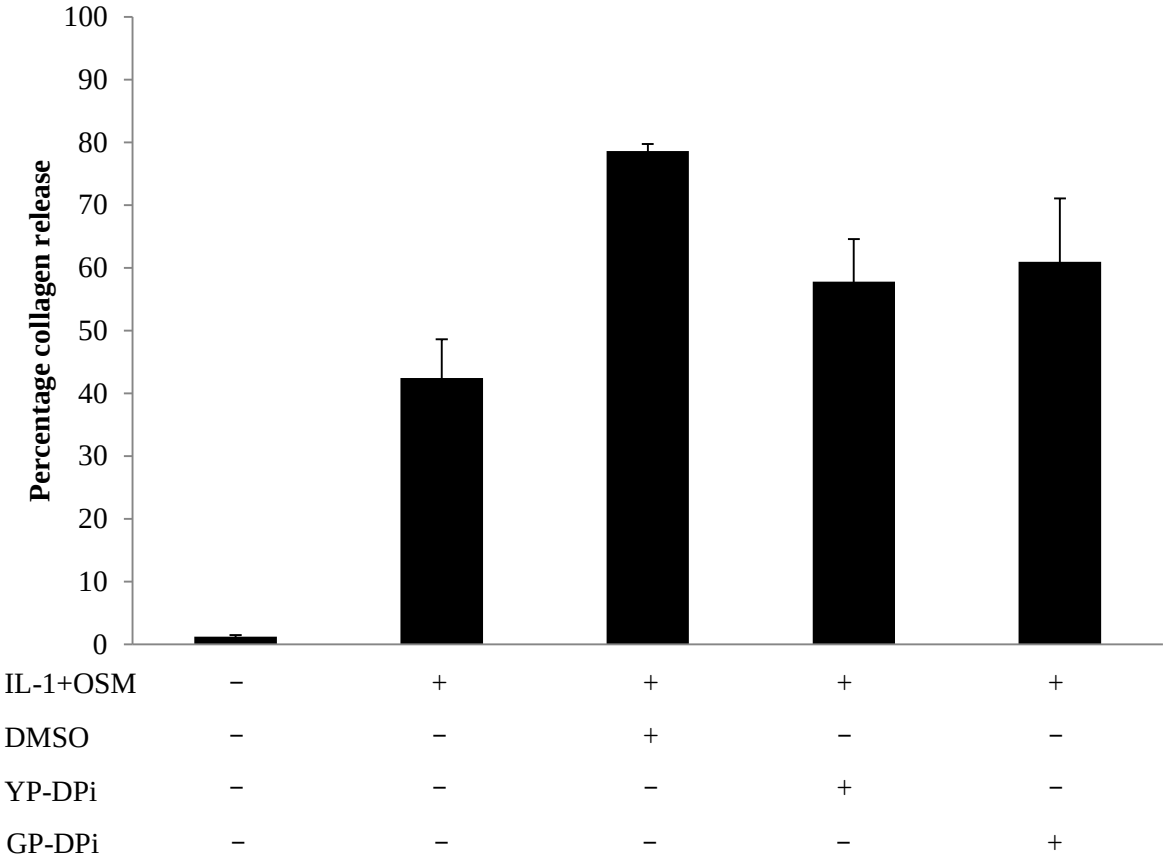


Figure 4.10 Multiple additions of diphenylphosphonate inhibitors to cartilage show no protective effect

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively) ± GP-DPi or YP-DPi or DMSO control. Media were removed on day 7 and fresh reagents were added. Either GP-DPi or YP-DPi (150 µM final concentration) were added to the cartilage between days 8 to 12 (inclusive). The experiment was ceased at day 14. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 14), expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 4 separate experiments.

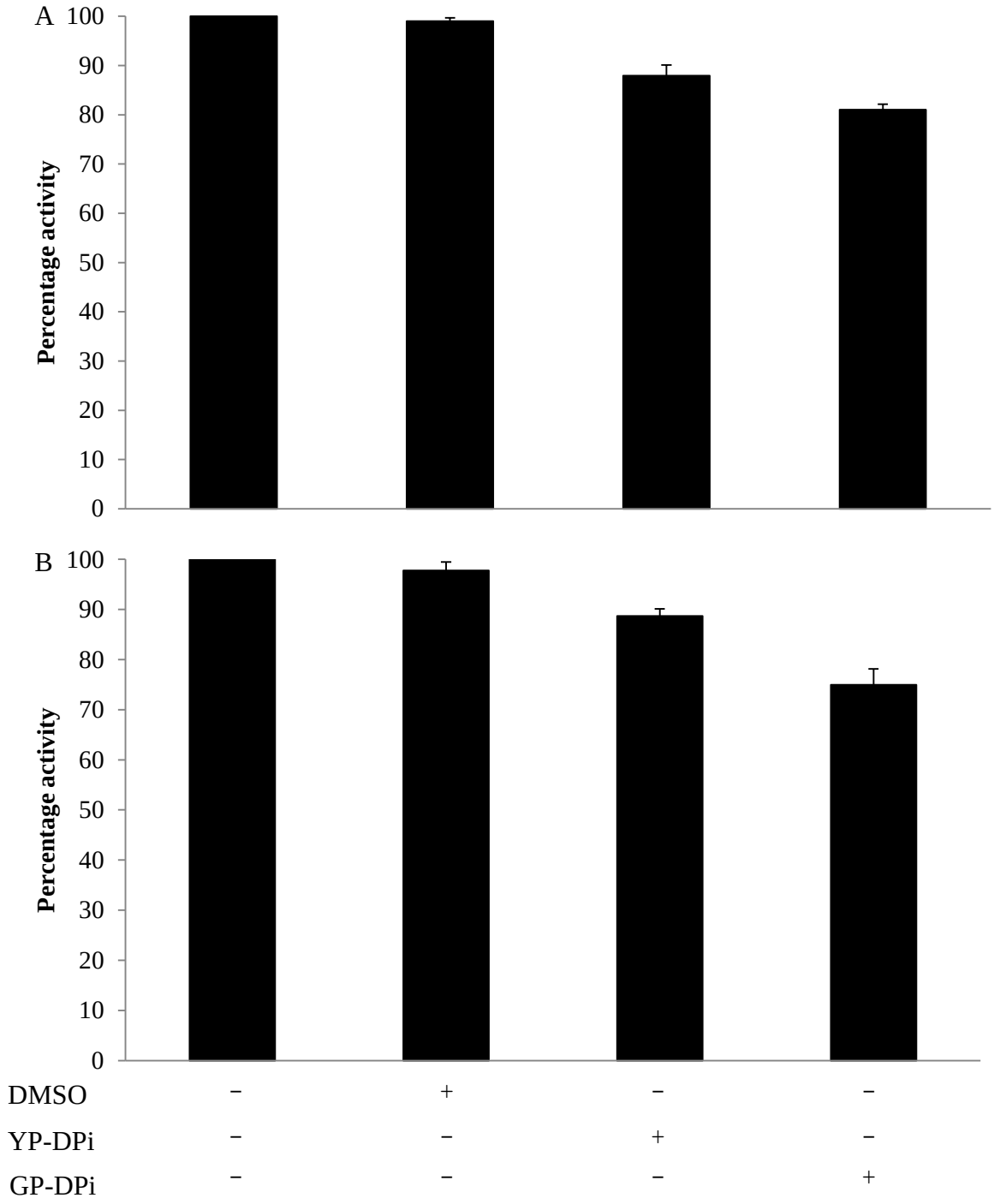


Figure 4.11 Effect of diphenylphosphonate inhibitors on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) ± DPi (150 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments.

4.2.2.3 Collagen release upon addition of UAMC-374, vildagliptin and AB192 to resorbing cartilage

There was no inhibition seen for any of the inhibitors tested (Figure 4.12). Recombinant human DPPIV was used to test if any of the inhibitors showed any potential to reduce the enzymatic activity of DPPIV *in vitro* (Figure 4.13). All of the inhibitors significantly decreased DPPIV activity compared to DMSO control, confirming the inhibitors were active when added to bovine cartilage.

Moreover, none of the inhibitors tested showed any inhibitory affect against recombinant FAPα *in vitro* (Figure 4.14).

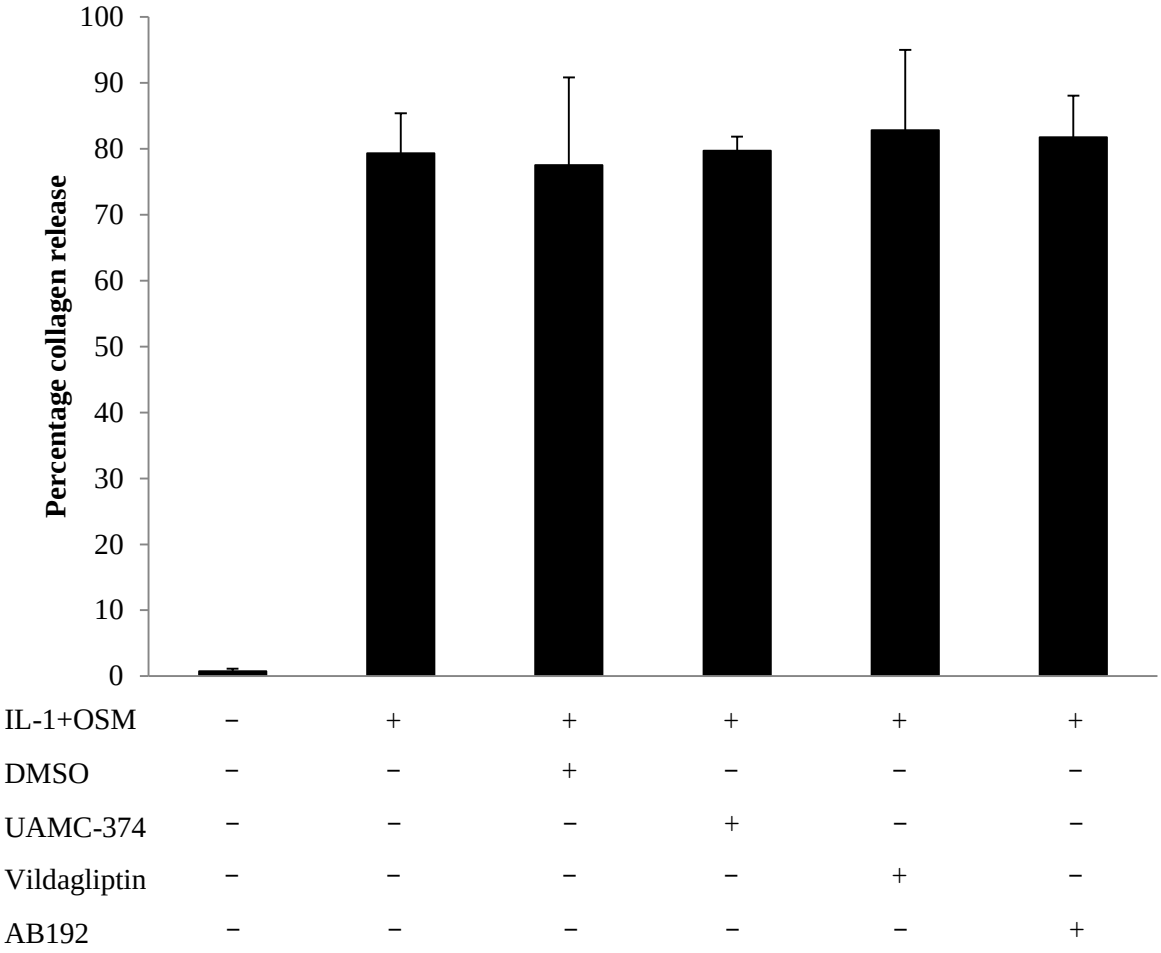


Figure 4.12 Effect of DPPIV inhibitors on cartilage breakdown.

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively). At days 0, 3, 7 and 10, the inhibitors UAMC-374, vildagliptin and AB192 (10 µM) or DMSO control were added to cartilage. Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release, expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 8 separate experiments.

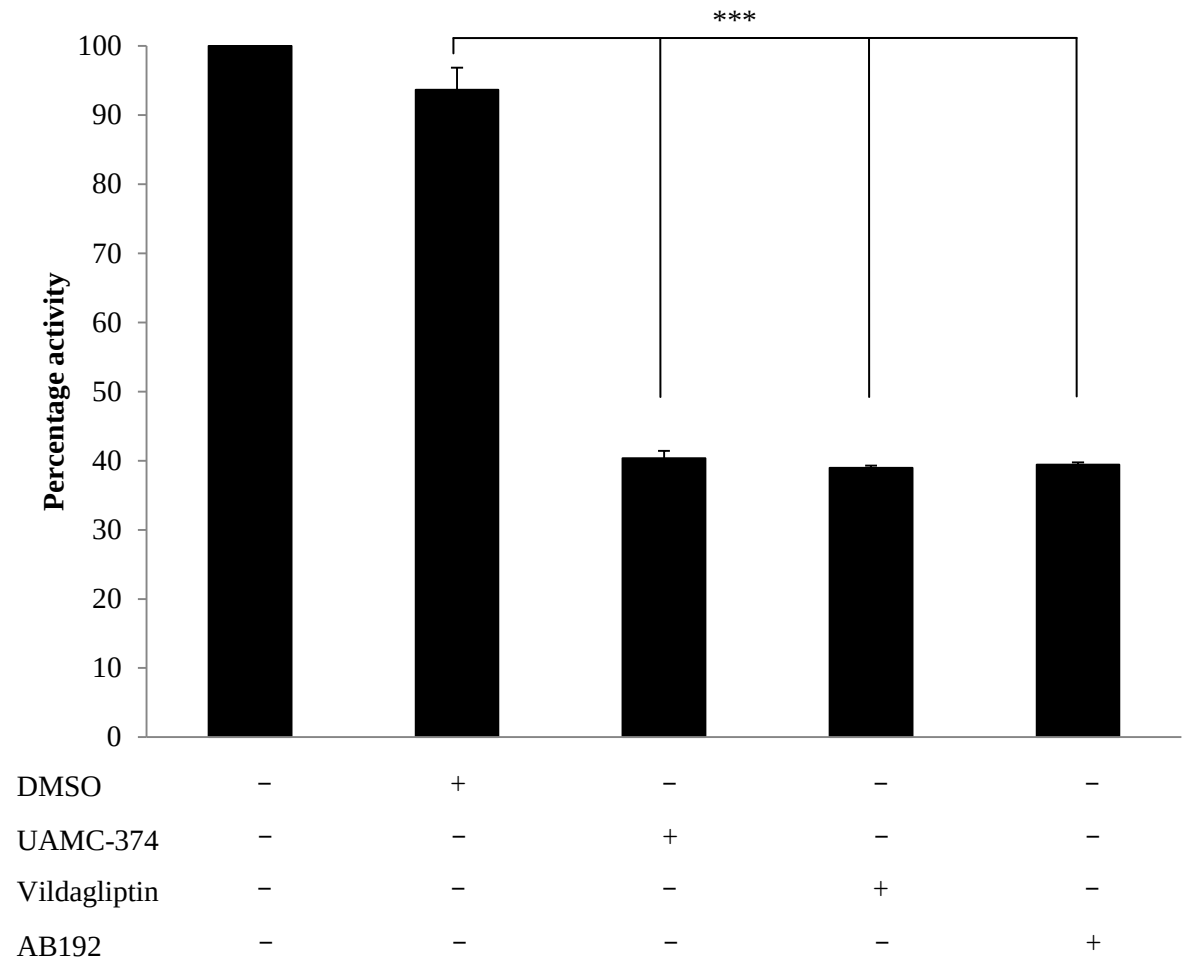


Figure 4.13 Modulation of DPPIV activity by DPPIV inhibitors

DPPIV activity was determined using a quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) with DPPIV inhibitors (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added to each well and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments. *** = $p < 0.001$ against DMSO control.

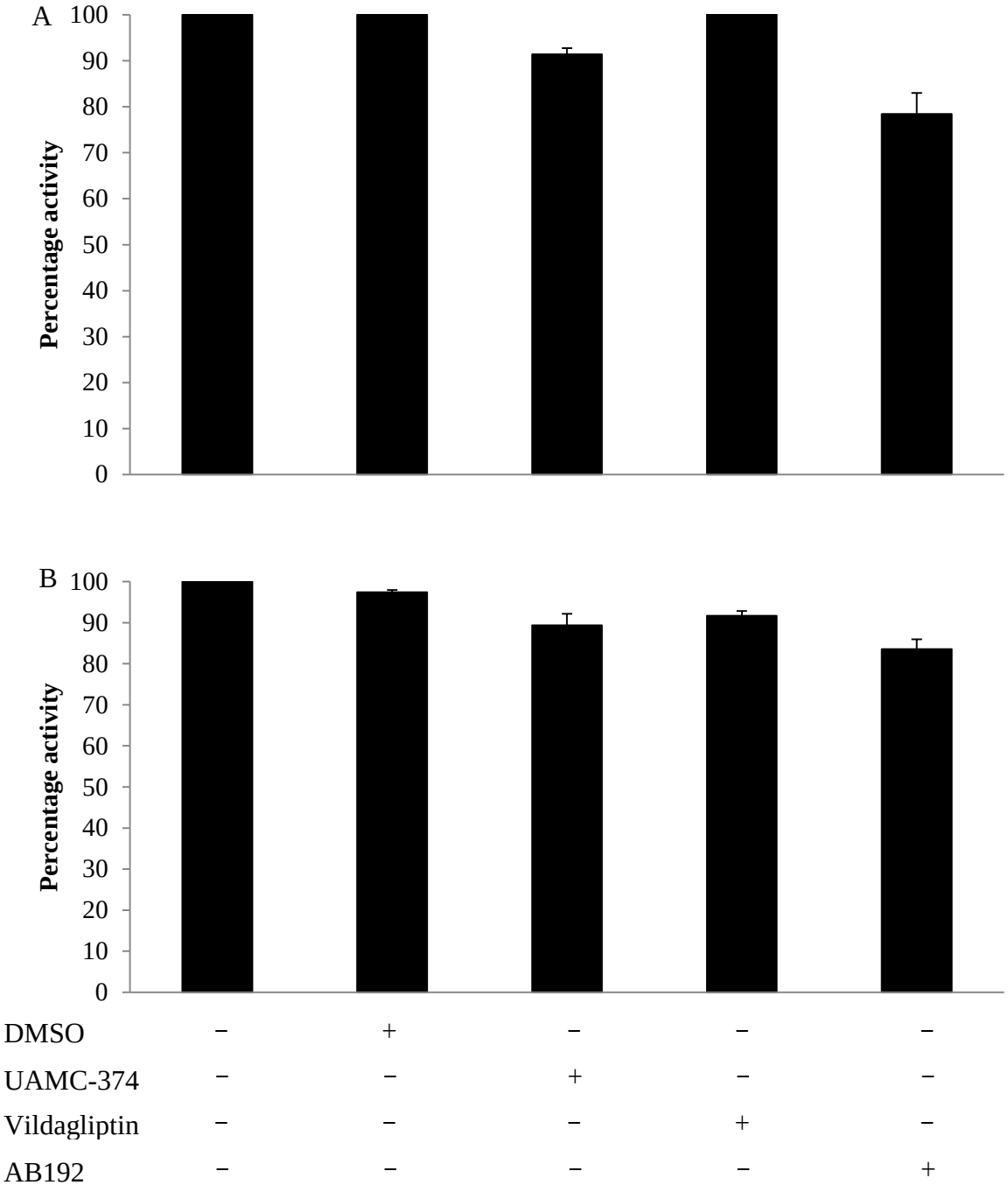


Figure 4.14 Effect of DPPIV inhibitors on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either DPPIV inhibitors (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activities were calculated. Results are expressed as mean ± SD (n=3) and are representative of 2 separate experiments.

4.2.2.4 Collagen release upon addition of DPPI 1c to resorbing cartilage

In the experiment shown (Figure 4.15), cartilage treated with IL-1+OSM yielded 79.16 ± 8.86% collagen release. The addition of a range of DPPI 1c concentrations did not affect cartilage matrix degradation after stimulation with IL-1+OSM.

Recombinant human DPPIV was used to test if the inhibitor had potential to reduce the enzymatic activity of DPPIV *in vitro* (Figure 4.16). The inhibitor significantly decreased DPPIV activity compared to control, confirming that the inhibitor was active when added to bovine cartilage. The lack of any biological effect *ex vivo* in bovine cartilage led to the discontinuation of the use of DPPI 1c. DPP 1c did not inhibit recombinant FAPα activity *in vitro* (Figure 4.17).

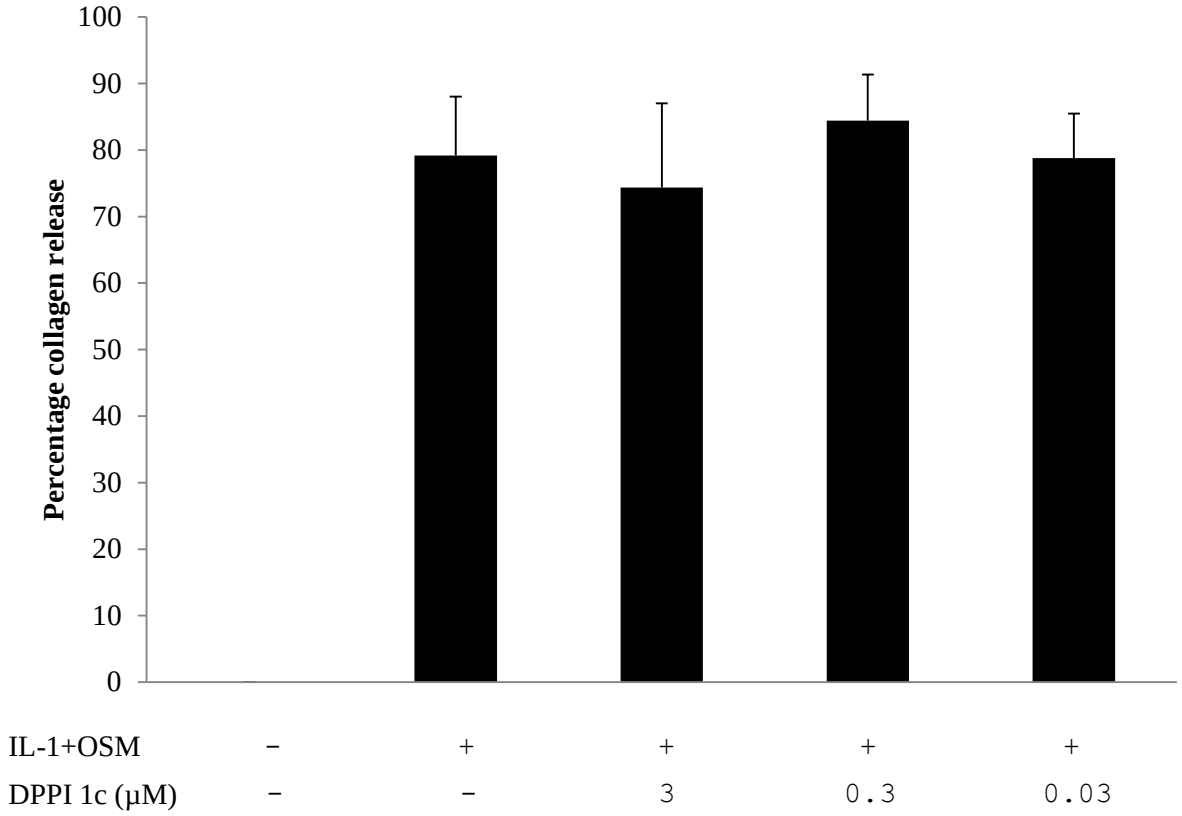


Figure 4.15 Effect of DPPI 1c inhibitor on cartilage breakdown.

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively) ± DPPI 1c. Media were removed on day 7 and fresh reagents added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release, expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 6 separate experiments.

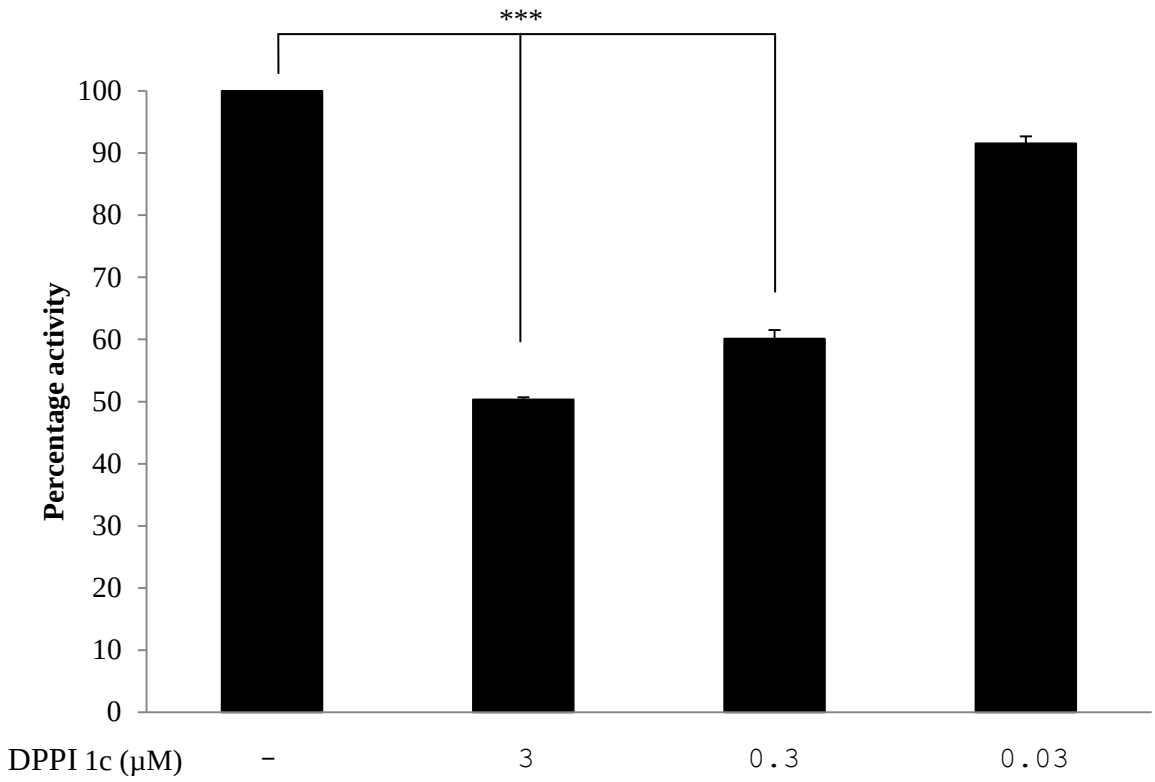


Figure 4.16 Effect of DPPI 1c on the activity of DPPIV.

DPPIV activity was determined using a quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) ± DPPI 1c were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 3 separate experiments. *** = $p < 0.001$ against control.

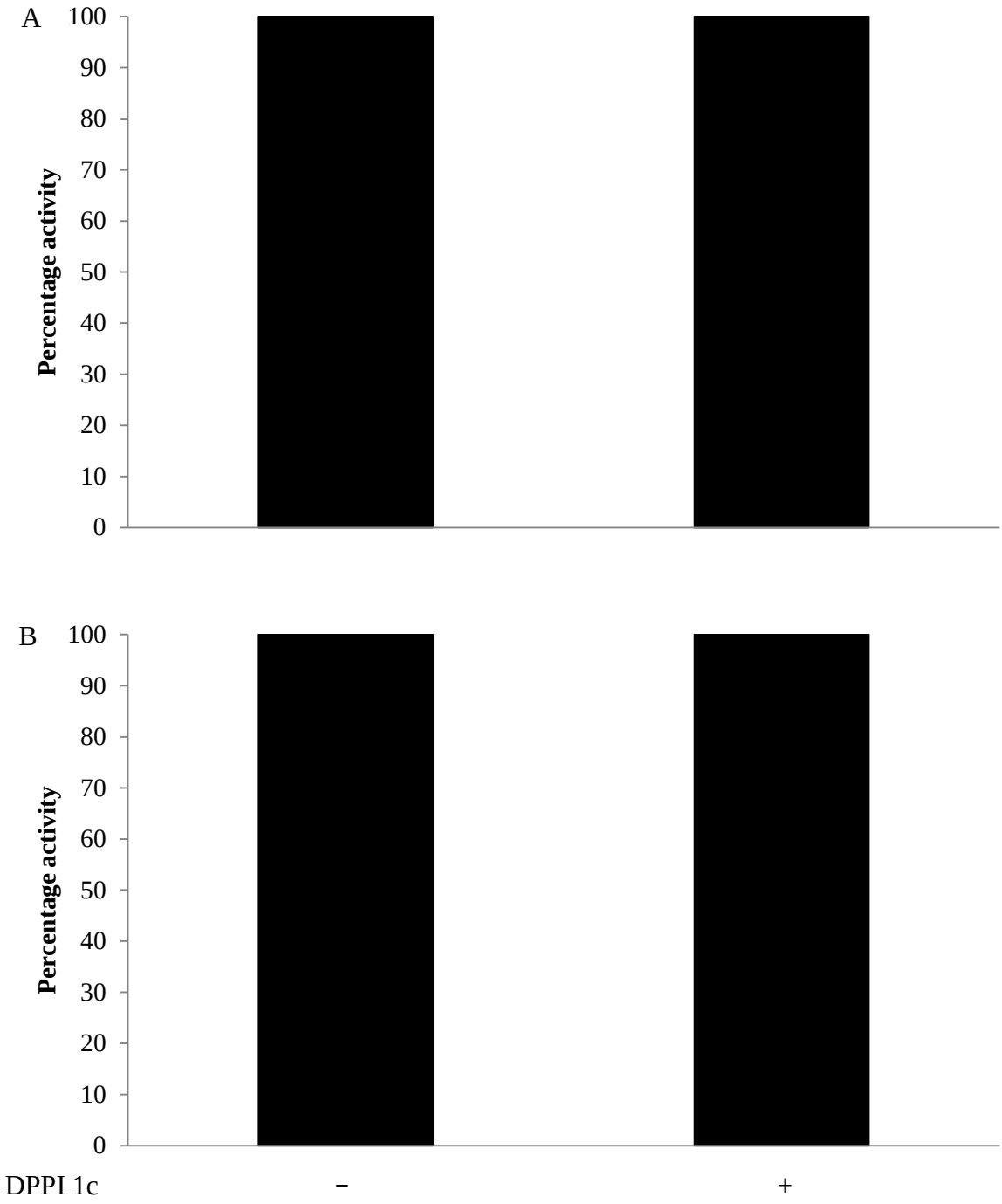


Figure 4.17 Effect of DPPI 1c on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) ± DPPI 1c (10 µM) were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activities were calculated. Results are expressed as mean ± SD (n=3) and are representative of 2 separate experiments.

4.2.2.5 Collagen release upon addition of K579 to resorbing cartilage

The addition of K579 significantly reduced cartilage matrix degradation after stimulation with IL-1+OSM, resulting in almost complete protection of the cartilage matrix (Figure 4.18).

Bovine cartilage treated with IL-1+OSM showed a significant increase in total collagenase levels at day 7 but no detectable increase in active collagenase levels (Figure 4.19a). The addition of K579 up to day 7 caused a significant decrease in total collagenase levels. There was a similar trend at day 14, as the addition of IL-1+OSM led to a significant increase in both total and active collagenase levels compared to control (Figure 4.19b). Again, the addition of K579 caused a significant decrease in total collagenase levels, and a significant decrease in active collagenase levels could be observed at day 14. Previous data have shown that inhibition of serine proteases leads to a significant decrease in collagenase levels (Milner et al., 2003). However, the mechanism behind this global reduction of collagenase levels has yet to be elucidated.

Recombinant human DPPIV was used to test the potential of the inhibitor K579 to reduce the enzymatic activity of DPPIV *in vitro* (Figure 4.20). The inhibitor significantly decreased DPPIV activity over a range of concentrations compared to control. Even at concentrations as low as 50 nM, K579 reduced activity by 70%. K579 showed no inhibition of recombinant FAPα activity *in vitro* (Figure 4.21).

K579 is a nitrile based inhibitor (Prof. B. Walker, Queen's University Belfast, UK, personal communication) and could potentially inhibit cathepsin K, a novel cysteine protease with collagenase activity (Dejica et al., 2008, Kafienah et al., 1998). L-873724 is a selective cathepsin K inhibitor that has an IC_{50} in the sub-nanomolar range (Li et al., 2006) and was added to IL-1+OSM-treated bovine cartilage. In the experiment shown, cartilage treated with IL-1+OSM yielded 81.56 ± 3.88% release at day 14. The addition

of L-873724 did not affect cartilage matrix degradation after stimulation with IL-1+OSM (Figure 4.22) and *in vitro* assays using K579 showed no efficacy against cathepsin K (Prof. J. Mort, McGill University, Québec, Canada, personal communication).

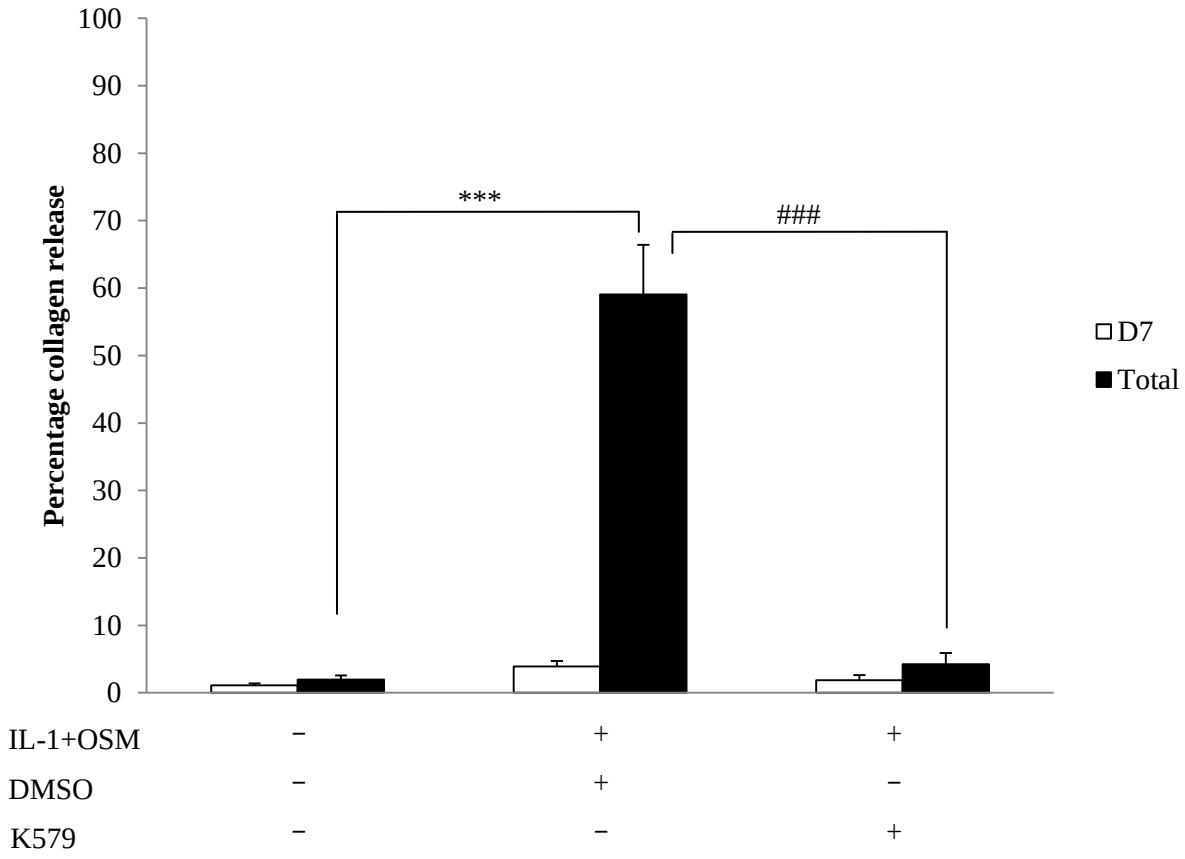


Figure 4.18 Effect of K579 inhibitor on cartilage breakdown.

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively). At days 0, 3, 7 and 10, K579 (5 µM) or DMSO control were added to cartilage. Media were removed on day 7 and fresh reagents added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown is the cumulative collagen release, expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 6 separate experiments. *** = $p < 0.001$ against untreated and ### = $p < 0.001$ against IL-1+OSM-treated cartilage.

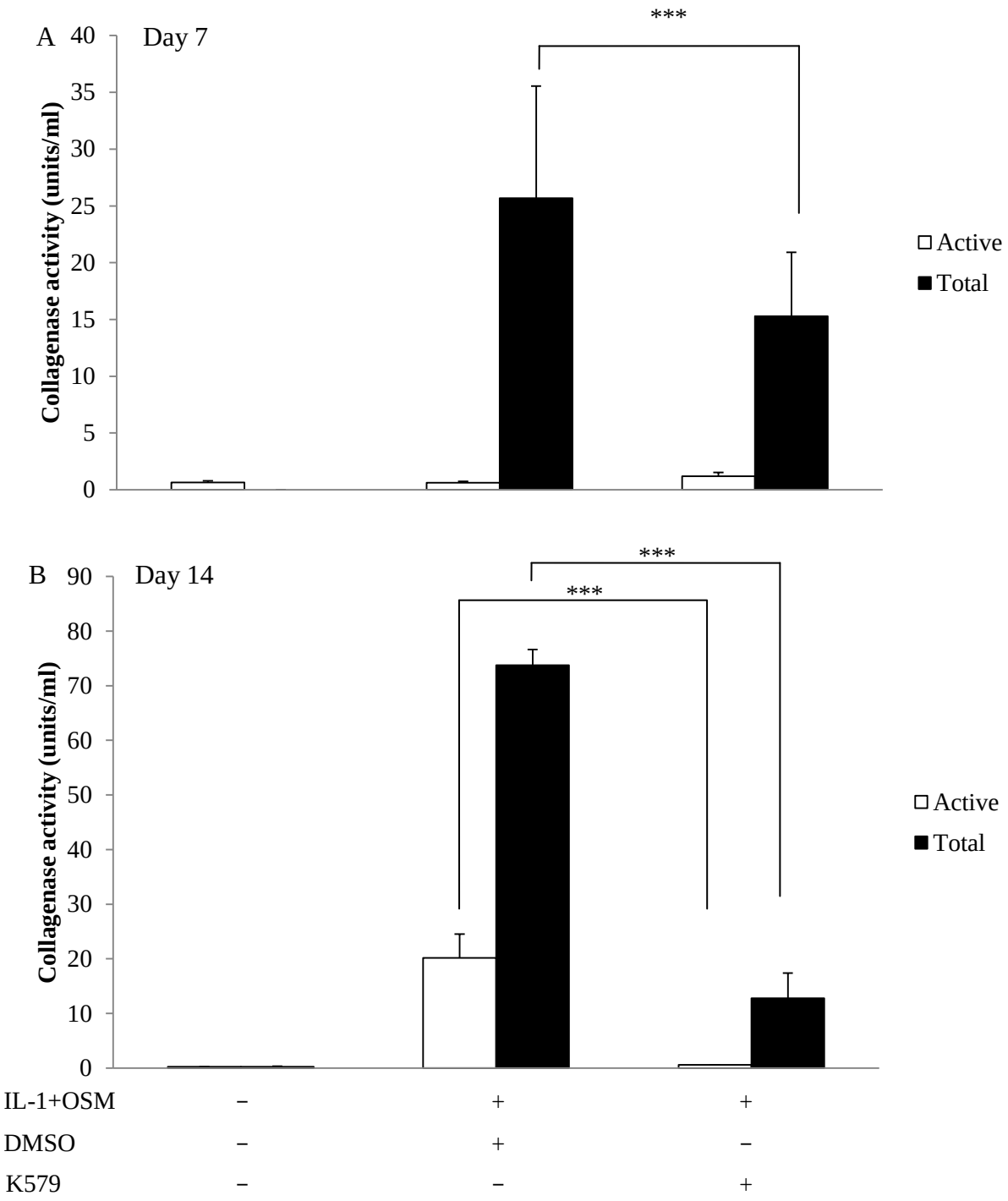


Figure 4.19 The effect of K579 on collagenase activity from resorbing cartilage.

Media from cartilage shown in Figure 4.18 were assayed for collagenase activity. Media were treated with APMA to test for total collagenase levels (pro- and active). A) Active and total collagenase activity for day 7 media and B) active and total collagenase activity for day 14 media. Results are expressed as mean ± SD (n=4) and representative of 2 separate experiments. *** = $p < 0.001$ against IL-1+OSM.

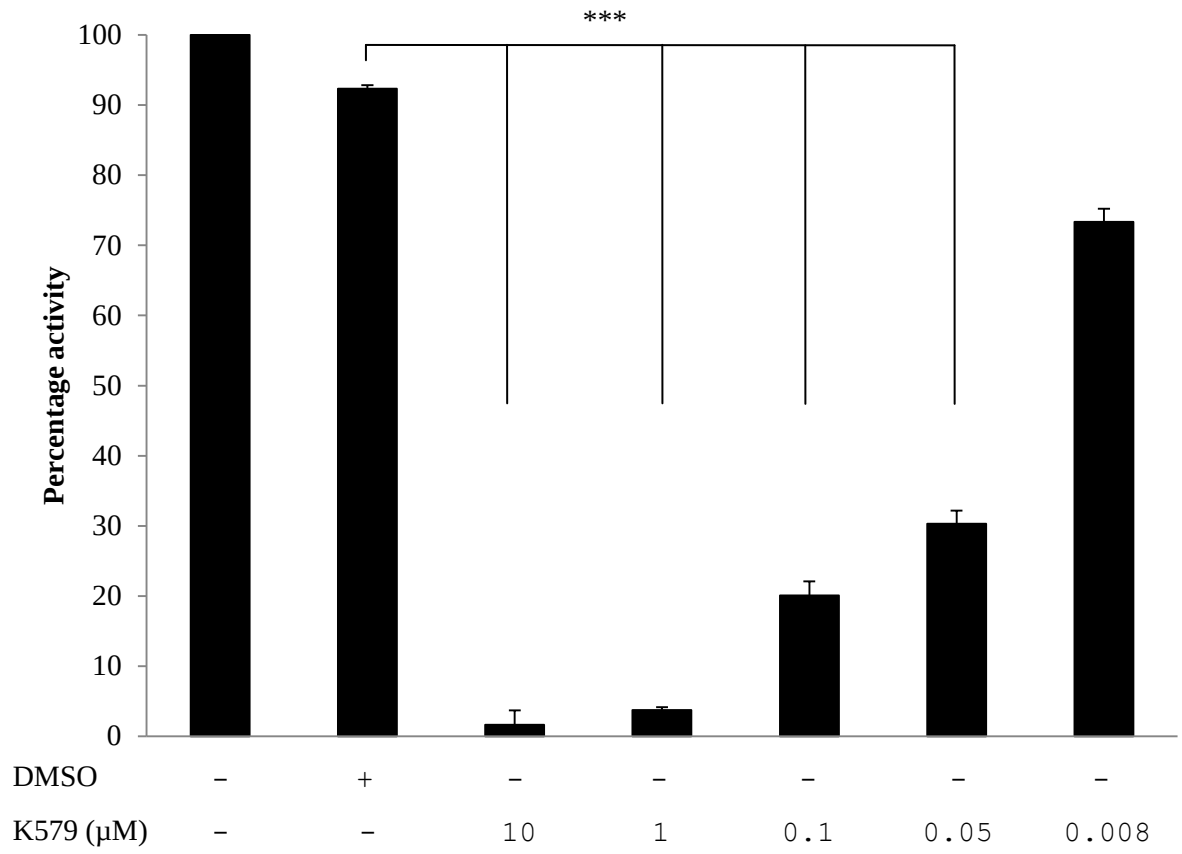


Figure 4.20 Effect of K579 on the activity of DPPIV.

DPPIV activity was determined using the quenched fluorescent substrate, GP-AMC. DPPIV (50 pM) ± K579 (IC_{50} = 8 nM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added to each well and left for 1 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The percentage activity was calculated. Results are expressed as mean ± SD (n=3) and representative of 2 separate experiments. *** = $p < 0.001$ against control.

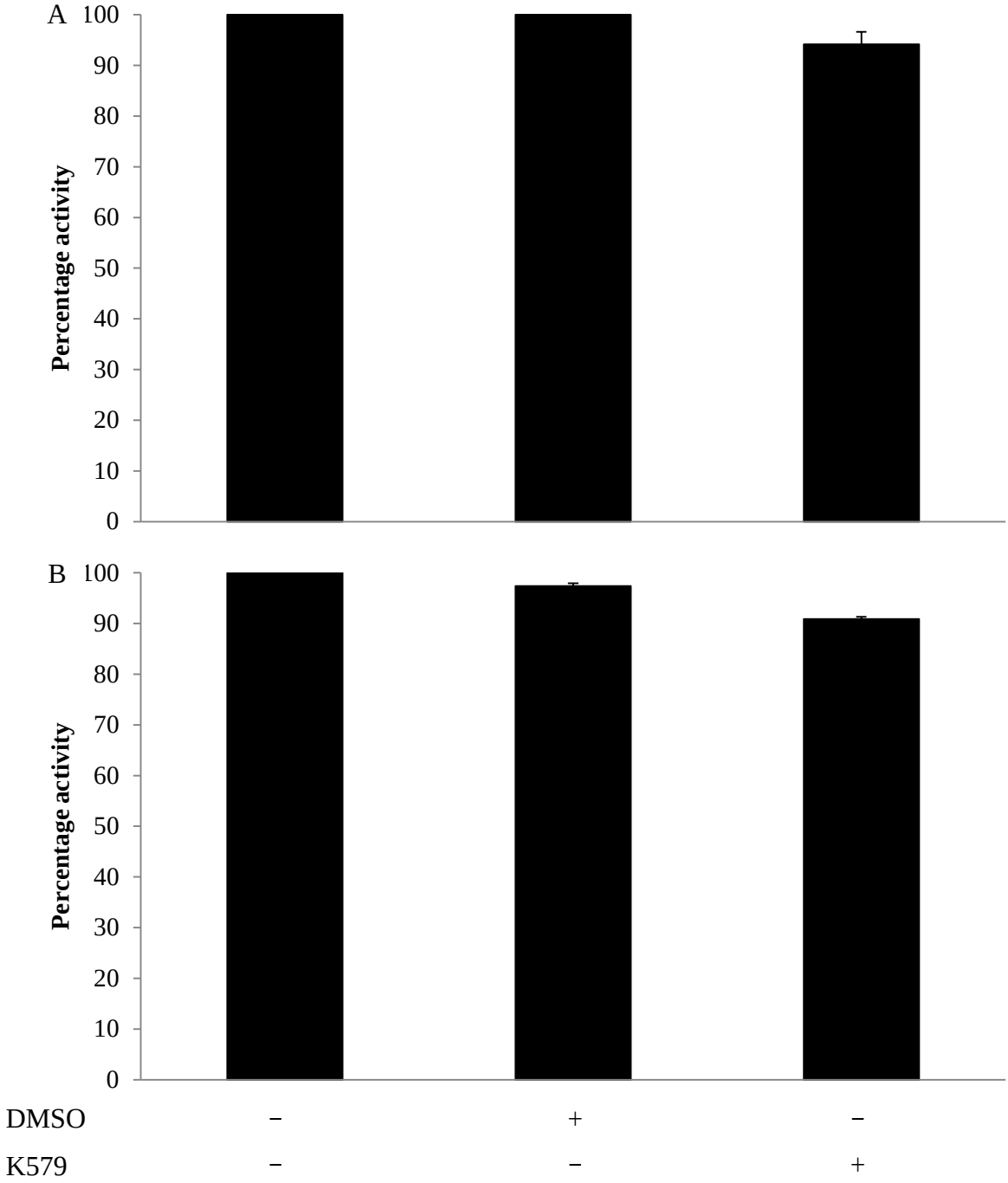


Figure 4.21 Effect of K579 on the activity of FAPα.

FAPα activity was determined using the quenched fluorescent substrate, A) Z-GP-AMC or B) GP-AMC. FAPα (120 pM) with either K579 (10 µM) or DMSO as a solvent control were incubated in assay buffer for 30 min at 37°C. Substrate (50 µM) was then added and left for 4 h at 37°C. Fluorescence was then measured immediately (λ_{ex} 360 nm, λ_{em} 460 nm). The A) endopeptidase or B) exopeptidase activities were calculated. Results are expressed as mean ± SD (n=3) and are representative of 2 separate experiments.

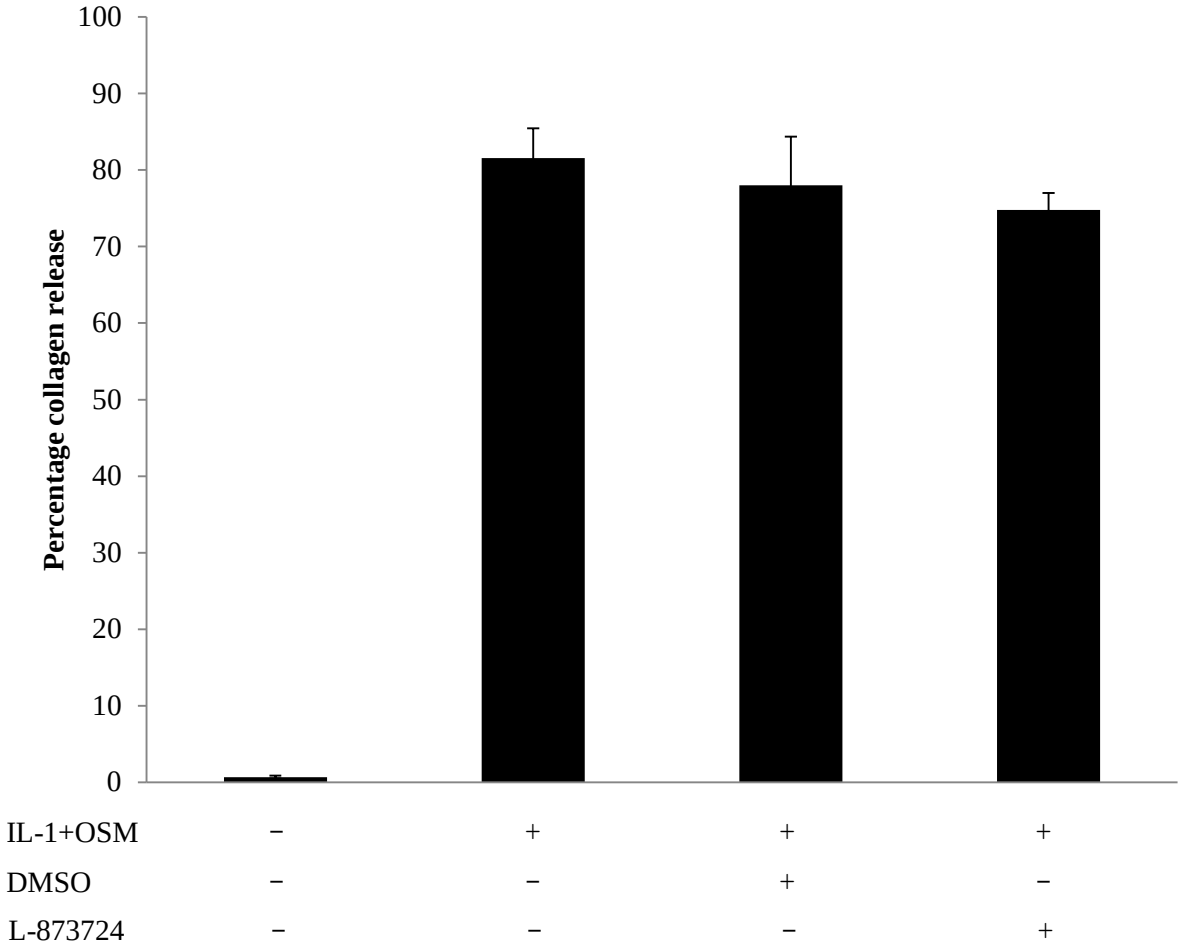


Figure 4.22 The effect of L-873724 on collagenase activity from resorbing bovine cartilage.

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively). At days 0, 3, 7 and 10, L-873724 (10 nM) or DMSO control were added to cartilage. Media were removed on day 7 and fresh reagents added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 14 media and in cartilage digests. Shown is the cumulative collagen release, expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 2 separate experiments.

4.2.3 Effect of DPPIV on IL-1+OSM-treated cartilage explant culture

The addition of K579 to IL-1+OSM-treated bovine cartilage showed a significant reduction in collagen release (Figure 4.18). Therefore, if DPPIV activity is playing a role in cartilage collagen breakdown, then does the addition of active DPPIV expedite collagen release for IL-1+OSM-treated cartilage?

There was no increase in collagen release with the addition of active DPPIV to IL-1+OSM-treated cartilage compared to IL-1+OSM alone (Figure 4.23).

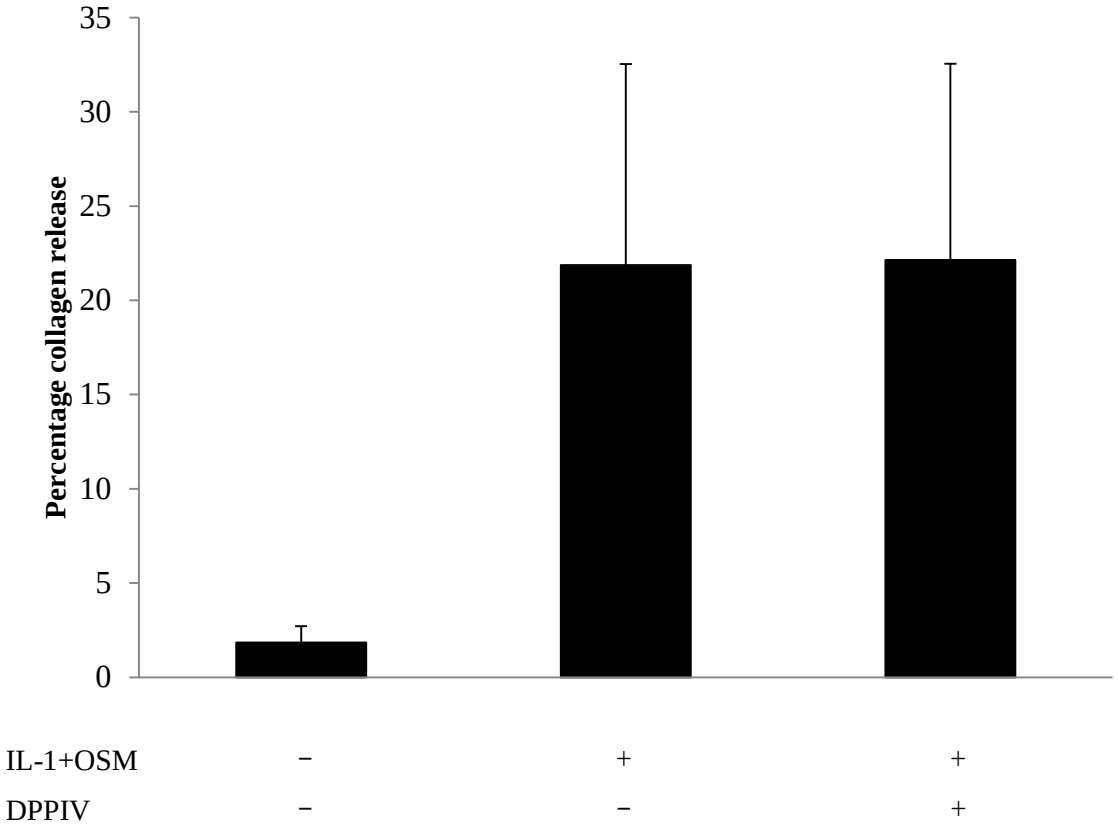


Figure 4.23 Effect of active DPPIV on cartilage breakdown

Bovine cartilage discs were cultured in medium ± IL-1+OSM (1 ng/ml and 10 ng/ml, respectively). Media were removed on day 7 and fresh media ± DPPIV (100 nM) were added until day 12 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 12 media and in cartilage digests. Shown is the cumulative collagen release (days 7 + day 12) expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=6) and representative of 4 separate experiments.

4.2.4 Effect of DPPIV enzyme activity on type II collagen

The addition of K579 to IL-1+OSM-treated bovine cartilage prevented collagen release (Figure 4.18). However, the addition of DPPIV to IL-1+OSM-treated cartilage failed to significantly increase collagen release (Figure 4.23).

The hypothesis being tested in this experiment was whether DPPIV could degrade type II collagen. There are conflicting reports in the literature that claim DPPIV possesses gelatinase activity (Bermpohl et al., 1998), while other data suggest that DPPIV does not possess endopeptidase activity (Aertgeerts et al., 2005).

Type II collagen was extracted from OA cartilage. To ensure that the type II collagen had retained its native triple helical state during the extraction, it was treated with trypsin, which confirmed that it still retained the native triple helical conformation: trypsin could not cleave the native collagen band but did hydrolyse the denatured collagen (Figure 4.24, lanes 4 and 5). In the experiment shown, DPPIV was not able to cleave native or denatured collagen (Figure 4.24, lanes 7 and 8). This confirms previous data that DPPIV does not have any gelatinase or endopeptidase activity (Aertgeerts et al., 2005).

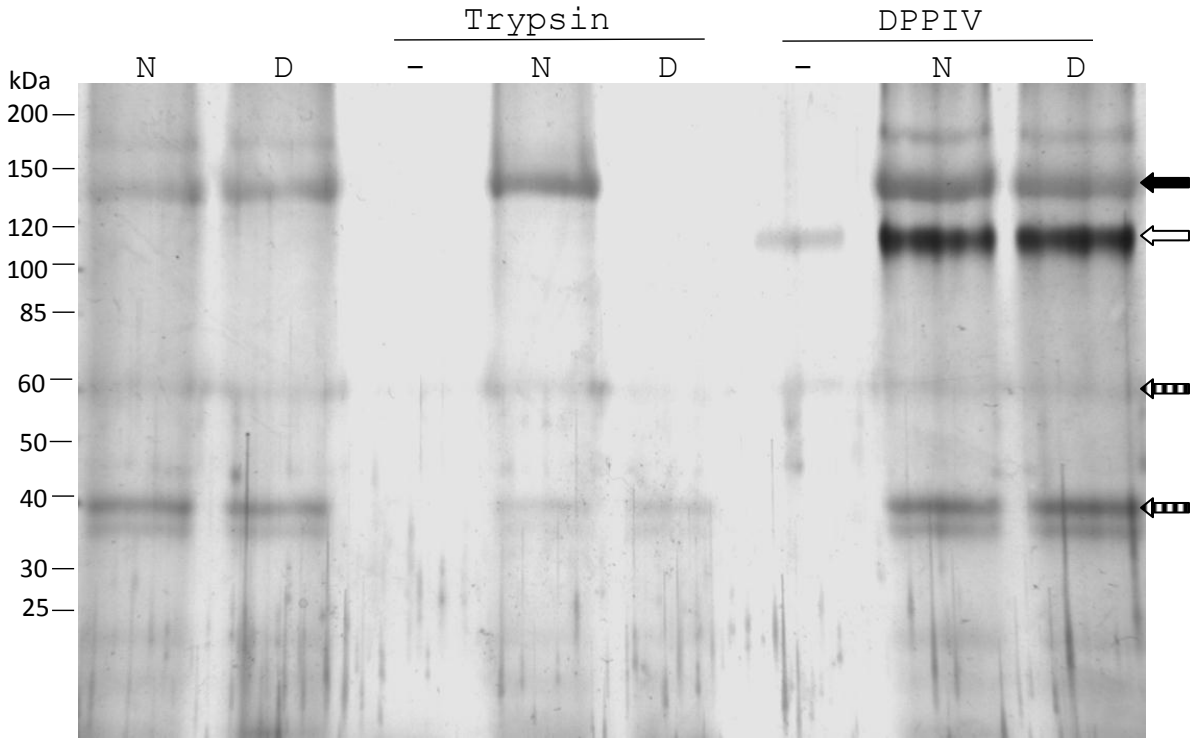


Figure 4.24 Proteolysis of type II collagen by DPPIV.

Type II collagen (10 µg) from human articular cartilage was incubated at 37°C for 24 h in the presence or absence of proteases in 50 mM Tris-HCl, pH 7.6, 1 M glucose, 200 mM NaCl, 5 mM $CaCl_2$ buffer. Lane: (1) Native collagen (N); (2) Denatured collagen (D); (3) 4 µM trypsin; (4) Native collagen, 0.4 µM trypsin; (5) Denatured collagen, 0.4 µM trypsin; (6) 0.1 µM DPPIV; (7) Native collagen, 0.4 µM DPPIV; (8) Denatured collagen, 0.4 µM DPPIV. Digests were reduced and analysed by 6.5 % SDS–PAGE. The closed arrow indicates the position of the intact α(II) chain of type II collagen. The open arrow indicates the position of DPPIV. The patterned arrows indicate the presence of unknown low molecular weight contaminants. Results are representative of 2 separate experiments.

4.3 Discussion

4.3.1 IL-1+OSM do not regulate DPPIV gene expression in primary chondrocytes

DPPIV gene expression has previously been reported to be increased in human OA cartilage compared to phenotypically normal cartilage (Swingler et al., 2009). As described in this chapter, DPPIV gene expression was increased in SW1353 chondrosarcoma cells by the addition of IL-1 and/or OSM compared to un-treated cells but the increase upon stimulation with IL-1+OSM was less than that seen with either cytokine alone. When TGF-β1, with or without pro-inflammatory cytokines, was added to SW1353 cells, a decrease in the expression of DPPIV could be observed. However, Gebauer et al. (2005) showed that SW1353 chondrosarcoma cells have limited potential as a model of primary chondrocyte gene expression. Therefore, primary chondrocytes from bovine cartilage were treated with combinations of IL-1, OSM and TGF-β1 and DPPIV gene expression was determined. The results differed between the two different populations of cells and this indicates that DPPIV gene expression is not robustly regulated by IL-1 or OSM or TGF-β1 in primary cells. Attempts to examine the expression of DPPIV in IL-1+OSM-treated bovine cartilage have failed. Primers that work when used with RNA isolated from chondrocytes grown in monolayer; fail to work with RNA isolated directly from bovine cartilage. Commercially available primers (PrimerDesign PerfectProbe assays) have also failed to amplify DPPIV transcripts from bovine cartilage mRNA but do show amplification from bovine chondrocyte mRNA. This could be due to contamination being carried over from RNA isolation that interferes with detection of the DPPIV transcript.

The promoter sequence of DPPIV has only been characterised in porcine cells and shows no consensus TATA-box sequence but two TATA-like sequences (Qvist et al., 1998) and indicates that the transcription factors that initiate DPPIV gene expression

have yet to be elucidated. However, recent data suggest that promoter methylation plays an important role in regulating DPPIV gene expression in a number of different carcinomas (Tsuji et al., 2004, McGuinness and Wesley, 2008). Data presented in this chapter show that DPPIV is not regulated directly by IL-1, OSM or TGF-β1. Therefore, there is the possibility that the DPPIV promoter is methylated in normal chondrocytes and the degradation of the ECM releases cryptic peptide fragments that bind to Toll-like receptors. This can lead to activation of intracellular pathways (Zhang et al., 2008) that result in the demethylation of the DPPIV promoter resulting in the increased gene expression in OA (Swingler et al., 2009).

4.3.2 Inhibition of DPPIV enzyme activity shows varied results

The inhibition of DPPIV enzyme activity in resorbing bovine cartilage shows mixed results with only K579 showing any inhibitory activity when added to resorbing cartilage. While the other inhibitors all show significant DPPIV inhibition *in vitro*, there is no statistically significant effect on collagen release when they are added to IL-1+OSM-treated bovine cartilage. An underlying issue could be that while the inhibitors show good *in vitro* inhibition, they cannot penetrate the cartilage matrix possibly due to binding to GAG or matrix proteins. Indeed, such a finding has previously been reported for MMP inhibitors (Janusz et al., 2006).

Recently, there has been a large increase in the number of DPPIV inhibitors that have been approved for clinical use to treat type II diabetes (Lankas et al., 2005). In this study, the authors highlighted that vildagliptin, in addition to standard treatments, stabilised glycaemic control in type II diabetes treatment compared to traditional treatments alone. Vildagliptin has now been approved for clinical treatment of type II diabetes in Europe (Yazbeck et al., 2009) and a recent meta-analyses study has shown that DPPIV inhibitors are well tolerated by patients (Ligueros-Saylan et al., 2010).

Vildagliptin is reported to be a slow binding inhibitor of DPPIV with a low K_i (Hughes et al., 1999). However, vildagliptin showed no efficacy in preventing collage release from IL-1+OSM-treated cartilage, but K579 treatment did show significantly reduced collagen release. The DPPIV inhibitor K579 has a similar structure to vildagliptin (Takasaki et al., 2004) but displays better inhibitory constants as K579 forms longer lasting complexes with DPPIV.

The *in vivo* efficacy of DPPIV inhibitors is greatly increased when supplied intravenously (De Meester et al., 1997), implying that the available inhibitor concentration at the target tissue is important. Therefore, if the other reversible inhibitors tested in this chapter, such as vildagliptin and UAMC-374, form short-lived complexes then their available concentration would a deciding factor for their potency in cartilage at physiological temperature. Brandt et al. (2005) showed incubating DPPIV with increasing concentration of vildagliptin showed an increase in the half-life of GLP-1 and other DPPIV substrates. Therefore, if the inhibitor cannot penetrate the cartilage matrix, this would decrease the concentration of available inhibitor near the cell membrane. Additionally, if the inhibitor only forms short-lived complexes, the half-life of DPPIV substrates would be decreased to levels similar to uninhibited DPPIV half-lives. Taken together this means that cartilage in the presence of an inhibitor that better penetrates the matrix and forms longer-lasting complexes, such as K579, could elicit different responses to inhibitors that cannot do either, possibly like vildagliptin. Therefore, K579 may affect the half-life of an unknown substrate and results in the protection of the cartilage matrix. But the other reversible inhibitors used in this study do not affect the half-life and therefore seem to have no effect in IL-1+OSM-treated cartilage.

K579 was shown to cause a decrease in pro- MMP levels seen, and this can be explained by a previous study showing that DPPIV inhibitors increase TGF-β1 production (Reinhold et al., 2006). One possible mechanism for the increased TGF-β1 production could be that DPPIV inhibitors could cause a conformational change and disrupt or prevent DPPIV binding to other cell surface proteins. Ishii *et al*. (2001) have shown that DPPIV co-localises with CD45 at lipid rafts and leads to the activation of ZAP70. Therefore, disruption of DPPIV-protein binding could elicit different responses, one consequence of this leading to increased TGF-β1 secretion. This phenomenon is likely to require DPPIV to still be bound to the membrane, as the addition of soluble DPPIV to IL-1+OSM-treated bovine cartilage did not show any increase in collagen release.

In addition to this signalling role, DPPIV could form a "super-complex" on the surface of the chondrocyte in a similar fashion to FAPα (section 3.3.3) (Figure 4.25). FAPα has been shown to localise with a number of molecules, such as MMP-2, MMP-14, uPAR and integrins (Monsky et al., 1994). Ghersi et al. (2002) highlighted that DPPIV and FAPα dimers associate to form a complex on the invadopodia of WI-38 lung fibroblasts as they migrate over a collagen matrix. Additionally, DPPIV has 52% sequence homology to FAPα (Goldstein et al., 1997) with a similar β-propeller domain fold (Aertgeerts et al., 2004). Therefore, it is possible that DPPIV could be in close proximity to the same proteins which in turn associate with other membrane proteins, such as uPAR binding the gp130 receptor (Koshelnick et al., 1997). However, there are data to suggest that DPPIV binds to other proteins not reported to bind FAPα. Plasminogen binds to DPPIV and promotes an invasive phenotype in prostrate carcinoma cells (Gonzalez-Gronow et al. 2005a, b) , and DPPIV has also been shown to bind collagen through a cysteine-rich region located in the β-propeller domain (Hanski et al., 1988, Loster et al., 1995). In particular, the binding of collagen by DPPIV is

important as it would increase the rate of collagen turnover in the proximity of this complex.

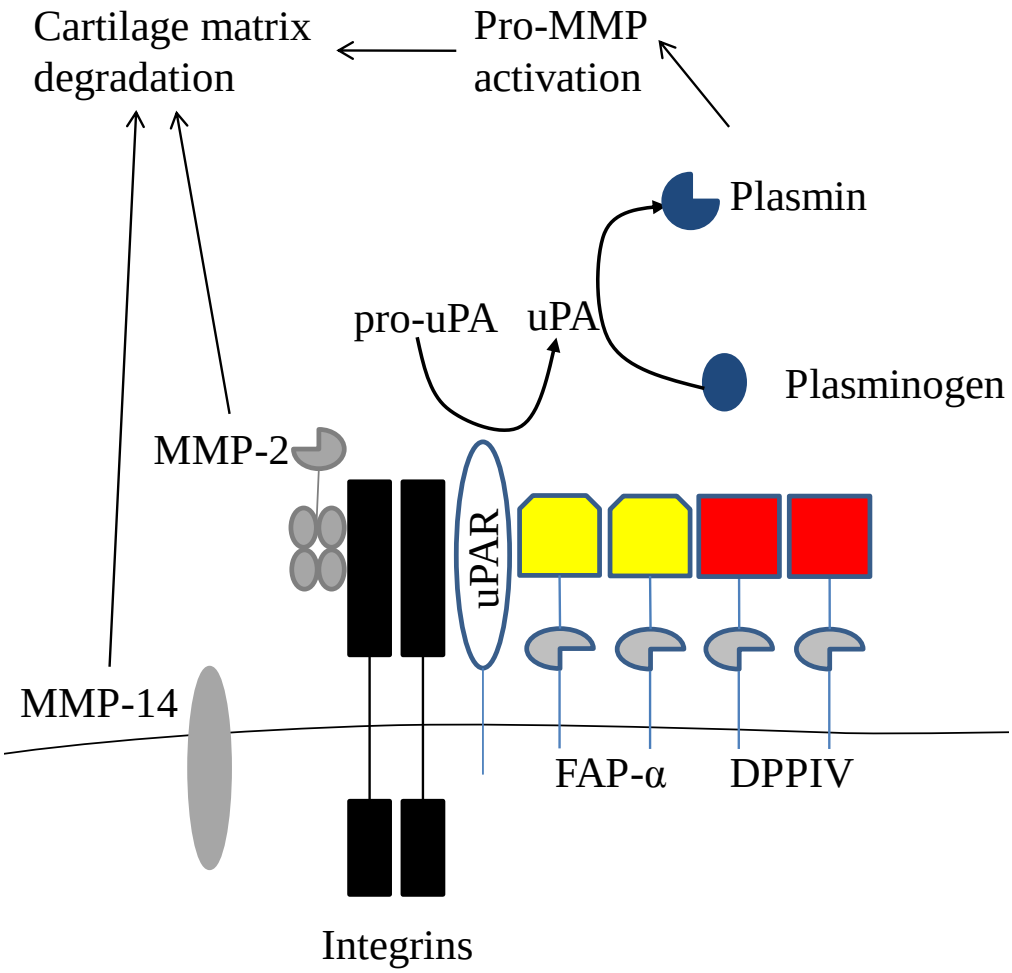


Figure 4.25 Model of a putative DPPIV "super-complex" on the chondrocyte cell surface

DPPIV has been shown to bind collagen (Hanski et al., 1988) and associate with FAPα to form a tetramer on the surface of melanoma cells (Ghersi et al., 2002). This would bring the membrane in close proximity to the collagen network. FAPα localises with α3β1 integrin (Zhang et al., 2003) and this is likely to increase association to the collagen network (Takada et al., 2007). Localisation of MMP-2 and MMP-14 (Monsky et al., 1994) as well as uPAR (Artym et al., 2002) to this area increases the rate of ECM proteolysis.

Through the mechanisms highlighted above, it does not seem likely that DPPIV plays any direct role on the proteolysis of matrix proteins. Rather, DPPIV acts either as a scaffold to localise catabolic factors to sites of tissue remodelling or facilitates the degradation of *de novo* fragments produced after the action of MMPs or other serine proteases. It is unlikely that DPPIV can degrade larger peptides or proteins as Asp-663 in the active site of DPPIV causes deprotonation of the glutamate-206 side chain that facilitates binding of charged amino terminal groups (Aertgeerts et al., 2005). In fact, larger peptides could be degraded by DPPIV only if they possess an unfolded N-terminal region that can be stabilised by the negative charge of the glutamate side-chain. However, conflicting data show that DPPIV can degrade type I through to type V collagen with approximately 50% of the collagen degraded within 24 hours (Bermpohl et al., 1998). The data presented in this chapter show that DPPIV cannot cleave type II collagen and conform to data from Aertgeerts et al. (2005) that DPPIV does not possess endopeptidase activity.

4.4 Summary

- DPPIV gene expression is not regulated by IL-1 or OSM or TGF-β1 in primary chondrocytes.
- K579 decreases cartilage matrix degradation in the presence of IL-1+OSM.
- K579 significantly reduces total collagenase levels from IL-1+OSM-treated cartilage.
- Exogenous active DPPIV does not expedite collagen breakdown from IL-1+OSM-treated bovine cartilage.
- DPPIV is unable to cleave denatured type II collagen.

CHAPTER 5

Elucidating the role of matriptase in cartilage homeostasis

5.1 Introduction

Recently, matriptase (section 1.7) expression was found to be significantly increased in OA compared to normal cartilage (Figure 3.1). Additionally, the increase in matriptase gene expression was detectable at the protein level as well.

Matriptase is a well characterised target in cancer as it can promote metastasis through a number of different mechanisms, such as activation of pro-hepatocyte growth factor (HGF) and pro-uPA (section 1.7.1.3). Furthermore, matriptase has been demonstrated to activate pro-MMP-3 (Jin et al., 2006), and addition of active MMP-3 to IL-1+OSM-treated cartilage has previously shown to expedite collagenolysis (Milner et al., 2001). Therefore, the hypothesis was that up-regulation of matriptase would increase cartilage matrix degradation.

The aim of this chapter was to:

- determine the role matriptase plays in collagen degradation of human OA cartilage.

5.2 Results

5.2.1 The effect of matriptase on IL-1+OSM-treated bovine cartilage

In the experiment shown (Figure 5.1), the addition of matriptase to IL-1+OSM-treated cartilage significantly increased collagen release compared to IL-1+OSM-treated cartilage alone. The addition of matriptase alone to bovine cartilage has been shown to cause no collagen release (data not shown).

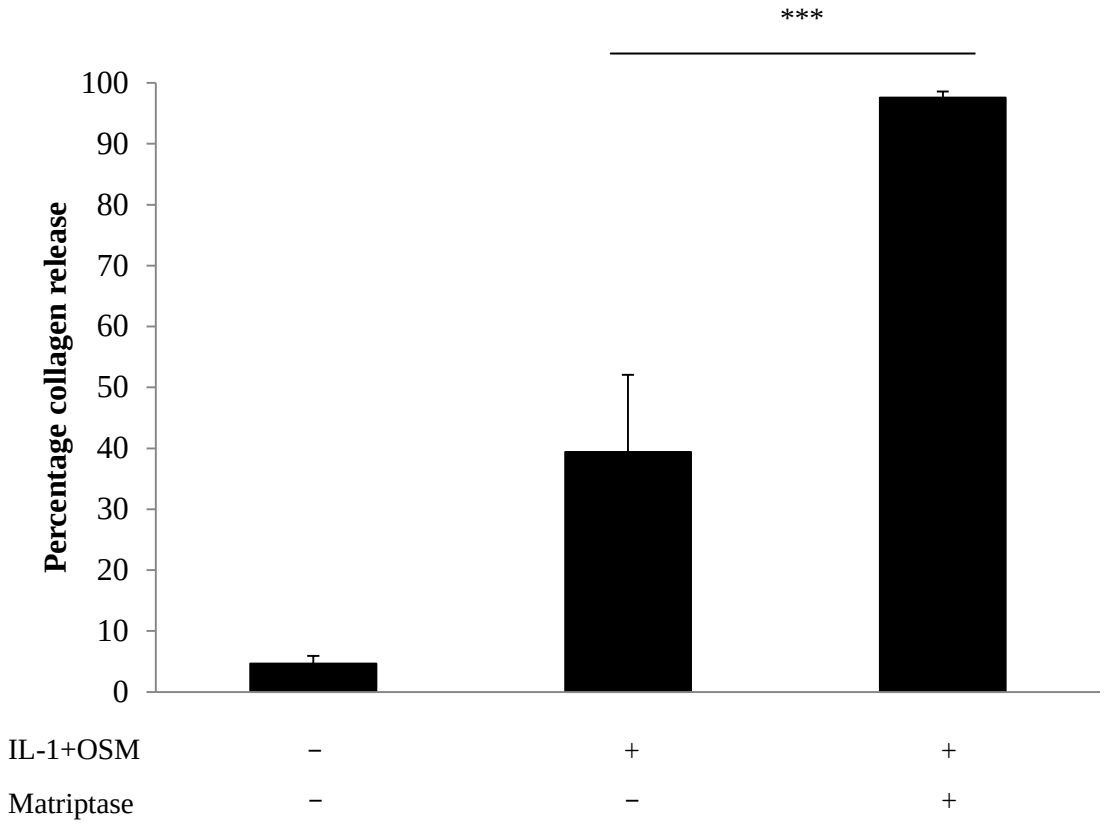


Figure 5.1 Matriptase expedites collagen release from IL-1+OSM-treated bovine cartilage

Bovine cartilage was cultured in medium ± matriptase (100 nM) ± IL-1+OSM (1 and 10 ng/ml, respectively). Media were removed on day 7 and fresh reagents were added until day 12 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 12 media and in cartilage digests. Shown is the cumulative collagen release (day 7 + day 12) expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=6) and representative of 4 separate experiments. *** = $p < 0.001$.

5.2.2 Matriptase-mediated activation of pro-MMPs

The addition of matriptase to IL-1+OSM-treated cartilage expedited collagen release (Figure 5.1). Milner et al. (2006a) highlighted that IL-1+OSM treatment produces a large quantity of pro-MMPs from day 3 but activation of these does not typically occur until after day 10.

Jin et al. (2006) has reported that matriptase activates pro-MMP-3. However, there is no evidence reported for matriptase-activation of other pro-MMPs. The increased collagen release by matriptase was likely to be due to the activation of a pro-collagenase or pro-collagenase activator, such as MMP-3. Therefore, I assessed if matriptase could directly activate pro-MMP-1or -13 to cause the increase in collagen release.

5.2.2.1 MMP activity after incubation with matriptase

In both experiments matriptase-activated MMP-1 showed significantly less activity than APMA-activated MMP-1. This indicates that matriptase does activate pro-MMP-1; however, the resulting MMP-1 species is less active than the one generated by APMA (Figure 5.2). Matriptase was not able to cleave either substrate tested in this experiment (data not shown).

There was little change between APMA-activated MMP-3 and matriptase-activated MMP-3, when tested with FS-6 (data not shown). Matriptase was not able to activate pro-MMP-13 (Dr J. M. Milner, personal communication).

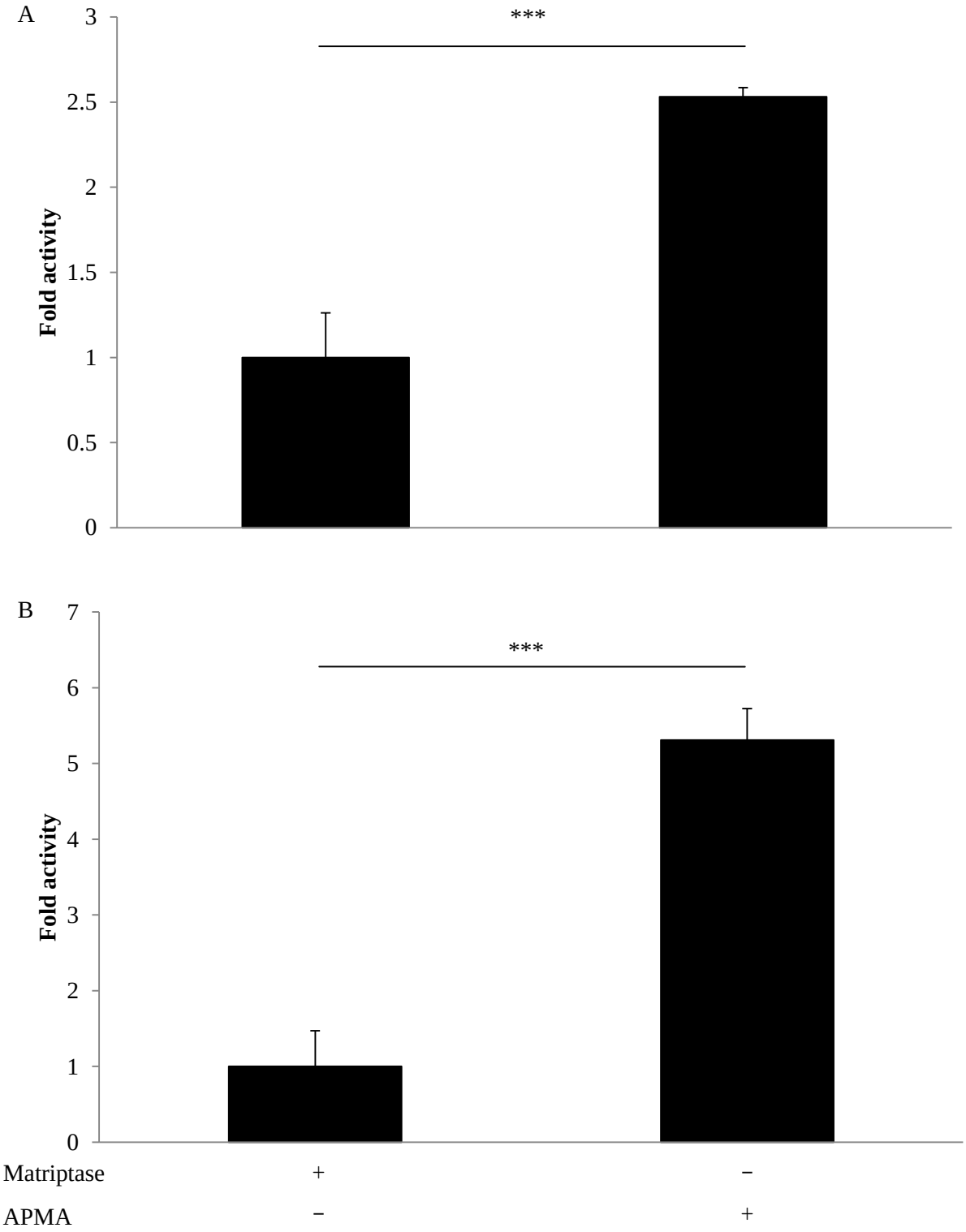


Figure 5.2 Influence of matriptase on MMP-1 activity *in vitro*.

Pro-MMP-1 (1.4 µM) was incubated ± matriptase (100 nM) or ± APMA (0.67 mM) for 4 h at 37°C. Activated MMP-1 was then incubated with either A) FS-6 (50 µM) or B) hide powder azure (3% w/v). The data from two separate experiments are shown and presented as activity relative to matriptase-activated MMP-1. Results are expressed as mean ± SD (n=5). *** = $p < 0.001$

5.2.2.2 Direct activation of MMPs by matriptase *in vitro*

Matriptase has been previously reported to activate pro-MMP-3 (Jin et al., 2006), but the exact site(s) of matriptase cleavage has not been elucidated. The general metalloprotease inhibitor GM6001 was used to abrogate MMP activity, therefore any cleavage detected in the presence of GM6001 would be due to matriptase activity. In the presence of GM6001 matriptase was found to cleave MMP-3 after arginine-37, -74 and -84 (Figure 5.3). Cleavage after arginine-37 and -84 by matriptase is to be expected as they are the consensus cleavage sites for other members of the S1 serine protease family, specifically plasma kallikrein and trypsin, respectively (Woessner and Nagase, 2000 and references therein). Cleavage after arginine-74 occurs within the cysteine switch domain. In the absence of GM6001, the only sequence found was that of the full-length active protease, starting at phenylalanine-83, indicating the presence of some active MMP in the preparation. This corroborates the result seen in section 5.2.2.1 as MMP-3 auto-activation occurs at a faster rate than matriptase activation of MMP-3. Therefore, there would be no difference between APMA-activated and matriptase-activated pro-MMP-3 when tested with FS-6, as the fully active species would be generated in both cases.

Matriptase only cleaved MMP-1 after arginine-72 in the presence of GM6001 (Figure 5.3); again showing that matriptase is able to cleave within the cysteine switch domain of MMPs. In the absence of GM6001, cleavage after threonine-64 and phenylalanine-81 occurred as reported previously (Woessner and Nagase, 2000 and references therein).

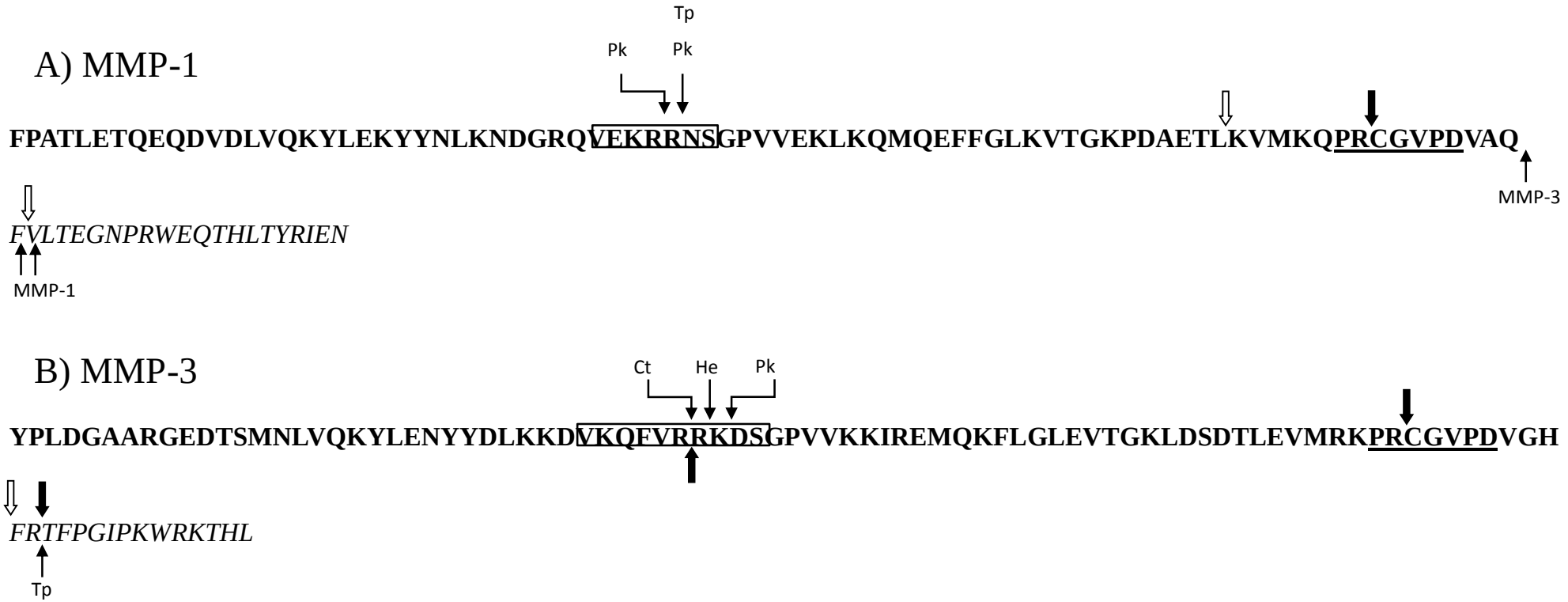


Figure 5.3 Activation of pro-MMPs by matriptase.

N-terminal sequence analyses were performed on MMP-1 and -3 after 1 – 4 h of incubation with matriptase. The data presented show several known serine protease cleavage sites (Woessner and Nagase, 2000) within the propeptide regions. The bait region is boxed whilst the cysteine switch region is underlined, whilst the sequence in *italics* represents the full-length mature MMP. Cleavage sites marked with large closed arrows are for matriptase (+GM6001), whilst those denoted by the large open arrows are matriptase (-GM6001). Ct = chymotrypsin; He = human neutrophil elastase; Pk = plasma kallikrein; Tp = trypsin.

5.2.3 The effect of matriptase on human OA cartilage

The activation of pro-MMP-1 and -3 by matriptase causes increased collagen release from IL-1+OSM treated cartilage (Figure 5.1). Although Cawston et al. (1998) has shown that treatment of OA cartilage with IL-1+OSM is unable to stimulate collagen release, this is probably due to a lack of pro-MMP activation as there are large quantities of MMP-1 produced. Therefore, matriptase was added to human OA cartilage in explant culture in an attempt to stimulate collagen release as it would be able to activate pro-MMPs.

5.2.3.1 The effect of matriptase on collagen release from OA cartilage

Addition of matriptase to human OA cartilage resulted in significant collagen release at day 7 and day 14 (Figure 5.4).

The addition of IL-1 and OSM to human OA cartilage showed no significant collagen release at day 7 or at day 14. These data were in-line with previous literature stating that only about 25% of all OA cartilage responds to pro-inflammatory cytokine stimuli (Cawston et al., 1998). However, the addition of matriptase to IL-1+OSM-treated cartilage caused significant collagen release at both time points (Figure 5.4). Interestingly, the collagen release was very similar between untreated and IL-1+OSM-treated cartilage incubated with matriptase implicating that the collagen release mediated by matriptase was independent of the pro-inflammatory stimulus.

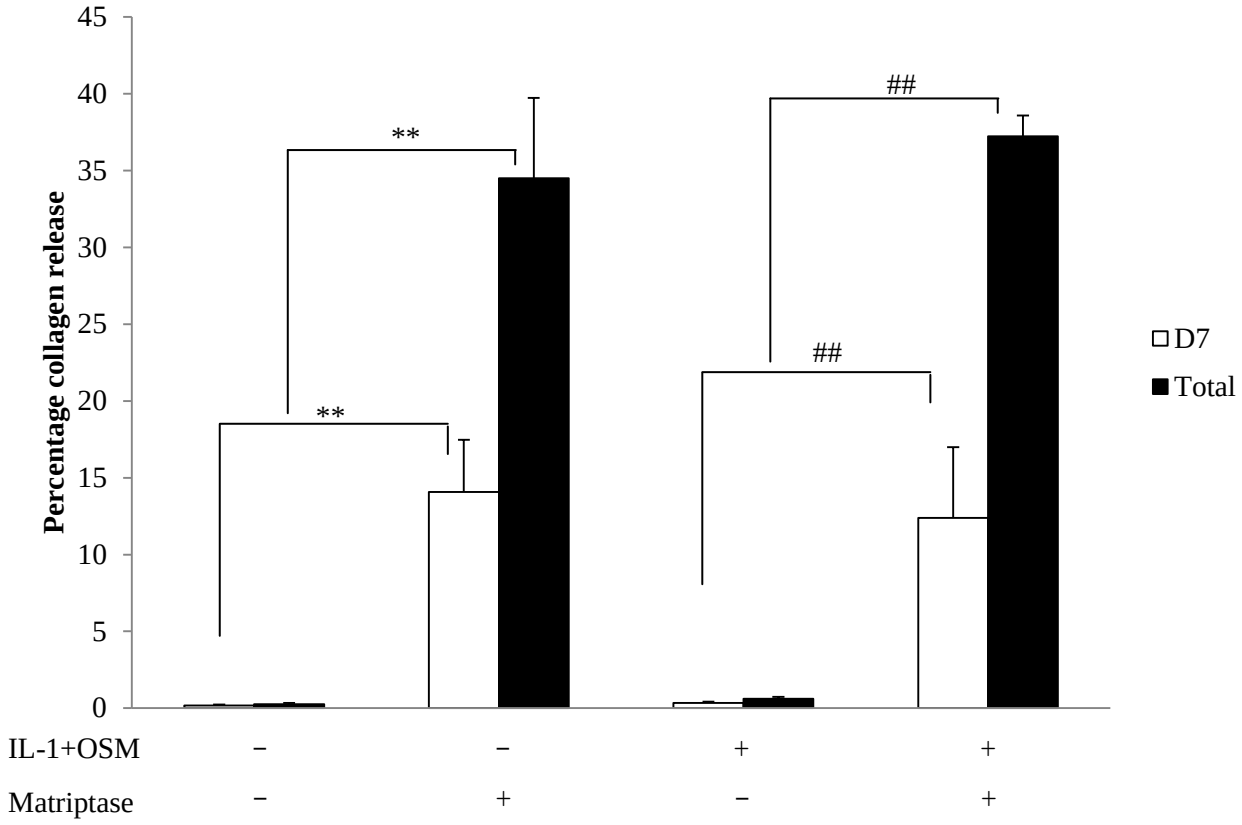


Figure 5.4 The effect of matriptase on collagen release from human OA cartilage. Human OA cartilage was cultured in medium ± matriptase (100 nM) ± IL-1+OSM (1 and 10 ng/ml, respectively). Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown are the collagen release at day 7 and cumulative collagen release expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 4 separate experiments. ** = $p < 0.01$ against untreated and ## = $p < 0.01$ against IL-1+OSM-treated cartilage.

5.2.3.2 The effect of matriptase on collagenase activity in OA cartilage

Untreated OA cartilage showed no increase in active or total collagenase activity at day 7 or day 14. However, the addition of matriptase to the culture medium showed a significant increase in active and total collagenase activity at day 7 (Figure 5.5a) and correlated with the increase in collagen release (Figure 5.4). A similar increase was not seen at day 14 for either active or total collagenase levels (Figure 5.5b), even though an increase in collagen release could be observed.

IL-1+OSM-treated OA cartilage showed significant levels of total collagenase activity at both day 7 and day 14, but little or no detectable active collagenase activity was observable at either time point. This result shows that while pro-MMPs are synthesised by OA chondrocytes, they are not activated to cause collagenolysis. This is in-line with previously published findings that show a minority of all OA cartilages respond to treatment with IL-1+OSM (Cawston et al., 1998). The addition of matriptase to IL-1+OSM-treated cartilage showed an interesting trend. At day 7, a significant increase in active collagenase levels was noted and this corresponds with the increase in collagen release (Figure 5.4). However, total collagenase activity was significantly decreased at day 7 when compared to IL-1+OSM-treated cartilage. 15.55 ± 1.86 units/ml of total collagenase activity were in the media from IL-1+OSM+matriptase-treated cartilage, in comparison to the 30.71 ± 5.81 units/ml in the media from IL-1+OSM treated cartilage. There was a slight difference in active collagenase levels at day 14 but this did not reach significance. The total collagenase activity in both media samples showed no observable difference.

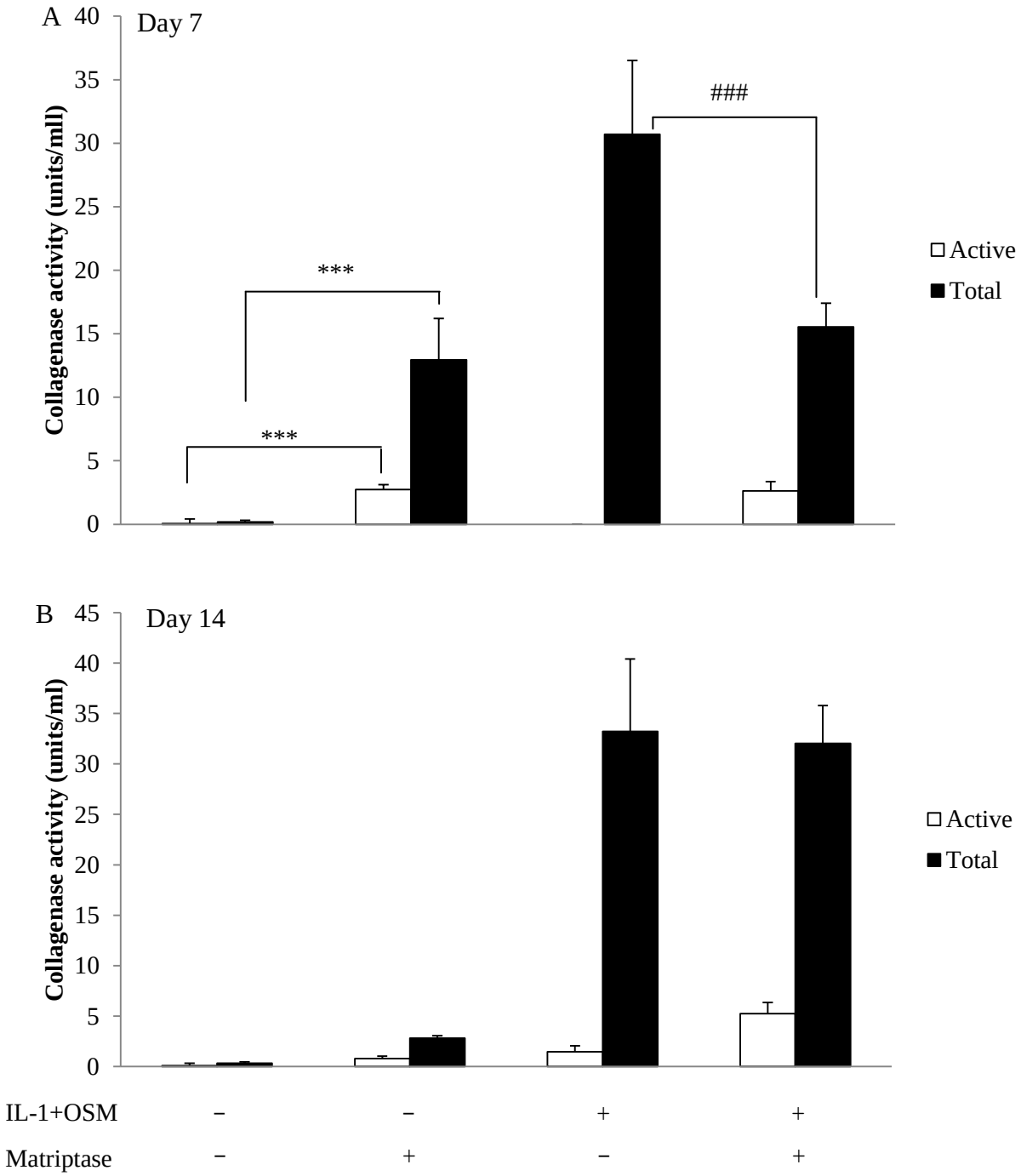


Figure 5.5 The effect of matriptase on collagenase activity from human OA cartilage.

Media from matriptase-treated cartilage shown in Figure 5.4 were assayed for collagenase activity. Media were treated with APMA to test for total collagenase levels (pro and active). A) Active and total collagenase activity for day 7 media and B) active and total collagenase activity for day 14 media. Results are expressed as mean ± SD (n=4) and representative of 2 separate experiments. *** = $p < 0.001$ against untreated and ### = $p < 0.001$ against IL-1+OSM-treated cartilage.

5.2.3.3 The effect of matriptase on MMP-1 and MMP-13 protein levels in OA cartilage

The addition of matriptase to untreated cartilage caused a significant increase in MMP-1 protein levels at both day 7 and 14 (Figure 5.6a), but there was no significant change in the MMP-13 levels at either time point (Figure 5.6b). Treatment of cartilage with IL-1+OSM caused a significant increase in both MMP-1 and MMP-13 protein levels at day 7 and 14. This result corroborates findings with the collagenase activity (section 5.2.3.2).

When matriptase was added to IL-1+OSM-treated cartilage, there was a significant decrease in MMP-1 levels at both day 7 and 14, but little change in MMP-13 protein levels at both time points. The low protein level observed for MMP-1 at day 7 may explain the significant decrease in collagenase activity (Figure 5.5).

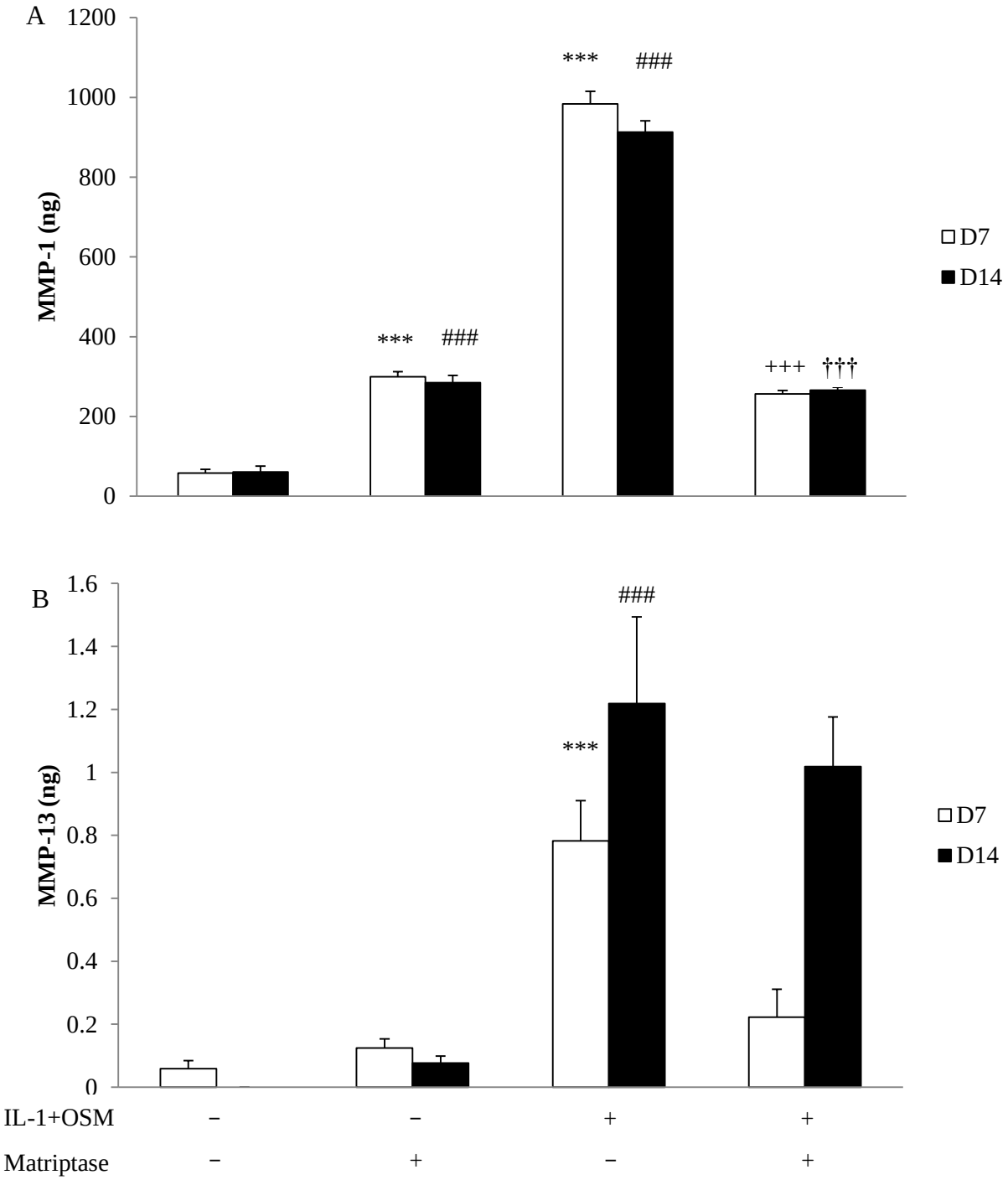


Figure 5.6 The effect of matriptase on total collagenase protein levels from human OA cartilage.

Media from matriptase-treated cartilage shown in Figure 5.4 were assayed for total collagenase protein levels. A) MMP-1 and B) MMP-13 protein levels were determined by sandwich ELISA. Results are expressed as mean ± SD (n=4) and representative of 2 separate experiments. *** = $p < 0.001$ against day 7 untreated cartilage, ### = $p < 0.001$ against day 14 untreated cartilage, +++ = $p < 0.001$ against day 7 IL-1+OSM-treated cartilage and ††† = $p < 0.001$ against day 14 IL-1+OSM-treated cartilage

5.2.4 The effect of metalloprotease and cathepsin K specific inhibitor on matriptase-mediated cartilage degradation

The aim of these experiments was to test if a novel type II collagenase, such as cathepsin K (Dejica et al., 2008), is mediating the degradation of the matrix or if this was solely MMP dependent. GM6001 is a metalloprotease-specific inhibitor with *in vitro* MMP inhibition in the low nanomolar range (Levy et al., 1998). L-873724 is a selective cathepsin K inhibitor that has an IC_{50} in the sub-nanomolar range (Li et al., 2006).

Addition of GM6001 to matriptase-treated OA cartilage (Figure 5.7) resulted in significantly decreased collagen release at day 7 and day 14. But GM6001 negative control did not affect collagen release (data not shown).

Cathepsin K is a cysteine protease that can cleave the triple helix of type II collagen (Kafienah et al., 1998). Previous work implicates that it has a role in collagen turnover in OA (Dejica et al., 2008). The inhibitor L-873724 is a potent inhibitor of cathepsin K (Li et al., 2006) and has been shown to decrease the generation of type II collagen fragments *in situ* (Dejica et al., 2008). The addition of L-873724 to OA cartilage caused no change in collagen release at day 7 or day 14 (Figure 5.7).

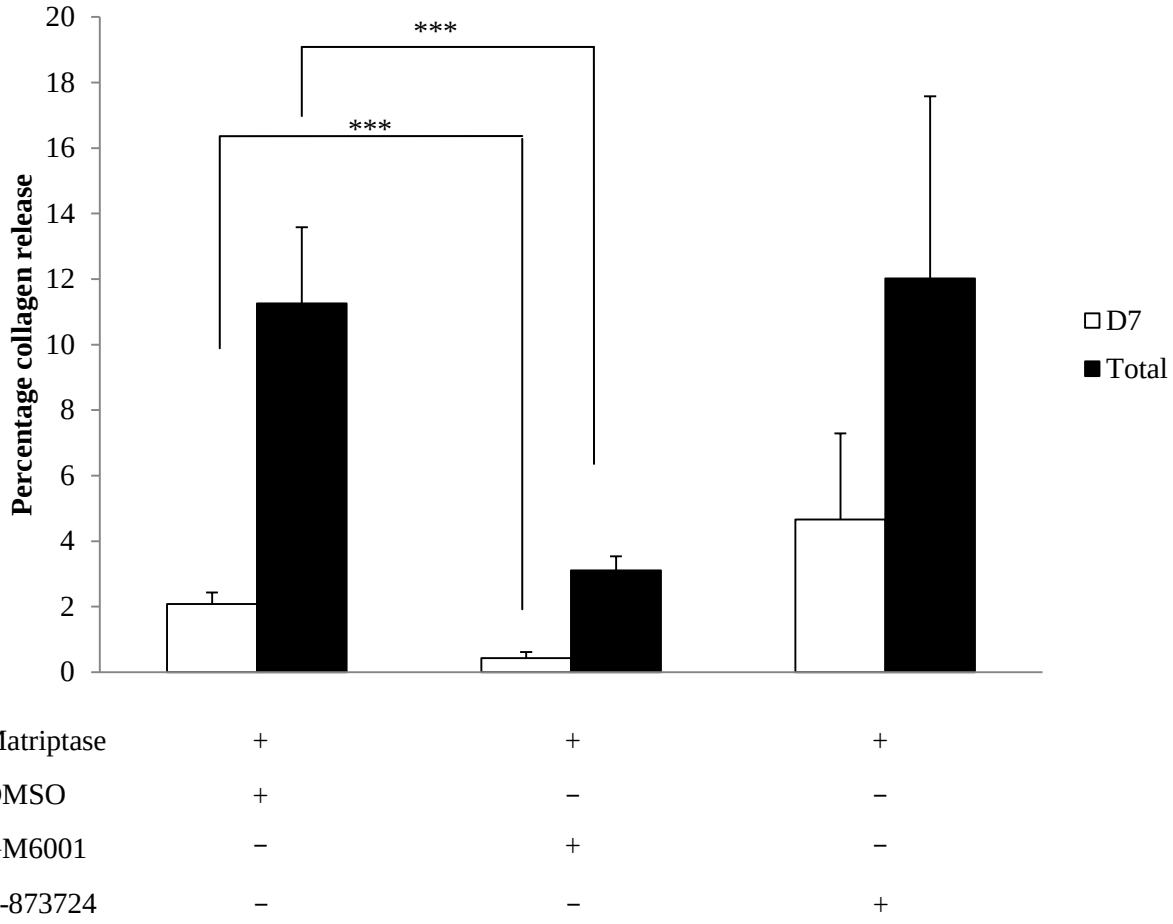


Figure 5.7 The effect of MMP and cathepsin K specific inhibitors on matriptase mediated collagen release.

Human OA cartilage was cultured ± matriptase (100 nM). The general MMP inhibitor GM6001 (10 µM) and the cathepsin K inhibitor L-873724 (10 nM) were added to the cartilage at days 0 and 7. DMSO was added as a solvent control. Media were removed on day 7 and fresh reagents added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown are the collagen release at day 7 and cumulative collagen release expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 4 separate experiments. *** = $p < 0.001$

5.2.5 Matriptase induced gene expression in OA cartilage

Matriptase is able to cause collagen release in the absence of pro-inflammatory stimuli (Figure 5.4) and significantly increase collagenase levels (Figure 5.5 and 5.6) from OA cartilage. This collagen release has been shown to be metalloprotease-dependent (Figure 5.7). The aim of these experiments was to assess if matriptase activity could directly induce gene expression in OA cartilage.

The addition of matriptase to "live" cartilage showed significant collagen release as seen previously (Figure 5.8), but the addition of matriptase to "dead" cartilage did not show any increase in collagen release compared to untreated cartilage.

Treatment of OA cartilage with matriptase significantly increased MMP-1 and MMP-3 mRNA levels compared to control cartilage (Figure 5.9). There was an increase in MMP-13 mRNA levels but this did not reach significance. This increase in MMP-1 and MMP-3 gene expression indicates that matriptase is able to activate cell signalling cascades either directly or indirectly.

The addition of di-isopropyl phosphorofluoridate (DFP)-inactivated matriptase to OA cartilage did not cause an increase in any MMP mRNA levels (data not shown). This result highlights that matriptase activity is required to cause collagenase gene expression in the absence of pro-inflammatory stimuli.

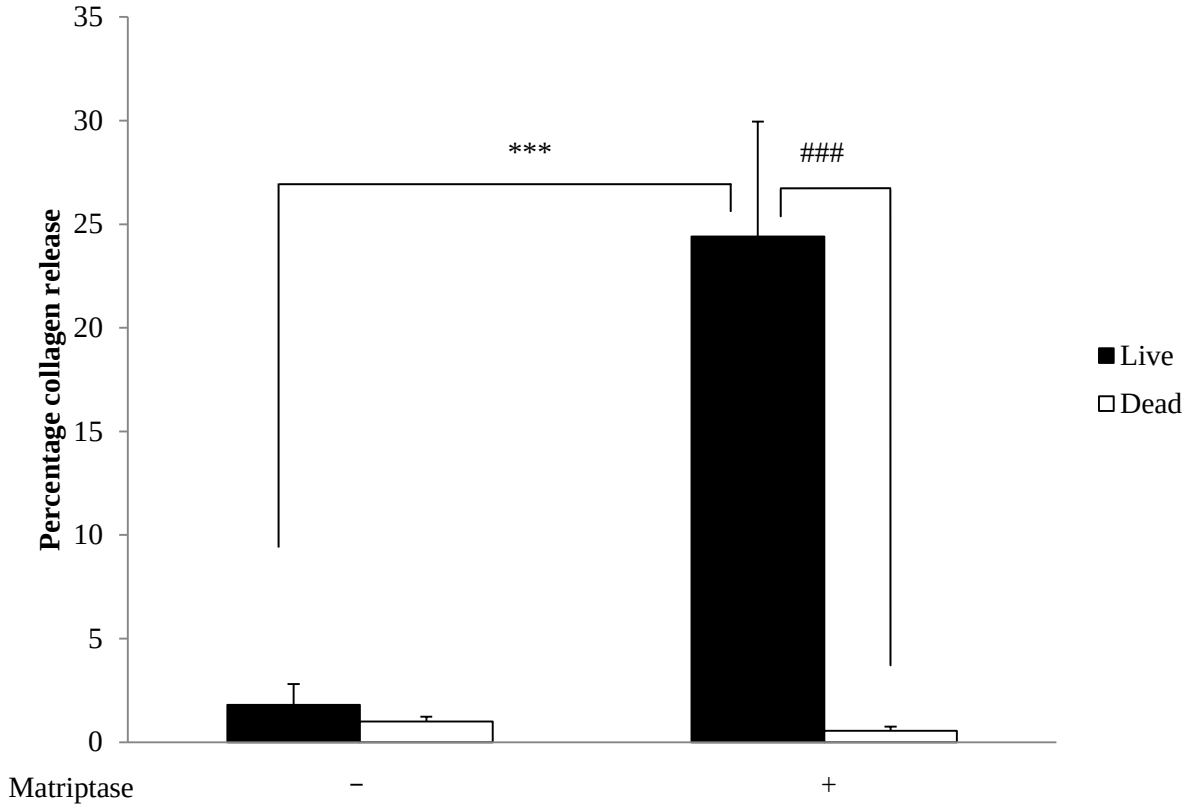


Figure 5.8 Intracellular signalling is required for matriptase-mediated degradation of OA cartilage.

OA cartilage was isolated on the day of surgery and either placed immediately at 37°C (Live) or freeze-thawed three times (Dead) and then left at 37°C for 24 h. Cartilage ± matriptase (100 nM) was left to culture for 7 days. Shown is the collagen release at day 7 with collagen release expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 1 experiment. *** = $p < 0.001$ against control, ### = $p < 0.001$ against live cartilage.

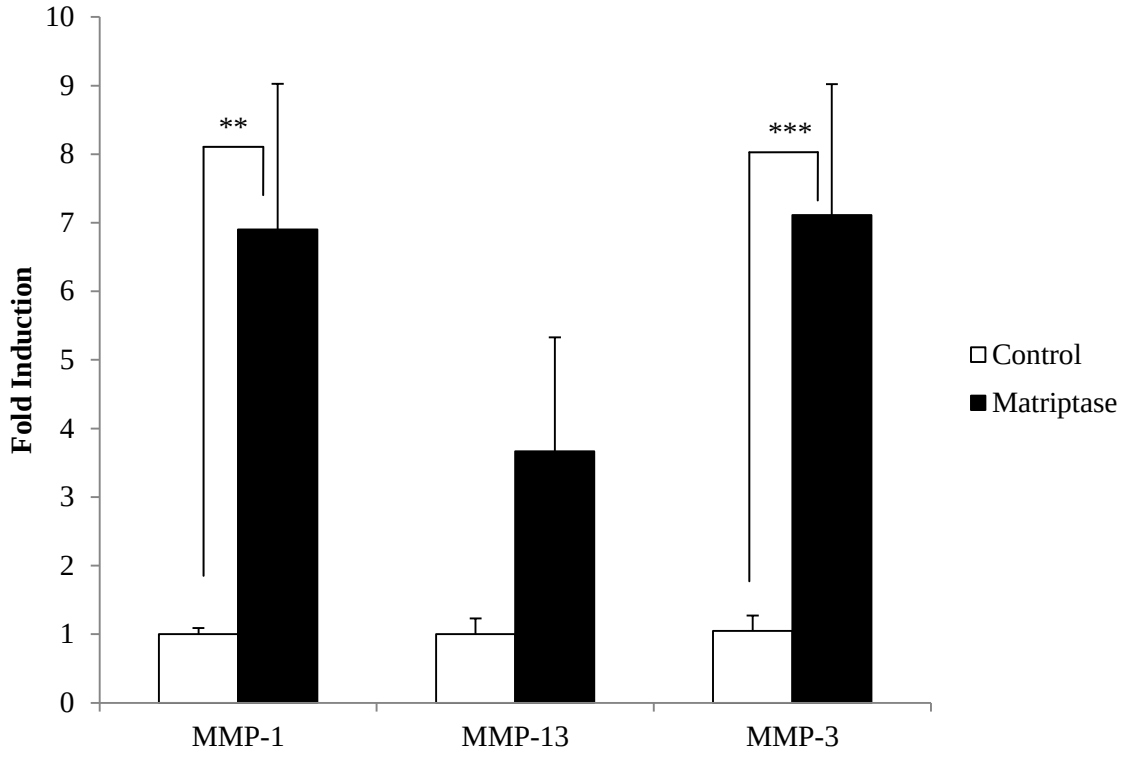


Figure 5.9 Matriptase induced MMP gene expression in OA cartilage.

OA cartilage was treated with matriptase (100 nM) for 7 days. Total RNA was extracted and MMP-1, -3 and -13 gene expression was determined by real-time PCR. 18S rRNA levels were determined for normalisation. The data from 2 separate experiments are shown and presented as fold induction relative to control. Results are expressed as mean ± SEM (n=4). ** = $p < 0.01$ and *** = $p < 0.001$.

5.2.6 Matriptase activates PAR-2 in OA cartilage to mediate the degradation of the collagen matrix

In the absence of pro-inflammatory cytokines, matriptase alone could not induce significant collagen degradation in bovine cartilage (Dr J. M. Milner, personal communication) or human NOF cartilage (Figure 5.10). Therefore, matriptase must induce collagenase gene expression (Figure 5.9) through activation of a receptor found only in OA cartilage.

PAR-2 is a well characterised substrate for matriptase (Takeuchi et al., 2000, Wang et al., 2008) and activation of PAR-2 has been shown to increase MMP expression in OA osteoblasts (Amiable et al., 2009). As PAR-2 expression is reported to be up-regulated in OA cartilage (Xiang et al., 2006), I hypothesised that matriptase-driven cartilage degradation was mediated through PAR-2 activation.

Addition of matriptase to the cartilage resulted in significant collagen release at day 7 as previously noted (Figure 5.11). The addition of both PAR-2 inhibitors significantly reduced the observed matriptase-mediated collagen release almost to basal levels, indicating that matriptase does act through PAR-2 to mediate breakdown of OA cartilage.

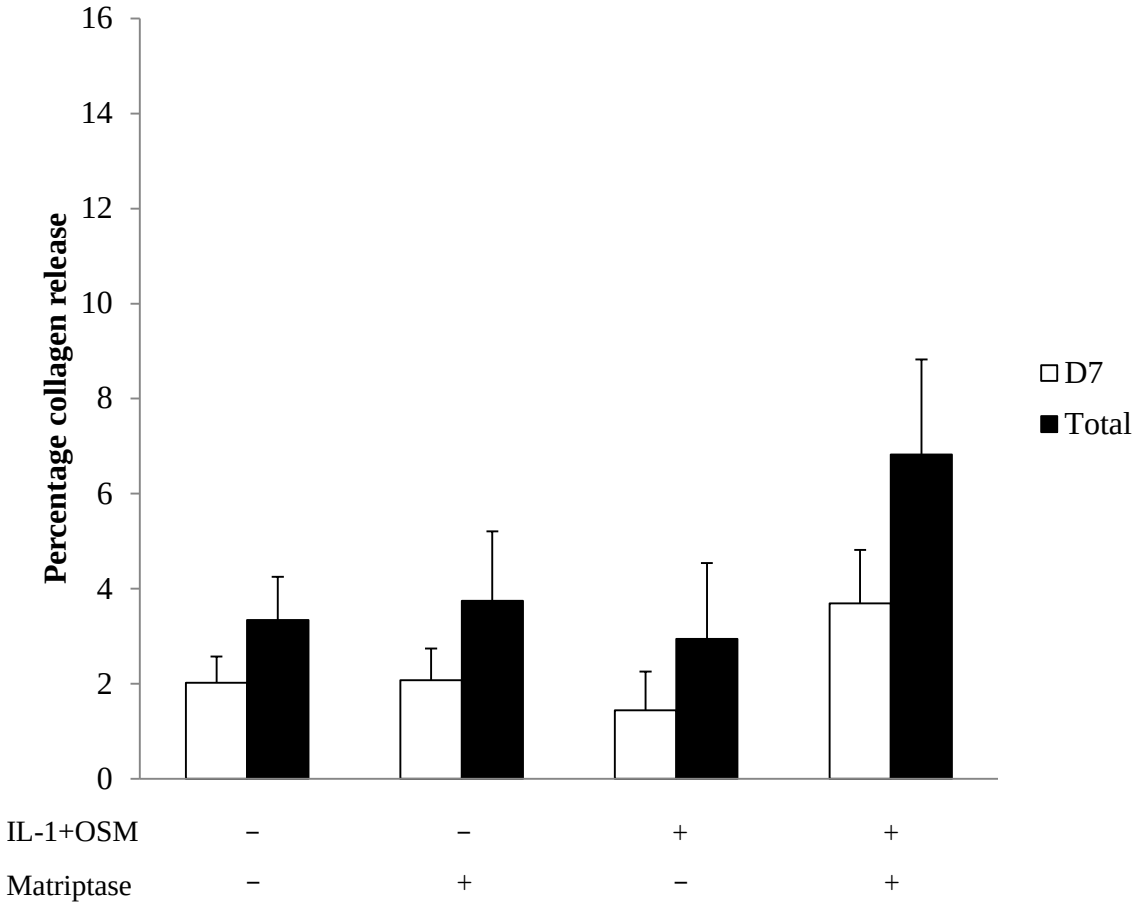


Figure 5.10 The effect of matriptase on collagen release from human NOF cartilage

Human NOF cartilage was cultured in medium ± matriptase (100 nM) ± IL-1+OSM (1 and 10 ng/ml, respectively). Media were removed on day 7 and fresh reagents were added until day 14 when the experiment was ceased. As a measure of collagen release, the levels of hydroxyproline were assayed in day 7 and day 14 media and in cartilage digests. Shown are the collagen release at day 7 and cumulative collagen release expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 1 experiment.

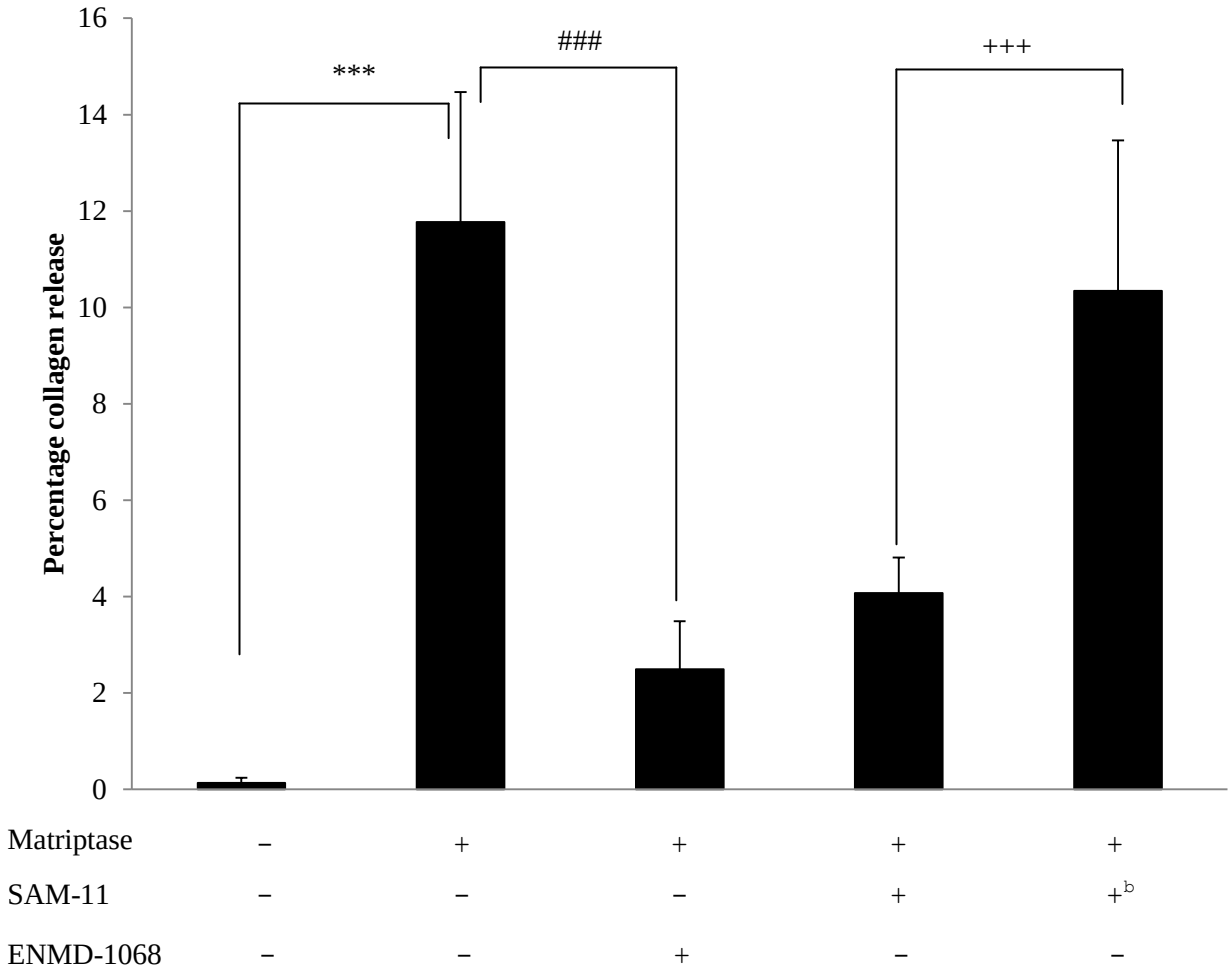


Figure 5.11 The effect of PAR-2 specific inhibitors on matriptase-mediated collagen release.

Human OA cartilage explants were pre-incubated ± SAM-11 (400 ng/ml final concentration) or the same antibody that had been previously heat-denatured (SAM-11^b), or ± ENMD-1068 (10 mM final concentration) for 72 h. Subsequently, matriptase (100 nM) was added such that the SAM-11 antibody and ENMD-1068 were at final concentrations of 200 ng/ml and 5 mM, respectively, for 7 days. Shown is the collagen release at day 7 and collagen release expressed as a percentage of the total collagen. Results are expressed as mean ± SD (n=4) and representative of 3 separate experiments. *** = $p < 0.001$ against control, ### = $p < 0.01$ against matriptase-treated cartilage and +++ = $p < 0.001$ against heat-denatured antibody control.

5.3 Discussion

5.3.1 Matriptase activation of pro-MMPs

Milner et al. (2001) showed that activation of the pro-collagenases in IL-1+OSM-treated cartilage was a key rate-limiting step in collagen breakdown. Plasminogen addition at day 0 to IL-1+OSM-treated bovine cartilage resulted in increased breakdown of the collagen network by day 7 compared to IL-1+OSM alone. Data in this chapter show that addition of matriptase at day 7 accelerated collagen release from IL-1+OSM-treated bovine cartilage. Therefore, matriptase is likely to activate pro-MMPs that are synthesised upon stimulation with IL-1+OSM and this expedites collagen release.

Jin et al. (2006) demonstrated that matriptase could directly activate pro-MMP-3 within 3 h. However, they found that matriptase non-specifically degraded pro-MMP-1, which conflicts with the data presented here. Our group found that pro-MMP-1 was not degraded in a non-specific manner in the presence of matriptase over 24 h (Dr J. M. Milner, personal communication) and has significantly greater activity compared to pro-MMP-1 alone (data not shown). In fact, N-terminal sequencing showed that matriptase activated MMP-1 and MMP-3 by cleaving the Arg-Cys bond in the cysteine switch region. After activation of pro-MMP-3 by matriptase, active MMP-3 can then cleave the N-terminus of pro-MMP-1 to generate a species that has higher activity (He et al., 1989, Murphy et al., 1987, Windsor et al., 1993). These data indicate that matriptase can directly activate pro-MMP-1 and pro-MMP-3. This in turn can lead to degradation of the collagen matrix as MMP-1 can directly cleave type II collagen (section 1.5.2) and MMP-3 can directly activate other pro-collagenases as well as other pro-MMPs (section 1.5.3).

5.3.2 Effect of matriptase on human OA cartilage

The breakdown of human OA cartilage by matriptase was described in this chapter. Human OA cartilage has previously been described to be highly resistant to pro-inflammatory-mediated breakdown despite increased expression of collagenolytic MMPs (Cawston et al., 1998). Although this observation could never be fully explained, it is likely that there is a failure in MMP activation.

Addition of matriptase to OA cartilage led to a significant increase in collagen release. These data show that matriptase activity in IL-1+OSM-treated cartilage is in-line with previous studies from our group (Milner et al., 2001, 2006b, 2003). However, the significant increase in collagen release by matriptase in the absence of pro-inflammatory cytokines is an extremely important finding. Matriptase represents the most potent stimulus for OA cartilage described to date. In the absence of pro-inflammatory cytokines, matriptase-mediated matrix breakdown could be due to the activation of latent growth factors or proteases. Previous *in vitro* data have shown that pro-HGF, pro-uPA and PAR-2 are matriptase substrates (Lee et al., 2000, Takeuchi et al., 2000), and further studies have shown that HGF induced MMP expression in endothelial (Wang and Keiser, 2000) and cancer cells (Monvoisin et al., 2002). The matriptase-mediated increase in collagenase activity and MMP protein levels in the absence of pro-inflammatory cytokines does highlight the possibility that activation of bioactive molecules might play an important role.

5.3.3 Matriptase induced collagen release is dependent on MMP activity

The addition of pro-inflammatory cytokines to human OA cartilage showed a significant increase in both total collagenase activity and protein levels. However, when matriptase was added in the presence of pro-inflammatory cytokines, there was a significant decrease in MMP-1 protein levels and collagenase activity. These data

implicate that the degradation of type II collagen could be mediated through a novel collagenase.

Kafienah et al. (1998) demonstrated that cathepsin K, a cysteine protease, could cleave native type I and II collagens and that this cleavage occurred at the N-terminus of the triple helix. Further data generated by Dejica et al. (2008) showed that a cathepsin K-specific neoepitope was detectable in OA cartilage. However, the data presented here indicate that matriptase-mediated collagen release was dependent on MMP activity and that inhibition of cathepsin K had little effect.

5.3.4 Matriptase directly induces gene expression

The data generated so far show that matriptase is a potent mediator of collagen degradation in OA cartilage. So far, the activation of pro-MMPs has been highlighted as one mechanism matriptase acts through to effect this collagen degradation. However, data show that matriptase can stimulate similar levels of collagen release in the presence or absence of pro-inflammatory cytokines. In addition, the MMP-1 protein levels are similar between the two conditions. The data presented here support intracellular signalling to be a key requirement for matriptase-mediated collagen degradation.

MMP-1 and MMP-3 gene expression are significantly up-regulated upon the addition of matriptase. MMP-13 expression is also up-regulated, although this was not statistically significant. As stated earlier, HGF is shown to increase MMP expression in a number of different cell types (Monvoisin et al., 2002, Wang and Keiser, 2000) and is activated by matriptase (Lee et al., 2000). Increased levels of HGF have been shown in OA cartilage (Pfander et al., 1999) and HGF can cause induction of MMP-13 gene transcription through the activation of MAPK pathways (Reboul et al., 2001). However, Guevremont et al. (2003) showed that HGF is not expressed by OA chondrocytes but by subchondral bone osteoblasts and diffuses from there to the radial zone of the cartilage.

Pro-uPA is another substrate activated by matriptase (Lee et al., 2000) and plays an important role in arthritis (Li et al., 2005, Busso and So, 1997). Matriptase activating pro-uPA is a possible mechanism that could cause the observed collagen breakdown in OA cartilage. However, data from our group have shown that addition of plasminogen to OA cartilage has little effect on collagen release without pro-inflammatory stimuli (Milner et al., 2001). Furthermore, unpublished data from our group show that plasminogen is not expressed by OA cartilage (Dr J. M. Milner, personal communication). These data indicate that in our cartilage explant model the uPA-plasmin system would cause neither collagen release nor *de novo* synthesis of MMP directly, but would be able to activate any pro-MMP present.

Previous reports have shown that activation of PAR-2 by a PAR-2-activating peptide induces MMP-1 and MMP-13 gene expression in OA chondrocytes (Boileau et al., 2007), correlating with the results presented in this chapter.

5.3.5 Matriptase activates PAR-2 in OA cartilage

The induction of collagenase gene expression by matriptase must involve the activation of intracellular signalling pathways to initiate gene transcription. Reports in the literature show that the expression of PARs (section 1.9.1) is significantly higher in OA cartilage compared to normal cartilage. One report states that all four known PARs are expressed by OA chondrocytes (Kirilak et al., 2006) while another just detected PAR-2 expression (Xiang et al., 2006).

PAR-2 activation involves proteolytic release of a tethered ligand which can be mimicked by use of a PAR-2-activating peptide (Hollenberg et al., 1997). Takeuchi et al. (2000) demonstrated that matriptase activated PAR-2 using PAR-2 overexpressing *Xenopus* oocytes but not with any other PAR expressing *Xenopus* oocytes. Our group confirmed that matriptase can activate PAR-2 *in vivo* (Prof. A. D. Rowan, personal

communication) using PAR-2 knock-out mice in synovial perfusions assays as described previously for β-tryptase (Palmer et al., 2007).

ENMD-1068 is a synthetic inhibitor that has been shown to inhibit proteolytic activation of PAR-2 (Kelso et al., 2006) and decreased IL-1β and TNF-α expression from rheumatoid synovium (Kelso et al., 2007). The use of ENMD-1068 or a PAR-2 neutralising antibody (SAM-11) significantly blocked matriptase-induced collagen release, further substantiating that PAR-2 activation is required for matriptase-mediated collagenolysis.

Finally, when matriptase was added to phenotypically normal cartilage, there was no detectable change in collagen release even in the presence of pro-inflammatory stimuli. As PAR-2 expression is higher in OA than normal chondrocytes (Kirilak et al., 2006), these data indicate that the activation of PAR-2 is a key point in arthritis.

The destabilisation of the medial meniscus (DMM) surgical murine model of OA has been reported to result in arthritic lesions similar to those observed in aged mice (Glasson et al., 2007). Due to the sensitivity of the DMM model in regards to disease modification, this model has been proposed to be a good choice for challenging gene knock-out mice with OA when compared to established models such as anterior cruciate ligament transection. Using this model, Ferrell et al. (2010) highlighted that PAR-2-deficient mice do not exhibit any signs of pathology compared to wild-type mice.

Interestingly, when OA cartilage was pre-incubated for 72 hours before the addition of matriptase, there was a reduction in collagen release (Figure 5.11) compared to OA cartilage treated immediately (Figure 5.8). This implies that upon removal of the mechanical forces generated during abnormal joint loading, PAR-2 expression decreases rapidly to a similar level found in NOF cartilage (Figure 5.10). In fact, this finding was corroborated using the DMM model as PAR-2 was detectable in cartilage

from sham-operated mice but significantly increased in cartilage from DMM mice. But matriptase was only detectable in the latter (Prof. A. D. Rowan, personal communication).

5.4 Conclusion

Although the factors that lead to the development of OA lesions have yet to be fully identified, biomechanical instability of the joint is thought to be a major contributor. The data presented in this chapter show that in an OA cartilage explant model, matriptase can act at a number of levels to mediate collagen breakdown (Figure 5.12). There is an increase in expression of matriptase and PAR-2 after injury to the joint. Activation of PAR-2 by matriptase activates stress pathways within the chondrocyte (Boileau et al., 2007) and leads to MMP-1, -3 and -13 gene expression. Subsequently, matriptase can activate MMP-1 and -3 and this finally leads to the breakdown of the collagen matrix.

Inhibitors targeted to PAR-2 or matriptase are novel therapeutic directions for the treatment of arthritis. Takeuchi et al. (1999) showed that inhibition of matriptase activity decreased PC3 prostate carcinoma size. Further work showed that a synthetic matriptase inhibitor, CVS-3983, displayed a similar trend in another mouse model of prostate cancer (Uhland, 2006). Recently, Darragh et al. (2010) developed an inhibitory antibody that was specific for matriptase and matriptase-specific cancers. Further development of inhibitors, against PAR-2 and matriptase, which can overcome the specific issues presented by cartilage, will be highly beneficial.

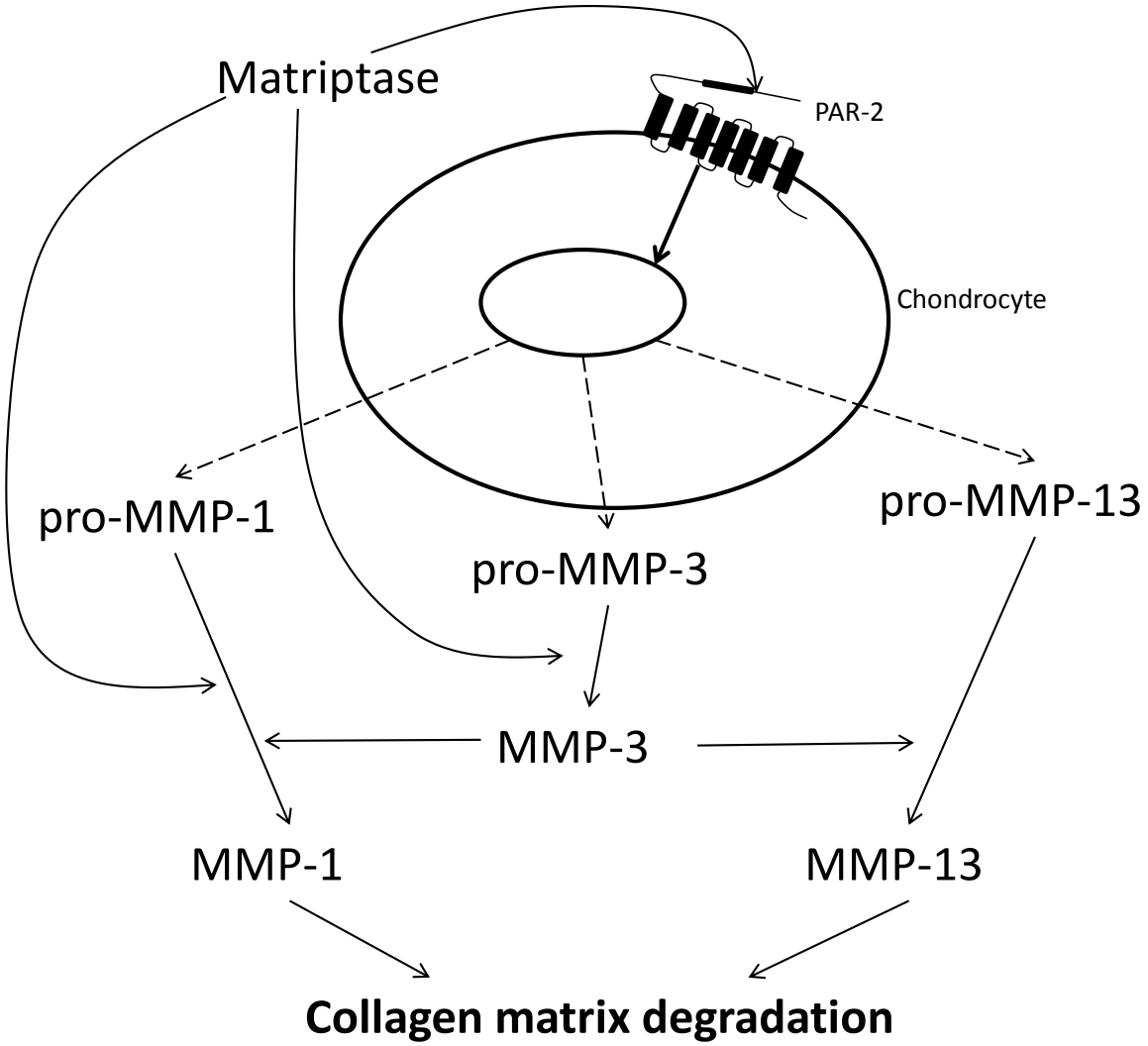


Figure 5.12 Matriptase-mediated pathways leading to collagen matrix degradation. The final common pathway of collagen degradation is MMP dependent as GM6001 completely blocks collagen release. Inhibition of PAR-2 signalling blocks cartilage degradation suggesting this to be a major pathway in the absence of pro-inflammatory stimuli. The activation mechanisms involved *in vivo* are likely to be more complex than shown here, possibly including other growth factors and serine protease zymogens.

5.5 Summary

- Matriptase instigated collagen release independent of IL-1+OSM.
- Collagenase activity and protein levels were reduced in the presence of matriptase.
- The collagen release was solely dependent on MMP activity.
- Matriptase activated pro-MMP-1 and pro-MMP-3 as determined by N-terminal sequencing.
- Matriptase induced MMP-1 and MMP-3 gene expression in OA cartilage.
- Inhibition of PAR-2 significantly reduced matriptase-mediated collagen degradation from OA cartilage.

CHAPTER 6

General Discussion

In the context of arthritis pathology, the main aim of this thesis has been to examine whether the activity of FAPα, DPPIV or matriptase promote collagen release and collagenase activation in resorbing bovine and human cartilages.

6.1 General perspective

Articular cartilage provides a friction-free surface to permit joint articulation and is an unusual tissue since it is hypocellular being populated by a single cell-type, the chondrocyte. During disease states, abnormal stimuli including abnormal loading, as well as pro-inflammatory stimuli such as IL-1 (Goldring and Goldring, 2007), prevail that lead to uncontrolled ECM turnover.

Although, there are a large number of different forms of arthritis, RA and OA are the most common afflictions. RA is a disease characterised by systemic activation of the immune system, which is possibly initiated by the recognition of auto-antigens (de Vries et al., 2005). The chronic activation of the immune system in the joint space leads to the release of inflammatory cytokines, such as IL-1 and OSM, which have been shown to synergistically increase levels of the MMPs (Cawston et al., 1998). OA is the most common form of arthritis, affecting the majority of individuals over the age of 65 years. Cartilage damage in OA is thought to occur due to a number of different reasons such as ageing, genetic factors as well as excessive loading. The degeneration of cartilage occurs due to an imbalance between the anabolic and catabolic pathways. Excessive loading of cartilage can cause increased collagen degradation and up-regulation of MMPs. In both diseases there is an increase in the levels of MMPs leading to the enhanced turnover of the cartilage matrix.

MMPs are the key rate-limiting enzymes that degrade the collagen matrix in arthritis and inhibition of these enzymes would be an attractive prospect. However, broad-spectrum MMP inhibitors have shown a clear lack of efficacy in clinical trials for the treatment of arthritis (Cawston and Young, 2010 and references therein). These inhibitors exhibited severe musculoskeletal side-effects, which were reversed when the compound was withdrawn (Nemunaitis et al., 1998).

Although, synthetic MMP inhibitors have proven to be ineffective, several new treatments have been approved for use in arthritis. One treatment in clinical use is anti-TNF therapy that dampens the inflammatory response, and therefore halts cartilage degradation (Maini and Feldmann, 2002). However, despite its clinical use, a substantial fraction of patients have no meaningful clinical response to anti-TNF treatments (Kievit et al., 2007).

Previous data from our group showed that serine proteases play an important role in cartilage degradation through the activation of pro-MMPs (Milner et al., 2001, 2003). While sequencing of the human genome has identified over one hundred and seventy serine protease genes (Quesada et al., 2009), the exact serine proteases expressed in cartilage, and the role they play, during disease progression have yet to be fully elucidated. Recently, FAPα, DPPIV and matriptase expression were found to be increased in OA cartilage compared to NOF cartilage (Milner et al., 2006b, Swingler et al., 2009). The aim of this thesis was to determine the role of these novel transmembrane serine proteases in the context of cartilage degradation.

6.2 Dipeptidyl peptidases in cartilage breakdown

FAPα and DPPIV are both members of the DPP family (section 1.8) and both are therapeutic targets in a number of disease models. Inhibition of DPPIV has been a very successful strategy for the treatment of type II diabetes (Pratley and Salsali, 2007).

Although, targeting FAPα directly in cancer has not proven as successful (Huang et al., 2004, Ostermann et al., 2008), its use as a biomarker for anti-cancer drug delivery shows promise (Bauer et al., 2004).

Previous data implied a protective role for DPPIV in disease pathology. DPPIV is up-regulated on the surface of OA chondrocytes (Lapadula et al., 1995) and expression is decreased in areas where there is progressive worsening of the osteoarthritic lesions. In RA, DPPIV activity is shown to decrease in both the synovial membrane and fluid of patients (Gotoh et al., 1989, Kamori et al., 1991). Furthermore, there is a decrease in the serum levels of DPPIV that is inversely proportional to the degree of inflammation experienced in the joint (Busso et al., 2005). FAPα expression has recently been detected on the cell surface of chondrocytes upon stimulation with IL-1 and OSM (Milner et al., 2006b). FAPα expression has also been detected on the surface of rheumatoid arthritis synovial fibroblasts (Bauer et al., 2006). FAPα has gelatinase activity (Aoyama and Chen, 1990, Monsky et al., 1994, Pineiro-Sanchez et al., 1997) as well as dipeptidyl peptidase activity (Park et al., 1999) and both of these activities occur in the same catalytic pocket located in the α/β-hydrolase domain. FAPα expression is involved with cell migration through collagen matrices (Ghersi et al., 2002) and correlates with collagen turnover in IL-1+OSM treated cartilage (Milner et al., 2006b).

The loss of DPPIV in both RA and OA is associated with increased disease pathology and implies that inactivation of chemokines within the joint space is an important protective mechanism against cartilage matrix breakdown. However, the expression of FAPα during collagen remodelling implicates a catabolic role. Taken together these findings implicate that DPPIV and FAPα activity play different but important roles in cartilage biology.

The molecular mechanisms of how DPPIV activity affects the cartilage matrix during arthritis are unclear. In this thesis, soluble DPPIV has not been shown to have an effect on IL-1+OSM-treated cartilage (section 4.2.3). However, experiments also show that inhibition of membrane-bound DPPIV activity decreases collagen degradation (section 4.2.2). Data suggest that DPPIV inhibitors play a role in the up-regulation of TGF-β1 secretion in T cells (Reinhold et al., 2006). Ishii *et al*. (2001) have shown that DPPIV co-localises with CD45 at lipid rafts and leads to the activation of ZAP70. Therefore, DPPIV inhibitors could cause a conformational change and disrupt or prevent DPPIV binding to other cell surface proteins, such as CD45, and this leads to TGF-β1 secretion. TGF-β1 (section 1.3.3) has been shown to have a differential role in cartilage (Blaney Davidson et al., 2007). Previous data from our group show that TGF-β1 can significantly reduce collagen release and collagenase levels from cytokine-treated cartilage (Hui et al., 2003a, Hui et al., 2001) and this is thought to occur through the receptor ALK5 (Blaney Davidson et al., 2009). However, during aging there is a reduction in the levels of ALK5 and an increase in ALK1 levels and this correlates with increased MMP-13 production in OA. Therefore, the global reduction of collagenase levels seen in this thesis when K579 was added to IL-1+OSM-treated bovine cartilage (section 4.2.2.5) could be caused by ALK5 activation by TGF-β1 as bovine cartilage is a model of healthy cartilage. Nonetheless, measuring changes in TGF-β1 levels would be problematic as large quantities of latent TGF-β1 are stored in the normal cartilage matrix (Pedrozo et al., 1998). This implies that DPPIV is involved in chondrocyte signalling if increased TGF-β1 levels are the mechanism of cartilage protection for DPPIV inhibition.

As mentioned earlier, DPPIV inhibitors are in clinical use in the treatment of type II diabetes. In this thesis, it has been demonstrated that inhibition of DPPIV is chondroprotective, but the use of DPPIV inhibitors to treat arthritis may be problematic.

Busso et al. (2005) highlighted that collagen-induced arthritis in $DPPIV^{-/-}$ mice was more severe than in wild-type $DPPIV^{+/+}$ mice. Furthermore, DPPIV expression is decreased in areas where there is progressive worsening of the osteoarthritic lesions (Lapadula et al., 1995).

The endopeptidase activity of DPPIV has been evaluated in this thesis and it was found that DPPIV could not cleave denatured type II collagen (section 4.2.4). This contradicts the study by Bermphol et al. (1998) that highlighted DPPIV could cleave denatured collagens and rules this out as a mechanism for the decreased collagen release from IL-1+OSM-treated cartilage by DPPIV inhibition (section 4.2.2.5).

The role of FAPα in cartilage matrix breakdown during the arthritides remains unclear. Experiments in this thesis showed that inhibition of FAPα activity does not affect collagen degradation (section 3.2.3). Chen et al. (2006a) speculate that N-terminal truncation is required to enhance gelatinase activity but not the dipeptidyl peptidase activity of FAPα and this truncation might change the active site *in vivo* to alter inhibitor binding. This finding might also suggest why soluble FAPα does not affect collagen degradation in IL-1+OSM-treated cartilage (section 3.2.4). However, there is no direct evidence for FAPα activity to be involved in ECM degradation other than its localisation at the invadopodia of melanoma cells (Artym et al., 2002, Ghersi et al., 2002) and close proximity to proteins such as MMPs. To date only a small number of FAPα substrates have been identified. In this thesis FAPα has been demonstrated to degrade denatured type II collagen (section 3.2.2). This finding is in-line with data published by Christiansen et al. (2007) that show degradation of denatured type I and III collagens, and α_2-antiplasmin has been described to be another natural FAPα substrate (Lee et al., 2004).

Work presented in this thesis shows that the regulation of DPPIV (section 4.2.1) and FAPα (section 3.2.1) gene expression in chondrocytes does not involve pro-inflammatory cytokines. DPPIV and FAPα have been described to be up-regulated in OA cartilage compared to normal (Milner et al., 2006b, Swingler et al., 2009). The increased gene transcription in cartilage could be due to a number of different pathways. Cartilage has to sustain extreme loads (section 1.1) and therefore mechanical loading may regulate DPPIV and/or FAPα gene expression in chondrocytes. A number of studies have shown that mechanical loading of chondrocytes activates a number of signalling pathways (Pingguan-Murphy et al., 2005, Bougault et al., 2008). Another pathway could be the TLR pathway and it has recently been described to play a role in OA chondrocytes (Zhang et al., 2008). Endogenous ligands recognised by the TLRs could include ECM catabolites such as heparin sulphate (Johnson et al., 2002). This pathway could play an important role in the bovine model as previous data from our group showed elevation of FAPα during breakdown of the collagen matrix (Milner et al., 2006b). In this model, the addition of IL-1+OSM to cartilage would initiate breakdown of the cartilage matrix. Pro-MMPs would be synthesised and activated, ECM proteins would then be degraded to reveal cryptic epitopes that could bind to an unidentified TLR and drive FAPα expression.

In conclusion, experiments in this thesis have been undertaken to broaden the understanding of the role FAPα and DPPIV play in cartilage homeostasis. The data suggest that FAPα enzyme activity has little effect in our cartilage model but can degrade denatured type II collagen. Although FAPα can degrade denatured type II collagen, the importance of this finding is unclear. In the bovine model, FAPα gene expression is found to increase after day 10 (Milner et al., 2006b), but MMP-2 and MMP-9 are expressed at 10- to 100-fold higher levels than FAPα (Milner et al., 2006a)

at this time point. Consequently, is FAPα adding to this gelatinolytic activity at the cell surface?

Inhibition of DPPIV enzyme activity shows mixed results and needs to be explored further. However, questions still remain about the molecular mechanisms involved in all of these observations. One possibility is that the β-propeller domain of FAPα or DPPIV could play an important role in recruiting receptors and proteases to increase matrix breakdown (Figure 4.26).

6.3 Matriptase in cartilage breakdown

Matriptase is a member of the TTSP family (section 1.7) that is becoming a target for therapeutic intervention in a number of cancers (Bugge et al., 2009, Szabo and Bugge, 2008). Matriptase expression has been shown to be up-regulated in OA cartilage compared to normal (Swingler et al., 2009). In a murine model of OA, matriptase expression is increased when the knee joint is destabilised (Prof. A. D. Rowan, personal communication). In this thesis matriptase was shown to enhance the degradation of IL-1+OSM-treated cartilage (section 5.2.1) in a manner similar to plasmin (Milner et al., 2001). Matriptase was also demonstrated to directly activate pro-MMP-1 and -3 (section 5.2.2). Previous data have shown that if active MMP-3 is added at day 0 to IL-1+OSM-treated cartilage significant collagen release is seen by day 7 (Milner et al., 2001).

Matriptase has been shown to be potent mediator of collagen degradation in human OA cartilage (section 5.2.3). Human OA cartilage has previously been described to be highly resistant to pro-inflammatory-mediated breakdown despite the increased expression of collagenolytic MMPs (Cawston et al., 1998). In the presence of IL-1+OSM, matriptase is likely to activate pro-MMPs analogous to the IL-1+OSM-treated bovine cartilage. However, the most striking finding was that matriptase is able to cause collagen degradation in human OA cartilage in the absence of pro-inflammatory

cytokines (section 5.2.3). The extent of collagen release, typically around 30% by day 14, was unprecedented compared to any previously used stimulus. This therefore represents the most potent stimulus for OA cartilage to be described to date. This result has not been seen previously, as plasmin was only able to cause collagen degradation in OA cartilage in concert with IL-1+OSM (Milner et al., 2001).

The degradation of OA cartilage by matriptase is metalloprotease-dependent, most probably via MMPs (section 5.2.4) but interestingly the collagenase levels in samples treated with matriptase were lower than in samples treated with IL-1+OSM (sections 5.2.3.2 and 5.2.3.3). Previous data have shown that MMPs can localise to the cell membrane via interaction with cell surface receptors (Emonard et al., 2005). Furthermore, data have recently shown that the CUB domains of matriptase facilitate protein-protein interactions to transmembrane proteins such as TMEFF1 and HAI-1 (Ge et al., 2006, Inouye et al., 2010). The functions of the non-catalytic domains of matriptase have not been fully elucidated in the context of arthritis, but could play an important role in substrate recognition or binding. Conversely, matriptase-mediated cartilage breakdown could cause distinct gene expression compared to IL-1+OSM treatment, such that uncharacterised proteins are localising MMPs to the chondrocyte surface and expedite collagen degradation.

Matriptase-induced collagen breakdown is dependent on intracellular signalling events in chondrocytes to cause *de novo* MMP production (section 5.2.5). The signalling was via activation of PAR-2 by matriptase (section 5.2.6) as PAR-2 inhibition blocked collagen release from OA cartilage. PAR-2 has been shown to be elevated in murine models of arthritis (Busso et al., 2005). However, I have demonstrated that PAR-2 activation has a profound impact on the cartilage matrix. This is corroborated by Ferrell

et al. (2010) as PAR-2$^{-/-}$ mice show significantly less cartilage and bone erosion than wild-type mice in two experimental murine models of OA.

Experiments in this thesis have shown that matriptase plays an important role in the breakdown of the cartilage collagen matrix by causing MMP synthesis, activation and finally collagen breakdown. One of the key observations has been that matriptase-mediated activation of the PAR-2 signalling pathway is a key requirement for collagen breakdown from arthritic cartilage in the absence of pro-inflammatory cytokines. The PAR-2 pathway needs to be examined in greater detail as there is little information on the genes that are regulated upon PAR-2 activation. Only a report by Tanaka et al. (2008) has shown that IL-8 is up-regulated upon PAR-2 activation in epithelial cell lines.

Matriptase inhibitors are currently being designed to suppress tumour growth and metastasis. However, the catalytic domain of matriptase shares a similar fold to chymotrypsin and therefore an inhibitor against matriptase would have to be extremely specific to avoid off-target effects. The matriptase inhibitor CVS-3983 has been shown to supress prostate cancer growth but shows slight cross-reactivity with trypsin and factor Xa proteases (Galkin et al., 2004). Schweinitz et al. (2009) have shown a similar trend where the most potent matriptase inhibitor (sub-nanomolar K_i) also displayed potent cross-reactivity with factor Xa. Another problem regarding the clinical use of matriptase inhibitors is that matriptase has been shown to play an important role in epithelial barrier formation and function (List et al., 2002, Buzza et al., 2010). These studies have used constitutive matriptase knockouts or low matriptase expression mice and provide strong evidence for a role in development. Recently, List et al. (2009) generated conditional matriptase knockout mice and showed that ablation of matriptase expression between 8 to 28 weeks postnatal caused a loss in epithelial integrity,

resulting in severe epithelial organ dysfunction. Taken together these data highlight the requirement for matriptase inhibitors specific and designed so as not to adversely affect epithelial tissues and organs in order to be of clinical relevance in the treatment of arthritis.

6.4 Future work

The work in this thesis has raised a number of questions about the role of transmembrane serine proteases in cartilage matrix homeostasis. Further work will be required to address these questions as well as confirm some of the conclusions drawn in this study.

- Develop an *in vitro* transgenic cartilage model to assess protease function on the cartilage matrix. Treatment of cartilage with siRNA is prohibitively expensive but retroviral gene transfer has been shown to allow long-term expression of transgenes (Kafienah et al., 2003). In theory, chondrocytes could be transfected with two plasmid constructs; one plasmid would be under TET-off control and express TGF-β3 to help promote cartilage matrix formation *in vitro*. The second plasmid would be under the control of the enhancer cumate (Mullick et al., 2006) and express a transgene or small homologous (sh) RNA. Initially, transduced chondrocytes would be cultured in the presence of cumate and doxycycline. This would allow TGF-β3 expression and the generation of engineered "tissue", and not the transgene. Withdrawal of cumate and doxycycline would stop TGF-β3 expression and permit transgene or shRNA expression.
- Use bimolecular fluorescence complementation to assess if DPPIV or FAPα associate with other proteases at the cell surface of OA chondrocytes. Bimolecular fluorescence complementation is based upon complementation of

two separately expressed fragments of GFP or derivates which are fused to putative interacting proteins.

- Grover and Roughley (2006) developed a conditional cartilage-specific knockout mouse model. As the expression of Cre recombinase was under control of both the type II collagen promoter and a doxycycline response element, this overcame the embryonic lethal phenotype that universal gene knockout mice often displayed. This mouse is then crossed with another strain of mouse where the gene of interest lies between two loxP sites. The resulting progeny could then be fed on a diet supplemented with doxycycline to repress target gene expression. Therefore, if we generate cartilage-specific DPPIV and FAPα knockout mice and then initiate arthritis either by injection of collagen into their tail-base or by destabilization of the medial meniscus. This would show whether these enzymes play a role in either inflammatory arthritis or in an OA-like model.
- Characterise the role of the non-catalytic domains of matriptase to test whether they are important in cartilage matrix breakdown. The CUB domains have been shown to facilitate the binding of matriptase to other transmembrane proteins. Inouye et al. (2010) highlight that the interaction between CUB domain II of matriptase and the second Kunitz domain of HAI-1 facilitates the binding of the first Kunitz domain of HAI-1 to the catalytic domain of matriptase. Therefore, expression of the individual tagged domains will allow for the identification of novel binding partners of matriptase and determine their functions in the context of arthritis.
- Generate cartilage-specific matriptase knockout mice and initiate arthritis by destabilization of the medial meniscus. This would highlight whether matriptase

knockout would protect cartilage from degradation as shown for PAR-2 knockout mice (Ferrell et al., 2010).

6.5 Summary

The most significant finding of this thesis is the breakdown of the cartilage collagen network by the transmembrane serine protease, matriptase. Studies within this department have provided evidence for the role of serine proteinases in the activation of pro-MMPs in cartilage (Milner et al., 2001, 2003). My data have demonstrated for the first time that matriptase plays an important role in cartilage breakdown by the activation of PAR-2 and pro-MMP-1 and -3. Data from our group have confirmed this finding *in vivo* using a murine model of OA. Finally, the data presented in this thesis show that investigating the role of dipeptidyl peptidases with pharmacological inhibitors is hindered by their lack of *ex vivo* efficacy in the context of arthritis. Consequently, the data from these studies require confirmation by alternative techniques.

The findings in this thesis highlight the need to further characterise the role of novel transmembrane serine proteases in cartilage. Such endeavour may well realise the potential of serine protease inhibitors as future therapeutic agents for the arthritides.

References

Aertgeerts, K., Levin, I., Shi, L., Snell, G. P., Jennings, A., Prasad, G. S., Zhang, Y., Kraus, M. L., Salakian, S., Sridhar, V., Wijnands, R. and Tennant, M. G. (2005) 'Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha', *J Biol Chem*, 280, (20), pp. 19441-4.

Aertgeerts, K., Ye, S., Tennant, M. G., Kraus, M. L., Rogers, J., Sang, B. C., Skene, R. J., Webb, D. R. and Prasad, G. S. (2004) 'Crystal structure of human dipeptidyl peptidase IV in complex with a decapeptide reveals details on substrate specificity and tetrahedral intermediate formation', *Protein Sci*, 13, (2), pp. 412-21.

Aggarwal, S., Brennen, W. N., Kole, T. P., Schneider, E., Topaloglu, O., Yates, M., Cotter, R. J. and Denmeade, S. R. (2007) 'Fibroblast Activation Protein Peptide Substrates Identified from Human Collagen I Derived Gelatin Cleavage Sites', *Biochemistry*, 47, (3), pp. 1076-1086.

Aigner, T. and Stove, J. (2003) 'Collagens--major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair', *Adv Drug Deliv Rev*, 55, (12), pp. 1569-93.

Ajami, K., Abbott, C. A., Obradovic, M., Gysbers, V., Kahne, T., McCaughan, G. W. and Gorrell, M. D. (2003) 'Structural requirements for catalysis, expression, and dimerization in the CD26/DPIV gene family', *Biochemistry*, 42, (3), pp. 694-701.

Al-Ayyoubi, M., Gettins, P. G. W. and Volz, K. (2004) 'Crystal Structure of Human Maspin, a Serpin with Antitumor Properties: Reactive Center Loop Of Maspin Is Exposed But Constrained', *J Biol Chem*, 279, (53), pp. 55540-55544.

Amiable, N., Tat, S. K., Lajeunesse, D., Duval, N., Pelletier, J. P., Martel-Pelletier, J. and Boileau, C. (2009) 'Proteinase-activated receptor (PAR)-2 activation impacts bone resorptive properties of human osteoarthritic subchondral bone osteoblasts', *Bone,* 44, (6), pp. 1143-50.

Aoyama, A. and Chen, W. T. (1990) 'A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells', *Proc Natl Acad Sci U S A,* 87, (21), pp. 8296-300.

Arend, W. P. (2002) 'The balance between IL-1 and IL-1Ra in disease', *Cytokine & Growth Factor Reviews,* 13, (4-5), pp. 323-340.

Ariga, N., Sato, E., Ohuchi, N., Nagura, H. and Ohtani, H. (2001) 'Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast', *International Journal of Cancer,* 95, (1), pp. 67-72.

Artym, V. V., Kindzelskii, A. L., Chen, W. T. and Petty, H. R. (2002) 'Molecular proximity of seprase and the urokinase-type plasminogen activator receptor on malignant melanoma cell membranes: dependence on beta1 integrins and the cytoskeleton', *Carcinogenesis,* 23, (10), pp. 1593-601.

Baker, A. H., Zaltsman, A. B., George, S. J. and Newby, A. C. (1998) 'Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death in vitro. TIMP-3 promotes apoptosis', *The Journal of Clinical Investigation,* 101, (6), pp. 1478-1487.

Barksby, H. E., Hui, W., Wappler, I., Peters, H. H., Milner, J. M., Richards, C. D., Cawston, T. E. and Rowan, A. D. (2006) 'Interleukin-1 in combination with oncostatin M up-regulates multiple genes in chondrocytes: Implications for cartilage destruction and repair', *Arthritis & Rheumatism,* 54, (2), pp. 540-550.

Bauer, S., Adrian, N., Williamson, B., Panousis, C., Fadle, N., Smerd, J., Fettah, I., Scott, A. M., Pfreundschuh, M. and Renner, C. (2004) 'Targeted Bioactivity of Membrane-Anchored TNF by an Antibody-Derived TNF Fusion Protein', *J Immunol,* 172, (6), pp. 3930-3939.

Bauer, S., Jendro, M. C., Wadle, A., Kleber, S., Stenner, F., Dinser, R., Reich, A., Faccin, E., Godde, S., Dinges, H., Muller-Ladner, U. and Renner, C. (2006) 'Fibroblast activation protein is expressed by rheumatoid myofibroblast-like synoviocytes', *Arthritis Res Ther,* 8, (6), pp. R171.

Bauvois, B., Sanceau, J. and Wietzerbin, J. (1992) 'Human U937 cell surface peptidase activities: characterization and degradative effect on tumor necrosis factor-alpha', *Eur J Immunol,* 22, (4), pp. 923-30.

Belcher, C., Fawthrop, F., Bunning, R. and Doherty, M. (1996) 'Plasminogen activators and their inhibitors in synovial fluids from normal, osteoarthritis, and rheumatoid arthritis knees', *Ann Rheum Dis,* 55, (4), pp. 230-6.

Benaud, C., Dickson, R. B. and Lin, C. Y. (2001) 'Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor', *Eur J Biochem,* 268, (5), pp. 1439-47.

Bergman, I. and Loxley, R. (1963) 'Two Improved and Simplified Methods for the Spectrophotometric Determination of Hydroxyproline', *Analytical Chemistry,* 35, (12), pp. 1961-1965.

Bermpohl, F., Loster, K., Reutter, W. and Baum, O. (1998) 'Rat dipeptidyl peptidase IV (DPP IV) exhibits endopeptidase activity with specificity for denatured fibrillar collagens', *FEBS Lett,* 428, (3), pp. 152-6.

Bhosale, A. M. and Richardson, J. B. (2008) 'Articular cartilage: structure, injuries and review of management', *Br Med Bull,* 87, (1), pp. 77-95.

Binder, B. R., Mihaly, J. and Prager, G. W. (2007) 'uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view', *Thromb Haemost,* 97, (3), pp. 336-42.

Blanc-Brude, O. P., Archer, F., Leoni, P., Derian, C., Bolsover, S., Laurent, G. J. and Chambers, R. C. (2005) 'Factor Xa stimulates fibroblast procollagen production, proliferation, and calcium signaling via PAR1 activation', *Exp Cell Res,* 304, (1), pp. 16-27.

Blaney Davidson, E. N., Remst, D. F. G., Vitters, E. L., van Beuningen, H. M., Blom, A. B., Goumans, M.-J., van den Berg, W. B. and van der Kraan, P. M. (2009) 'Increase in ALK1/ALK5 Ratio as a Cause for Elevated MMP-13 Expression in Osteoarthritis in Humans and Mice', *J Immunol,* 182, (12), pp. 7937-7945.

Blaney Davidson, E. N., van der Kraan, P. M. and van den Berg, W. B. (2007) 'TGF-beta and osteoarthritis', *Osteoarthritis and Cartilage,* 15, (6), pp. 597-604.

Blow, D. M., Birktoft, J. J. and Hartley, B. S. (1969) 'Role of a buried acid group in the mechanism of action of chymotrypsin', *Nature,* 221, (5178), pp. 337-40.

Bode, W., Gomis-Ruth, F. X. and Stockler, W. (1993) 'Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'', *FEBS Lett,* 331, (1-2), pp. 134-40.

Bogatkevich, G. S., Tourkina, E., Silver, R. M. and Ludwicka-Bradley, A. (2001) 'Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway', *J Biol Chem,* 276, (48), pp. 45184-92.

Boileau, C., Amiable, N., Martel-Pelletier, J., Fahmi, H., Duval, N. and Pelletier, J. P. (2007) 'Activation of proteinase-activated receptor 2 in human osteoarthritic cartilage upregulates catabolic and proinflammatory pathways capable of

inducing cartilage degradation: a basic science study', *Arthritis Res Ther*, 9, (6), pp. R121.

Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K. H., Bourguignon, J., Lasselin, C. and Freisinger, P. (1994) 'Reexpression of Cartilage-Specific Genes by Dedifferentiated Human Articular Chondrocytes Cultured in Alginate Beads', *Experimental Cell Research*, 212, (1), pp. 97-104.

Bord, S., Horner, A., Hembry, R. M. and Compston, J. E. (1998) 'Stromelysin-1 (MMP-3) and Stromelysin-2 (MMP-10) Expression in Developing Human Bone: Potential Roles in Skeletal Development', *Bone*, 23, (1), pp. 7-12.

Boris, V. S., Wen-Rong, L., Carlo, L. M., Ada, A. C., Susan, C. and Karen, A. H. (1997) 'Osteoarthritic Lesions. Involvement of three different collagenases', *Arthritis & Rheumatism*, 40, (11), pp. 2065-2074.

Bougault, C., Paumier, A., Aubert-Foucher, E. and Mallein-Gerin, F. (2008) 'Molecular analysis of chondrocytes cultured in agarose in response to dynamic compression', *BMC Biotechnology*, 8, (1), pp. 71.

Brandt, I., Joossens, J., Chen, X., Maes, M. B., Scharpe, S., De Meester, I. and Lambeir, A. M. (2005) 'Inhibition of dipeptidyl-peptidase IV catalyzed peptide truncation by Vildagliptin ((2S)-{[(3-hydroxyadamantan-1-yl)amino]acetyl}-pyrrolidine-2-carbonitrile)', *Biochem Pharmacol*, 70, (1), pp. 134-43.

Brandt, K. D., Radin, E. L., Dieppe, P. A. and van de Putte, L. (2006) 'Yet more evidence that osteoarthritis is not a cartilage disease', *Ann Rheum Dis*, 65, (10), pp. 1261-4.

Bugge, T. H., Antalis, T. M. and Wu, Q. (2009) 'Type II transmembrane serine proteases', *J Biol Chem*, 284, (35), pp. 23177-81.

Busek, P., Stremenova, J., Krepela, E. and Sedo, A. (2007) 'Modulation of substance P signaling by dipeptidyl peptidase-IV enzymatic activity in human glioma cell lines', *Physiol Res*.

Busso, N., Peclat, V., Van Ness, K., Kolodziesczyk, E., Degen, J., Bugge, T. and So, A. (1998) 'Exacerbation of antigen-induced arthritis in urokinase-deficient mice', *J Clin Invest,* 102, (1), pp. 41-50.

Busso, N. and So, A. (1997) 'Urokinase in rheumatoid arthritis: causal or coincidental?', *Ann Rheum Dis,* 56, (12), pp. 705-706.

Busso, N., Wagtmann, N., Herling, C., Chobaz-Peclat, V., Bischof-Delaloye, A., So, A. and Grouzmann, E. (2005) 'Circulating CD26 is negatively associated with inflammation in human and experimental arthritis', *Am J Pathol,* 166, (2), pp. 433-42.

Buzza, M. S., Netzel-Arnett, S., Shea-Donohue, T., Zhao, A., Lin, C.-Y., List, K., Szabo, R., Fasano, A., Bugge, T. H. and Antalis, T. M. (2010) 'Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine', *Proc Natl Acad Sci U S A,* 107, (9), pp. 4200-4205.

Cawston, T. E. (1996) 'Metalloproteinase inhibitors and the prevention of connective tissue breakdown', *Pharmacology & Therapeutics,* 70, (3), pp. 163-182.

Cawston, T. E. and Barrett, A. J. (1979) 'A rapid and reproducible assay for collagenase using [1-14C]acetylated collagen', *Analytical Biochemistry,* 99, (2), pp. 340-345.

Cawston, T. E., Curry, V. A., Summers, C. A., Clark, I. M., Riley, G. P., Life, P. F., Spaull, J. R., Goldring, M. B., Koshy, P. J. T., Rowan, A. D. and Shingleton, W. D. (1998) 'The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint', *Arthritis & Rheumatism,* 41, (10), pp. 1760-71.

Cawston, T. E., Ellis, A. J., Humm, G., Lean, E., Ward, D. and Curry, V. (1995) 'Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture', *Biochem Biophys Res Commun,* 215, (1), pp. 377-85.

Cawston, T. E., Koshy, P. and Rowan, A. D. (2001) 'Assay of matrix metalloproteinases against matrix substrates', *Methods Mol Biol,* 151, pp. 389-97.

Cawston, T. E., Mercer, E., Silva, M. D. and Hazleman, B. L. (1984) 'Metalloproteinases and collagenase inhibitors in rheumatoid synovial fluid', *Arthritis & Rheumatism,* 27, (3), pp. 285-290.

Cawston, T. E. and Tyler, J. A. (1979) 'Purification of pig synovial collagenase to high specific activity', *Biochem J,* 183, (3), pp. 647-56.

Cawston, T. E. and Young, D. A. (2010) 'Proteinases involved in matrix turnover during cartilage and bone breakdown', *Cell Tissue Res,* 339, (1), pp. 221-35.

Chan, D., Cole, W. G., Rogers, J. G. and Bateman, J. F. (1995) 'Type X Collagen Multimer Assembly in Vitro Is Prevented by a Gly to Val Mutation in the 1(X) NC1 Domain Resulting in Schmid Metaphyseal Chondrodysplasia', *J Biol Chem,* 270, (9), pp. 4558-4562.

Chen, D., Kennedy, A., Wang, J. Y., Zeng, W., Zhao, Q., Pearl, M., Zhang, M., Suo, Z., Nesland, J. M., Qiao, Y., Ng, A. K., Hirashima, N., Yamane, T., Mori, Y., Mitsumata, M., Ghersi, G. and Chen, W. T. (2006a) 'Activation of EDTA-resistant gelatinases in malignant human tumors', *Cancer Res,* 66, (20), pp. 9977-85.

Chen, H., Yang, W.-W., Wen, Q.-T., Xu, L. and Chen, M. (2009) 'TGF-[beta]-induced fibroblast activation protein expression, fibroblast activation protein expression increases the proliferation, adhesion, and migration of HO-8910PM', *Experimental and Molecular Pathology,* 87, (3), pp. 189-194.

Chen, S.-J., Ning, H., Ishida, W., Sodin-Semrl, S., Takagawa, S., Mori, Y. and Varga, J. (2006b) 'The Early-Immediate Gene EGR-1 Is Induced by Transforming Growth Factor-β and Mediates Stimulation of Collagen Gene Expression', *J Biol Chem,* 281, (30), pp. 21183-21197.

Chen, W. T. and Kelly, T. (2003) 'Seprase complexes in cellular invasiveness', *Cancer Metastasis Rev,* 22, (2-3), pp. 259-69.

Chen, W. T., Kelly, T. and Ghersi, G. (2003) 'DPPIV, seprase, and related serine peptidases in multiple cellular functions', *Curr Top Dev Biol,* 54, pp. 207-32.

Cheng, J. D., Dunbrack, R. L., Jr., Valianou, M., Rogatko, A., Alpaugh, R. K. and Weiner, L. M. (2002) 'Promotion of Tumor Growth by Murine Fibroblast Activation Protein, a Serine Protease, in an Animal Model', *Cancer Res,* 62, (16), pp. 4767-4772.

Chesler, L., Golde, D. W., Bersch, N. and Johnson, M. D. (1995) 'Metalloproteinase inhibition and erythroid potentiation are independent activities of tissue inhibitor of metalloproteinases-1', *Blood,* 86, (12), pp. 4506-4515.

Chiu, Y. C., Fong, Y. C., Lai, C. H., Hung, C. H., Hsu, H. C., Lee, T. S., Yang, R. S., Fu, W. M. and Tang, C. H. (2008) 'Thrombin-induced IL-6 production in human synovial fibroblasts is mediated by PAR1, phospholipase C, protein kinase Calpha, c-Src, NF-kappaB and p300 pathway', *Mol Immunol,* 45, (6), pp. 1587-99.

Choi, S. Y., Bertram, S., Glowacka, I., Park, Y. W. and Pohlmann, S. (2009) 'Type II transmembrane serine proteases in cancer and viral infections', *Trends Mol Med,* 15, (7), pp. 303-12.

Chomczynski, P. and Sacchi, N. (1987) 'Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction', *Analytical Biochemistry,* 162, (1), pp. 156-9.

Christiansen, V. J., Jackson, K. W., Lee, K. N. and McKee, P. A. (2007) 'Effect of fibroblast activation protein and alpha2-antiplasmin cleaving enzyme on collagen types I, III, and IV', *Arch Biochem Biophys,* 457, (2), pp. 177-86.

Cinzia, M., Riccardo, M., Luigi, F., Tania, S., Lia, P., Ilaria, M., Rosa Maria, B., Mariagrazia, U. and Andrea, F. (1998) 'Enhanced and coordinated in vivo expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis', *Arthritis & Rheumatism,* 41, (12), pp. 2165-2174.

Coimbra, I. B., Jimenez, S. A., Hawkins, D. F., Piera-Velazquez, S. and Stokes, D. G. (2004) 'Hypoxia inducible factor-1 alpha expression in human normal and osteoarthritic chondrocytes', *Osteoarthritis and Cartilage,* 12, (4), pp. 336-345.

Covas, M. J., Pinto, L. A. and Victorino, R. M. (1997) 'Effects of substance P on human T cell function and the modulatory role of peptidase inhibitors', *Int J Clin Lab Res,* 27, (2), pp. 129-34.

Creighton, T. E., Bagley, C. J., Cooper, L., Darby, N. J., Freedman, R. B., Kemmink, J. and Sheikh, A. (1993) 'On the biosynthesis of bovine pancreatic trypsin inhibitor (BPTI). Structure, processing, folding and disulphide bond formation of the precursor in vitro and in microsomes', *J Mol Biol,* 232, (4), pp. 1176-96.

Creighton, T. E. and Charles, I. G. (1987) 'Sequences of the genes and polypeptide precursors for two bovine protease inhibitors', *J Mol Biol,* 194, (1), pp. 11-22.

Darragh, M. R., Schneider, E. L., Lou, J., Phojanakong, P. J., Farady, C. J., Marks, J. D., Hann, B. C. and Craik, C. S. (2010) 'Tumor detection by imaging proteolytic activity', *Cancer Res,* 70, (4), pp. 1505-12.

David, D. W., Earl, J. I. and David, H. (1985) 'Release of interleukin-1 from human synovial tissue in vitro', *Arthritis & Rheumatism,* 28, (8), pp. 853-862.

de Caestecker, M. (2004) 'The transforming growth factor-[beta] superfamily of receptors', *Cytokine & Growth Factor Reviews,* 15, (1), pp. 1-11.

De Meester, I., Belyaev, A., Lambeir, A.-M., De Meyer, G. R. Y., Van Osselaer, N., Haemers, A. and Scharpé, S. (1997) 'In vivo inhibition of dipeptidyl peptidase IV activity by pro-pro-diphenyl-phosphonate (prodipine)', *Biochemical Pharmacology,* 54, (1), pp. 173-179.

De Meester, I. A., Kestens, L. L., Vanham, G. L., Vanhoof, G. C., Vingerhoets, J. H., Gigase, P. L. and Scharpe, S. L. (1995) 'Costimulation of CD4+ and CD8+ T cells through CD26: the ADA-binding epitope is not essential for complete signaling', *J Leukoc Biol,* 58, (3), pp. 325-30.

de Vries, R. R., Huizinga, T. W. and Toes, R. E. (2005) 'Redefining the HLA and RA association: to be or not to be anti-CCP positive', *J Autoimmun,* 25 Suppl, pp. 21-5.

DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D. and Bunnett, N. W. (2000) 'beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2', *J Cell Biol,* 148, (6), pp. 1267-81.

Dejica, V. M., Mort, J. S., Laverty, S., Percival, M. D., Antoniou, J., Zukor, D. J. and Poole, A. R. (2008) 'Cleavage of type II collagen by cathepsin K in human osteoarthritic cartilage', *Am J Pathol,* 173, (1), pp. 161-9.

Eager, R. M., Cunningham, C. C., Senzer, N., Richards, D. A., Raju, R. N., Jones, B., Uprichard, M. and Nemunaitis, J. (2009a) 'Phase II trial of talabostat and docetaxel in advanced non-small cell lung cancer', *Clin Oncol (R Coll Radiol),* 21, (6), pp. 464-72.

Eager, R. M., Cunningham, C. C., Senzer, N. N., Stephenson, J., Jr., Anthony, S. P., O'Day, S. J., Frenette, G., Pavlick, A. C., Jones, B., Uprichard, M. and

Nemunaitis, J. (2009b) 'Phase II assessment of talabostat and cisplatin in second-line stage IV melanoma', *BMC Cancer*, 9, pp. 263.

Edosada, C. Y., Quan, C., Wiesmann, C., Tran, T., Sutherlin, D., Reynolds, M., Elliott, J. M., Raab, H., Fairbrother, W. and Wolf, B. B. (2006) 'Selective Inhibition of Fibroblast Activation Protein Protease Based on Dipeptide Substrate Specificity', *J Biol Chem*, 281, (11), pp. 7437-7444.

Ekici, O. D., Paetzel, M. and Dalbey, R. E. (2008) 'Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration', *Protein Sci*, 17, (12), pp. 2023-37.

Ellingsen, T., Hornung, N., Moller, B. K., Hjelm-Poulsen, J. and Stengaard-Pedersen, K. (2007) 'In active chronic rheumatoid arthritis, dipeptidyl peptidase IV density is increased on monocytes and CD4(+) T lymphocytes', *Scand J Immunol*, 66, (4), pp. 451-7.

Elliott, P. R., Abrahams, J. P. and Lomas, D. A. (1998) 'Wild-type alpha 1-antitrypsin is in the canonical inhibitory conformation', *J Mol Biol*, 275, (3), pp. 419-25.

Emonard, H., Bellon, G., de Diesbach, P., Mettlen, M., Hornebeck, W. and Courtoy, P. J. (2005) 'Regulation of matrix metalloproteinase (MMP) activity by the low-density lipoprotein receptor-related protein (LRP). A new function for an "old friend"', *Biochimie*, 87, (3-4), pp. 369-76.

Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H. U. and Brandstetter, H. (2003) 'The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism', *Proc Natl Acad Sci U S A*, 100, (9), pp. 5063-8.

Ferrell, W. R., Kelso, E. B., Lockhart, J. C., Plevin, R. and McInnes, I. B. (2010) 'Protease-activated receptor 2: a novel pathogenic pathway in a murine model of osteoarthritis', *Ann Rheum Dis*.

Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1991) 'Cleavage of cartilage proteoglycan between G1 and G2 domains by stromelysins', *J Biol Chem*, 266, (24), pp. 15579-15582.

Freije, J. M., Díez-Itza, I., Balbín, M., SÃ¡nchez, L. M., Blasco, R., Tolivia, J. and López-Otín, C. (1994) 'Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas', *J Biol Chem*, 269, (24), pp. 16766-16773.

Friedrich, R., Fuentes-Prior, P., Ong, E., Coombs, G., Hunter, M., Oehler, R., Pierson, D., Gonzalez, R., Huber, R., Bode, W. and Madison, E. L. (2002) 'Catalytic domain structures of MT-SP1/matriptase, a matrix-degrading transmembrane serine proteinase', *J Biol Chem*, 277, (3), pp. 2160-8.

Galkin, A. V., Mullen, L., Fox, W. D., Brown, J., Duncan, D., Moreno, O., Madison, E. L. and Agus, D. B. (2004) 'CVS-3983, a selective matriptase inhibitor, suppresses the growth of androgen independent prostate tumor xenografts', *Prostate*, 61, (3), pp. 228-35.

Galloway, W. A., Murphy, G., Sandy, J. D., Gavrilovic, J., Cawston, T. E. and Reynolds, J. J. (1983) 'Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective-tissue components', *Biochem J*, 209, (3), pp. 741-52.

Garin-Chesa, P., Old, L. J. and Rettig, W. J. (1990) 'Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers', *Proc Natl Acad Sci U S A*, 87, (18), pp. 7235-9.

Ge, W., Hu, H., Ding, K., Sun, L. and Zheng, S. (2006) 'Protein interaction analysis of ST14 domains and their point and deletion mutants', *J Biol Chem*, 281, (11), pp. 7406-12.

Gebauer, M., Saas, J., Sohler, F., Haag, J., Söder, S., Pieper, M., Bartnik, E., Beninga, J., Zimmer, R. and Aigner, T. (2005) 'Comparison of the chondrosarcoma cell line SW1353 with primary human adult articular chondrocytes with regard to their gene expression profile and reactivity to IL-1[beta]', *Osteoarthritis and Cartilage,* 13, (8), pp. 697-708.

Gentili, C. and Cancedda, R. (2009) 'Cartilage and bone extracellular matrix', *Curr Pharm Des,* 15, (12), pp. 1334-48.

Ghersi, G., Dong, H., Goldstein, L. A., Yeh, Y., Hakkinen, L., Larjava, H. S. and Chen, W. T. (2002) 'Regulation of fibroblast migration on collagenous matrix by a cell surface peptidase complex', *J Biol Chem,* 277, (32), pp. 29231-41.

Gibson, G. J. and Flint, M. H. (1985) 'Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral development', *J Cell Biol,* 101, (1), pp. 277-284.

Gilmore, B. F., Lynas, J. F., Scott, C. J., McGoohan, C., Martin, L. and Walker, B. (2006) 'Dipeptide proline diphenyl phosphonates are potent, irreversible inhibitors of seprase (FAPalpha)', *Biochem Biophys Res Commun,* 346, (2), pp. 436-46.

Glasson, S. S., Blanchet, T. J. and Morris, E. A. (2007) 'The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse', *Osteoarthritis and Cartilage,* 15, (9), pp. 1061-1069.

Goldring, M. B. and Goldring, S. R. (2007) 'Osteoarthritis', *J Cell Physiol,* 213, (3), pp. 626-34.

Goldring, S. R. and Goldring, M. B. (2004) 'The role of cytokines in cartilage matrix degeneration in osteoarthritis', *Clin Orthop Relat Res,* (427 Suppl), pp. S27-36.

Goldstein, L. A. and Chen, W. T. (2000) 'Identification of an alternatively spliced seprase mRNA that encodes a novel intracellular isoform', *J Biol Chem,* 275, (4), pp. 2554-9.

Goldstein, L. A., Ghersi, G., Pineiro-Sanchez, M. L., Monica, S., Yeh, Y., Flessate, D. and Chen, W.-T. (1997) 'Molecular cloning of seprase: a serine integral membrane protease from human melanoma', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1361, (1), pp. 11-19.

Gomis-Rüth, F. X., Gohlke, U., Betz, M., Knäuper, V., Murphy, G., López-Otín, C. and Bode, W. (1996) 'The Helping Hand of Collagenase-3 (MMP-13): 2.7 Å Crystal Structure of its C-terminal Haemopexin-like Domain', *J Mol Biol,* 264, (3), pp. 556-566.

Gonzalez-Gronow, M., Grenett, H. E., Gawdi, G. and Pizzo, S. V. (2005a) 'Angiostatin directly inhibits human prostate tumor cell invasion by blocking plasminogen binding to its cellular receptor, CD26', *Experimental Cell Research,* 303, (1), pp. 22-31.

Gonzalez-Gronow, M., Hershfield, M. S., Arredondo-Vega, F. X. and Pizzo, S. V. (2004) 'Cell surface adenosine deaminase binds and stimulates plasminogen activation on 1-LN human prostate cancer cells', *J Biol Chem,* 279, (20), pp. 20993-8.

Gonzalez-Gronow, M., Misra, U. K., Gawdi, G. and Pizzo, S. V. (2005b) 'Association of Plasminogen with Dipeptidyl Peptidase IV and Na+/H+ Exchanger Isoform NHE3 Regulates Invasion of Human 1-LN Prostate Tumor Cells', *J Biol Chem,* 280, (29), pp. 27173-27178.

Gorrell, M. D. (2005) 'Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders', *Clin Sci (Lond),* 108, (4), pp. 277-92.

Gotoh, H., Hagihara, M., Nagatsu, T., Iwata, H. and Miura, T. (1989) 'Activities of dipeptidyl peptidase II and dipeptidyl peptidase IV in synovial fluid from patients with rheumatoid arthritis and osteoarthritis', *Clin Chem,* 35, (6), pp. 1016-8.

Gross, J. and Lapiere, C. M. (1962) 'Collagenolytic activity in amphibian tissues: a tissue culture assay', *Proc Natl Acad Sci U S A,* 48, pp. 1014-22.

Grover, J. and Roughley, P. J. (2006) 'Generation of a transgenic mouse in which Cre recombinase is expressed under control of the type II collagen promoter and doxycycline administration', *Matrix Biol,* 25, (3), pp. 158-65.

Guan, E., Wang, J. and Norcross, M. A. (2004) 'Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidyl-peptidase IV', *J Cell Biochem,* 92, (1), pp. 53-64.

Guevremont, M., Martel-Pelletier, J., Massicotte, F., Tardif, G., Pelletier, J. P., Ranger, P., Lajeunesse, D. and Reboul, P. (2003) 'Human adult chondrocytes express hepatocyte growth factor (HGF) isoforms but not HgF: potential implication of osteoblasts on the presence of HGF in cartilage', *J Bone Miner Res,* 18, (6), pp. 1073-81.

Hanski, C., Huhle, T., Gossrau, R. and Reutter, W. (1988) 'Direct evidence for the binding of rat liver DPP IV to collagen in vitro', *Experimental Cell Research,* 178, (1), pp. 64-72.

Hascall, V. C., Morales, T. I., Hascall, G. K., Handley, C. J. and McQuillan, D. J. (1983) 'Biosynthesis and turnover of proteoglycans in organ culture of bovine articular cartilage', *J Rheumatol Suppl,* 11, pp. 45-52.

Hasty, K. A., Jeffrey, J. J., Hibbs, M. S. and Welgus, H. G. (1987) 'The collagen substrate specificity of human neutrophil collagenase', *J Biol Chem,* 262, (21), pp. 10048-10052.

He, C. S., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z. and Goldberg, G. I. (1989) 'Tissue cooperation in a proteolytic cascade activating human interstitial collagenase', *Proc Natl Acad Sci U S A*, 86, (8), pp. 2632-6.

Heukeshoven, J. and Dernick, R. (1985) 'Characterization of a solvent system for separation of water-insoluble poliovirus proteins by reversed-phase high-performance liquid chromatography', *J Chromatogr*, 326, pp. 91-101.

Highberger, J. H., Corbett, C. and Gross, J. (1979) 'Isolation and characterization of A peptide containing the site of cleavage of the chick skin collagen [alpha]1[I] chain by animal collagenases', *Biochemical and Biophysical Research Communications*, 89, (1), pp. 202-208.

Hirano, F., Kobayashi, A., Hirano, Y., Nomura, Y., Fukawa, E. and Makino, I. (2002) 'Thrombin-induced expression of RANTES mRNA through protease activated receptor-1 in human synovial fibroblasts', *Ann Rheum Dis*, 61, (9), pp. 834-7.

Hollander, A. P., Pidoux, I., Reiner, A., Rorabeck, C., Bourne, R. and Poole, A. R. (1995) 'Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration', *J Clin Invest*, 96, (6), pp. 2859-69.

Hollenberg, M. D., Saifeddine, M., al-Ani, B. and Kawabata, A. (1997) 'Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides', *Can J Physiol Pharmacol*, 75, (7), pp. 832-41.

Hooper, J. D., Clements, J. A., Quigley, J. P. and Antalis, T. M. (2001) 'Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes', *J Biol Chem*, 276, (2), pp. 857-60.

Houssiau, F. A., Devogelaer, J.-P., Van Damme, J., Nagant De Deuxchaisnes, C. and Van Snick, J. (1988) 'Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides', *Arthritis & Rheumatism*, 31, (6), pp. 784-788.

Huang, S., Apasov, S., Koshiba, M. and Sitkovsky, M. (1997) 'Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion', *Blood*, 90, (4), pp. 1600-10.

Huang, Y., Wang, S. and Kelly, T. (2004) 'Seprase promotes rapid tumor growth and increased microvessel density in a mouse model of human breast cancer', *Cancer Res*, 64, (8), pp. 2712-6.

Hughes, T. E., Mone, M. D., Russell, M. E., Weldon, S. C. and Villhauer, E. B. (1999) 'NVP-DPP728 (1-[[[2-[(5-cyanopyridin-2-yl)amino]ethyl]amino]acetyl]-2-cyano-(S)- pyrrolidine), a slow-binding inhibitor of dipeptidyl peptidase IV', *Biochemistry*, 38, (36), pp. 11597-603.

Hui, W., Cawston, T. and Rowan, A. D. (2003a) 'Transforming growth factor beta 1 and insulin-like growth factor 1 block collagen degradation induced by oncostatin M in combination with tumour necrosis factor alpha from bovine cartilage', *Ann Rheum Dis*, 62, (2), pp. 172-4.

Hui, W., Rowan, A. D. and Cawston, T. (2001) 'Modulation of the expression of matrix metalloproteinase and tissue inhibitors of metalloproteinases by TGF-β1 and IGF-1 in primary human articular and bovine nasal chondrocytes stimulated with TNF-α', *Cytokine*, 16, (1), pp. 31-35.

Hui, W., Rowan, A. D., Richards, C. D. and Cawston, T. E. (2003b) 'Oncostatin M in combination with tumor necrosis factor alpha induces cartilage damage and matrix metalloproteinase expression in vitro and in vivo', *Arthritis & Rheumatism*, 48, (12), pp. 3404-18.

Ikemefuna, O., Mary, B. G. and Chisa, H. (2009) 'Chondrogenesis, joint formation, and articular cartilage regeneration', *Journal of Cellular Biochemistry,* 107, (3), pp. 383-392.

Inouye, K., Tsuzuki, S., Yasumoto, M., Kojima, K., Mochida, S. and Fushiki, T. (2010) 'Identification of the matriptase second CUB domain as the secondary site for interaction with hepatocyte growth factor activator inhibitor type-1', *J Biol Chem*.

Irving, J. A., Pike, R. N., Lesk, A. M. and Whisstock, J. C. (2000) 'Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function', *Genome Res,* 10, (12), pp. 1845-64.

Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T. and Coughlin, S. R. (1997) 'Protease-activated receptor 3 is a second thrombin receptor in humans', *Nature,* 386, (6624), pp. 502-6.

Ishii, T., Ohnuma, K., Murakami, A., Takasawa, N., Kobayashi, S., Dang, N. H., Schlossman, S. F. and Morimoto, C. (2001) 'CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO', *Proc Natl Acad Sci U S A,* 98, (21), pp. 12138-12143.

Ito, A. and Nagase, H. (1988) 'Evidence that human rheumatoid synovial matrix metalloproteinase 3 is an endogenous activator of procollagenase', *Arch Biochem Biophys,* 267, (1), pp. 211-6.

Iwaki-Egawa, S., WatanabeY and Matsuno, H. (2001) 'Correlations between matrix metalloproteinase-9 and adenosine deaminase isozymes in synovial fluid from patients with rheumatoid arthritis', *J Rheumatol,* 28, (3), pp. 485-9.

Iyer, S., Wei, S., Brew, K. and Acharya, K. R. (2007) 'Crystal Structure of the Catalytic Domain of Matrix Metalloproteinase-1 in Complex with the Inhibitory Domain of Tissue Inhibitor of Metalloproteinase-1', *J Biol Chem,* 282, (1), pp. 364-371.

Jamshid, K., Vadim, P. and Billy, G. H. (2008) 'Mammalian collagen IV', *Microscopy Research and Technique,* 71, (5), pp. 357-370.

Janciauskiene, S., Nita, I., Subramaniyam, D., Li, Q., Lancaster, J. R., Jr. and Matalon, S. (2008) 'Alpha1-antitrypsin inhibits the activity of the matriptase catalytic domain in vitro', *Am J Respir Cell Mol Biol,* 39, (6), pp. 631-7.

Janusz, M. J., Hookfin, E. B., Brown, K. K., Hsieh, L. C., Heitmeyer, S. A., Taiwo, Y. O., Natchus, M. G., Pikul, S., Almstead, N. G., De, B., Peng, S. X., Baker, T. R. and Patel, V. (2006) 'Comparison of the pharmacology of hydroxamate- and carboxylate-based matrix metalloproteinase inhibitors (MMPIs) for the treatment of osteoarthritis', *Inflamm Res,* 55, (2), pp. 60-5.

Jin, T., Tarkowski, A., Carmeliet, P. and Bokarewa, M. (2003) 'Urokinase, a constitutive component of the inflamed synovial fluid, induces arthritis', *Arthritis Res Ther,* 5, (1), pp. R9-R17.

Jin, X., Yagi, M., Akiyama, N., Hirosaki, T., Higashi, S., Lin, C. Y., Dickson, R. B., Kitamura, H. and Miyazaki, K. (2006) 'Matriptase activates stromelysin (MMP-3) and promotes tumor growth and angiogenesis', *Cancer Sci,* 97, (12), pp. 1327-34.

Johnson, G. B., Brunn, G. J., Kodaira, Y. and Platt, J. L. (2002) 'Receptor-Mediated Monitoring of Tissue Well-Being Via Detection of Soluble Heparan Sulfate by Toll-Like Receptor 4', *J Immunol,* 168, (10), pp. 5233-5239.

Kadler, K. E., Holmes, D. F., Trotter, J. A. and Chapman, J. A. (1996) 'Collagen fibril formation', *Biochem J,* 316, (1), pp. 1-11.

Kafienah, W., Al-Fayez, F., Hollander, A. P. and Barker, M. D. (2003) 'Inhibition of cartilage degradation: A combined tissue engineering and gene therapy approach', *Arthritis & Rheumatism,* 48, (3), pp. 709-718.

Kafienah, W., Bromme, D., Buttle, D. J., Croucher, L. J. and Hollander, A. P. (1998) 'Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix', *Biochem J,* 331 (Pt 3), pp. 727-32.

Kalliolias, G. D. and C Liossis, S.-N. (2008) 'The future of the IL-1 receptor antagonist anakinra: from rheumatoid arthritis to adult-onset Still's disease and systemic-onset juvenile idiopathic arthritis', *Expert Opinion on Investigational Drugs,* 17, (3), pp. 349-359.

Kamori, M., Hagihara, M., Nagatsu, T., Iwata, H. and Miura, T. (1991) 'Activities of dipeptidyl peptidase II, dipeptidyl peptidase IV, prolyl endopeptidase, and collagenase-like peptidase in synovial membrane from patients with rheumatoid arthritis and osteoarthritis', *Biochem Med Metab Biol,* 45, (2), pp. 154-60.

Kang, T., Nagase, H. and Pei, D. (2002) 'Activation of Membrane-type Matrix Metalloproteinase 3 Zymogen by the Proprotein Convertase Furin in the trans-Golgi Network', *Cancer Res,* 62, (3), pp. 675-681.

Kaslik, G., Patthy, A., Balint, M. and Graf, L. (1995) 'Trypsin complexed with alpha 1-proteinase inhibitor has an increased structural flexibility', *FEBS Lett,* 370, (3), pp. 179-83.

Kaufmann, R., Schulze, B., Krause, G., Mayr, L. M., Settmacher, U. and Henklein, P. (2005) 'Proteinase-activated receptors (PARs)--the PAR3 Neo-N-terminal peptide TFRGAP interacts with PAR1', *Regul Pept,* 125, (1-3), pp. 61-6.

Kelly, T. (2005) 'Fibroblast activation protein-alpha and dipeptidyl peptidase IV (CD26): cell-surface proteases that activate cell signaling and are potential targets for cancer therapy', *Drug Resist Updat,* 8, (1-2), pp. 51-8.

Kelso, E. B., Ferrell, W. R., Lockhart, J. C., Elias-Jones, I., Hembrough, T., Dunning, L., Gracie, J. A. and McInnes, I. B. (2007) 'Expression and proinflammatory role of proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using

a novel proteinase-activated receptor 2 antagonist', *Arthritis & Rheumatism,* 56, (3), pp. 765-71.

Kelso, E. B., Lockhart, J. C., Hembrough, T., Dunning, L., Plevin, R., Hollenberg, M. D., Sommerhoff, C. P., McLean, J. S. and Ferrell, W. R. (2006) 'Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation', *J Pharmacol Exp Ther,* 316, (3), pp. 1017-24.

Kemmink, J. and Creighton, T. E. (1993) 'Local conformations of peptides representing the entire sequence of bovine pancreatic trypsin inhibitor and their roles in folding', *J Mol Biol,* 234, (3), pp. 861-78.

Kerin, A., Patwari, P., Kuettner, K., Cole, A. and Grodzinsky, A. (2002) 'Molecular basis of osteoarthritis: biomechanical aspects', *Cellular and Molecular Life Sciences,* 59, (1), pp. 27-35.

Kevorkian, L., Young, D. A., Darrah, C., Donell, S. T., Shepstone, L., Porter, S., Brockbank, S. M. V., Edwards, D. R., Parker, A. E. and Clark, I. M. (2004) 'Expression profiling of metalloproteinases and their inhibitors in cartilage', *Arthritis & Rheumatism,* 50, (1), pp. 131-141.

Kiani, C., Chen, L., Wu, Y. J., Yee, A. J. and Yang, B. B. (2002) 'Structure and function of aggrecan', *Cell Res,* 12, (1), pp. 19-32.

Kievit, W., Fransen, J., Oerlemans, A. J., Kuper, H. H., van der Laar, M. A., de Rooij, D. J., De Gendt, C. M., Ronday, K. H., Jansen, T. L., van Oijen, P. C., Brus, H. L., Adang, E. M. and van Riel, P. L. (2007) 'The efficacy of anti-TNF in rheumatoid arthritis, a comparison between randomised controlled trials and clinical practice', *Ann Rheum Dis,* 66, (11), pp. 1473-8.

Kinne, R., Stuhlmuller, B. and Burmester, G.-R. (2007) 'Cells of the synovium in rheumatoid arthritis. Macrophages', *Arthritis Res Ther,* 9, (6), pp. 224.

Kirilak, Y., Pavlos, N. J., Willers, C. R., Han, R., Feng, H., Xu, J., Asokananthan, N., Stewart, G. A., Henry, P., Wood, D. and Zheng, M. H. (2006) 'Fibrin sealant promotes migration and proliferation of human articular chondrocytes: possible involvement of thrombin and protease-activated receptors', *International Journal of Molecular Medicine.*, 17, (4), pp. 551-558.

Kitamoto, Y., Yuan, X., Wu, Q., McCourt, D. W. and Sadler, J. E. (1994) 'Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains', *Proc Natl Acad Sci U S A*, 91, (16), pp. 7588-92.

Kjoller, L., Engelholm, L. H., Hoyer-Hansen, M., Dano, K., Bugge, T. H. and Behrendt, N. (2004) 'uPARAP/endo180 directs lysosomal delivery and degradation of collagen IV', *Exp Cell Res*, 293, (1), pp. 106-16.

Knäuper, V., López-Otin, C., Smith, B., Knight, G. and Murphy, G. (1996) 'Biochemical Characterization of Human Collagenase-3', in *J Biol Chem*. Vol. 271, pp. 1544-1550.

Kolacna, L., Bakesova, J., Varga, F., Kostakova, E., Planka, L., Necas, A., Lukas, D., Amler, E. and Pelouch, V. (2007) 'Biochemical and biophysical aspects of collagen nanostructure in the extracellular matrix', *Physiol Res*, 56 Suppl 1, pp. S51-60.

Koshelnick, Y., Ehart, M., Hufnagl, P., Heinrich, P. C. and Binder, B. R. (1997) 'Urokinase Receptor Is Associated with the Components of the JAK1/STAT1 Signaling Pathway and Leads to Activation of This Pathway upon Receptor Clustering in the Human Kidney Epithelial Tumor Cell Line TCL-598', *J Biol Chem*, 272, (45), pp. 28563-28567.

Koshy, P. J., Lundy, C. J., Rowan, A. D., Porter, S., Edwards, D. R., Hogan, A., Clark, I. M. and Cawston, T. E. (2002) 'The modulation of matrix metalloproteinase

and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M: a time-course study using real-time quantitative reverse transcription-polymerase chain reaction', *Arthritis & Rheumatism,* 46, (4), pp. 961-7.

Kuettner, K. E. (1992) 'Biochemistry of articular cartilage in health and disease', *Clinical Biochemistry,* 25, (3), pp. 155-163.

Kwan, A. P., Cummings, C. E., Chapman, J. A. and Grant, M. E. (1991) 'Macromolecular organization of chicken type X collagen in vitro', *J Cell Biol,* 114, (3), pp. 597-604.

Lambeir, A. M., Borloo, M., De Meester, I., Belyaev, A., Augustyns, K., Hendriks, D., Scharpe, S. and Haemers, A. (1996) 'Dipeptide-derived diphenyl phosphonate esters: mechanism-based inhibitors of dipeptidyl peptidase IV', *Biochim Biophys Acta,* 1290, (1), pp. 76-82.

Lankas, G. R., Leiting, B., Roy, R. S., Eiermann, G. J., Beconi, M. G., Biftu, T., Chan, C.-C., Edmondson, S., Feeney, W. P., He, H., Ippolito, D. E., Kim, D., Lyons, K. A., Ok, H. O., Patel, R. A., Petrov, A. N., Pryor, K. A., Qian, X., Reigle, L., Woods, A., Wu, J. K., Zaller, D., Zhang, X., Zhu, L., Weber, A. E. and Thornberry, N. A. (2005) 'Dipeptidyl Peptidase IV Inhibition for the Treatment of Type 2 Diabetes', *Diabetes,* 54, (10), pp. 2988-2994.

Lapadula, G., Iannone, F., Zuccaro, C., Covelli, M., Patella, V., Lobianco, G. and Pipitone, V. (1995) 'Expression of membrane-bound peptidases (CD10 and CD26) on human articular chondrocytes. Possible role of neuropeptidases in the pathogenesis of osteoarthritis', *Clin Exp Rheumatol,* 13, (2), pp. 143-8.

Lee, D. M., Friend, D. S., Gurish, M. F., Benoist, C., Mathis, D. and Brenner, M. B. (2002) 'Mast cells: a cellular link between autoantibodies and inflammatory arthritis', *Science,* 297, (5587), pp. 1689-92.

Lee, D. M. and Weinblatt, M. E. (2001) 'Rheumatoid arthritis', *The Lancet*, 358, (9285), pp. 903-911.

Lee, K. N., Jackson, K. W., Christiansen, V. J., Chung, K. H. and McKee, P. A. (2004) 'A novel plasma proteinase potentiates {alpha}2-antiplasmin inhibition of fibrin digestion', *Blood*, 103, (10), pp. 3783-3788.

Lee, K. N., Jackson, K. W., Christiansen, V. J., Lee, C. S., Chun, J.-G. and McKee, P. A. (2006) 'Antiplasmin-cleaving enzyme is a soluble form of fibroblast activation protein', *Blood*, 107, (4), pp. 1397-1404.

Lee, S.-L., Dickson, R. B. and Lin, C.-Y. (2000) 'Activation of Hepatocyte Growth Factor and Urokinase/Plasminogen Activator by Matriptase, an Epithelial Membrane Serine Protease', *J Biol Chem*, 275, (47), pp. 36720-36725.

Lefebvre, O., Regnier, C., Chenard, M. P., Wendling, C., Chambon, P., Basset, P. and Rio, M. C. (1995) 'Developmental expression of mouse stromelysin-3 mRNA', *Development*, 121, (4), pp. 947-955.

Levy, D. E., Lapierre, F., Liang, W., Ye, W., Lange, C. W., Li, X., Grobelny, D., Casabonne, M., Tyrrell, D., Holme, K., Nadzan, A. and Galardy, R. E. (1998) 'Matrix Metalloproteinase Inhibitors: A Structure−Activity Study', *Journal of Medicinal Chemistry*, 41, (2), pp. 199-223.

Levy, M. T., McCaughan, G. W., Abbott, C. A., Park, J. E., Cunningham, A. M., Müller, E., Rettig, W. J. and Gorrell, M. D. (1999) 'Fibroblast activation protein: A cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodelling interface in human cirrhosis', *Hepatology*, 29, (6), pp. 1768-1778.

Li, C. S., Deschenes, D., Desmarais, S., Falgueyret, J.-P., Gauthier, J. Y., Kimmel, D. B., Léger, S., Massé, F., McGrath, M. E., McKay, D. J., Percival, M. D., Riendeau, D., Rodan, S. B., Thérien, M., Truong, V.-L., Wesolowski, G.,

Zamboni, R. and Black, W. C. (2006) 'Identification of a potent and selective non-basic cathepsin K inhibitor', *Bioorganic & Medicinal Chemistry Letters,* 16, (7), pp. 1985-1989.

Li, J., Ny, A., Leonardsson, G., Nandakumar, K. S., Holmdahl, R. and Ny, T. (2005) 'The plasminogen activator/plasmin system is essential for development of the joint inflammatory phase of collagen type II-induced arthritis', *Am J Pathol,* 166, (3), pp. 783-92.

Ligueros-Saylan, M., Foley, J. E., Schweizer, A., Couturier, A. and Kothny, W. (2010) 'An assessment of adverse effects of vildagliptin versus comparators on the liver, the pancreas, the immune system, the skin and in patients with impaired renal function from a large pooled database of Phase II and III clinical trials', *Diabetes, Obesity and Metabolism,* 12, (6), pp. 495-509.

Lin, C. Y., Anders, J., Johnson, M., Sang, Q. A. and Dickson, R. B. (1999) 'Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity', *J Biol Chem,* 274, (26), pp. 18231-6.

Lin, C. Y., Wang, J. K., Torri, J., Dou, L., Sang, Q. A. and Dickson, R. B. (1997) 'Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. Monoclonal antibody production, isolation, and localization', *J Biol Chem,* 272, (14), pp. 9147-52.

List, K., Haudenschild, C. C., Szabo, R., Chen, W., Wahl, S. M., Swaim, W., Engelholm, L. H., Behrendt, N. and Bugge, T. H. (2002) 'Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis', *Oncogene,* 21, (23), pp. 3765-79.

List, K., Kosa, P., Szabo, R., Bey, A. L., Wang, C. B., Molinolo, A. and Bugge, T. H. (2009) 'Epithelial Integrity Is Maintained by a Matriptase-Dependent Proteolytic Pathway', *Am J Pathol,* 175, (4), pp. 1453-1463.

Loebermann, H., Tokuoka, R., Deisenhofer, J. and Huber, R. (1984) 'Human [alpha]1-proteinase inhibitor : Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function', *J Mol Biol,* 177, (3), pp. 531-557.

Longo, P. G., Laurenti, L., Gobessi, S., Sica, S., Leone, G. and Efremov, D. G. (2008) 'The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells', *Blood,* 111, (2), pp. 846-55.

Loster, K., Zeilinger, K., Schuppan, D. and Reutter, W. (1995) 'The Cysteine-Rich Region of Dipeptidyl Peptidase IV (CD 26) Is the Collagen Binding Site', *Biochemical and Biophysical Research Communications,* 217, (1), pp. 341-348.

Ludwig, A., Schiemann, F., Mentlein, R., Lindner, B. and Brandt, E. (2002) 'Dipeptidyl peptidase IV (CD26) on T cells cleaves the CXC chemokine CXCL11 (I-TAC) and abolishes the stimulating but not the desensitizing potential of the chemokine', *J Leukoc Biol,* 72, (1), pp. 183-91.

Lundy, S., Sarkar, S., Tesmer, L. and Fox, D. (2007) 'Cells of the synovium in rheumatoid arthritis. T lymphocytes', *Arthritis Res Ther,* 9, (1), pp. 202.

Lutzky, V., Hannawi, S. and Thomas, R. (2007) 'Cells of the synovium in rheumatoid arthritis. Dendritic cells', *Arthritis Res Ther,* 9, (4), pp. 219.

Luyten, F. P., Hascall, V. C., Nissley, S. P., Morales, T. I. and Reddi, A. H. (1988) 'Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants', *Arch Biochem Biophys,* 267, (2), pp. 416-25.

Lynne, C. T., Daman, J. A. and David, E. W. (2001) 'Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic

cartilage: Associations with degenerative changes', *Arthritis & Rheumatism*, 44, (3), pp. 585-594.

Ma, L., Perini, R., McKnight, W., Dicay, M., Klein, A., Hollenberg, M. D. and Wallace, J. L. (2005) 'Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets', *Proc Natl Acad Sci U S A*, 102, (1), pp. 216-20.

Maini, R. and Feldmann, M. (2002) 'How does infliximab work in rheumatoid arthritis?', *Arthritis Res*, 4, (Suppl 2), pp. S22 - S28.

Majithia, V. and Geraci, S. A. (2007) 'Rheumatoid Arthritis: Diagnosis and Management', *The American Journal of Medicine*, 120, (11), pp. 936-939.

Mankin, H. J. (1982) 'The response of articular cartilage to mechanical injury', *J Bone Joint Surg Am*, 64, (3), pp. 460-466.

Martel-Pelletier, J., Boileau, C., Pelletier, J.-P. and Roughley, P. J. (2008) 'Cartilage in normal and osteoarthritis conditions', *Best Practice & Research Clinical Rheumatology*, 22, (2), pp. 351-384.

Martin, J. A. and Buckwalter, J. A. (2001) 'Roles of articular cartilage aging and chondrocyte senescence in the pathogenesis of osteoarthritis', *Iowa Orthop J*, 21, pp. 1-7.

Martin, M., Huguet, J., Centelles, J. J. and Franco, R. (1995) 'Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex. Possible role of adenosine deaminase as costimulatory molecule', *J Immunol*, 155, (10), pp. 4630-43.

Materazzi, S., Pellerito, S., Di Serio, C., Paglierani, M., Naldini, A., Ardinghi, C., Carraro, F., Geppetti, P., Cirino, G., Santucci, M., Tarantini, F. and Massi, D. (2007) 'Analysis of protease-activated receptor-1 and -2 in human scar formation', *J Pathol*, 212, (4), pp. 440-9.

Mauri, C. and Ehrenstein, M. (2007) 'Cells of the synovium in rheumatoid arthritis. B cells', *Arthritis Res Ther,* 9, (2), pp. 205.

McGuinness, C. and Wesley, U. V. (2008) 'Dipeptidyl peptidase IV (DPPIV), a candidate tumor suppressor gene in melanomas is silenced by promoter methylation', *Front Biosci,* 13, pp. 2435-43.

McLaughlin, J. N., Patterson, M. M. and Malik, A. B. (2007) 'Protease-activated receptor-3 (PAR3) regulates PAR1 signaling by receptor dimerization', *Proc Natl Acad Sci U S A,* 104, (13), pp. 5662-7.

Menshikov, M., Torosyan, N., Elizarova, E., Plakida, K., Vorotnikov, A., Parfyonova, Y., Stepanova, V., Bobik, A., Berk, B. and Tkachuk, V. (2006) 'Urokinase induces matrix metalloproteinase-9/gelatinase B expression in THP-1 monocytes via ERK1/2 and cytosolic phospholipase A2 activation and eicosanoid production', *J Vasc Res,* 43, (5), pp. 482-90.

Mentlein, R. (1999) 'Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides', *Regul Pept,* 85, (1), pp. 9-24.

Miller, E. J. and Rhodes, R. K. (1982) 'Preparation and characterization of the different types of collagen', *Methods Enzymol,* 82 Pt A, pp. 33-64.

Milner, J., Rowan, A., Cawston, T. and Young, D. (2006a) 'Metalloproteinase and inhibitor expression profiling of resorbing cartilage reveals pro-collagenase activation as a critical step for collagenolysis', *Arthritis Res Ther,* 8, (5), pp. R142.

Milner, J. M., Elliott, S. F. and Cawston, T. E. (2001) 'Activation of procollagenases is a key control point in cartilage collagen degradation: interaction of serine and metalloproteinase pathways', *Arthritis & Rheumatism,* 44, (9), pp. 2084-96.

Milner, J. M., Kevorkian, L., Young, D. A., Jones, D., Wait, R., Donell, S. T., Barksby, E., Patterson, A. M., Middleton, J., Cravatt, B. F., Clark, I. M., Rowan, A. D.

and Cawston, T. E. (2006b) 'Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis', *Arthritis Res Ther,* 8, (1), pp. R23.

Milner, J. M., Patel, A. and Rowan, A. D. (2008) 'Emerging roles of serine proteinases in tissue turnover in arthritis', *Arthritis & Rheumatism,* 58, (12), pp. 3644-3656.

Milner, J. M., Rowan, A. D., Elliott, S. F. and Cawston, T. E. (2003) 'Inhibition of furin-like enzymes blocks interleukin-1alpha/oncostatin M-stimulated cartilage degradation', *Arthritis & Rheumatism,* 48, (4), pp. 1057-66.

Miyake, Y., Tsuzuki, S., Fushiki, T. and Inouye, K. (2010) 'Matriptase Does Not Require Hepatocyte Growth Factor Activator Inhibitor Type-1 for Activation in an Epithelial Cell Expression Model', *Biosci Biotechnol Biochem*.

Monsky, W. L., Lin, C. Y., Aoyama, A., Kelly, T., Akiyama, S. K., Mueller, S. C. and Chen, W. T. (1994) 'A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells', *Cancer Res,* 54, (21), pp. 5702-10.

Monvoisin, A., Bisson, C., Si-Tayeb, K., Balabaud, C., Desmoulière, A. and Rosenbaum, J. (2002) 'Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells', *International Journal of Cancer,* 97, (2), pp. 157-162.

Morgan, T. G., Rowan, A. D., Dickinson, S. C., Jones, D., Hollander, A. P., Deehan, D. and Cawston, T. E. (2006) 'Human nasal cartilage responds to oncostatin M in combination with interleukin 1 or tumour necrosis factor alpha by the release of collagen fragments via collagenases', *Ann Rheum Dis,* 65, (2), pp. 184-90.

Morris, R., Winyard, P. G., Brass, L. F., Blake, D. R. and Morris, C. J. (1996) 'Thrombin receptor expression in rheumatoid and osteoarthritic synovial tissue', *Ann Rheum Dis,* 55, (11), pp. 841-3.

Mott, J. D. and Werb, Z. (2004) 'Regulation of matrix biology by matrix metalloproteinases', *Current Opinion in Cell Biology,* 16, (5), pp. 558-564.

Mueller, S. C., Ghersi, G., Akiyama, S. K., Sang, Q. X., Howard, L., Pineiro-Sanchez, M., Nakahara, H., Yeh, Y. and Chen, W. T. (1999) 'A novel protease-docking function of integrin at invadopodia', *J Biol Chem,* 274, (35), pp. 24947-52.

Muir, H. (1995) 'The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules', *Bioessays,* 17, (12), pp. 1039-48.

Muller-Ladner, U., Ospelt, C., Gay, S., Distler, O. and Pap, T. (2007) 'Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts', *Arthritis Res Ther,* 9, (6), pp. 223.

Mullick, A., Xu, Y., Warren, R., Koutroumanis, M., Guilbault, C., Broussau, S., Malenfant, F., Bourget, L., Lamoureux, L., Lo, R., Caron, A., Pilotte, A. and Massie, B. (2006) 'The cumate gene-switch: a system for regulated expression in mammalian cells', *BMC Biotechnology,* 6, (1), pp. 43.

Murphy, G., Cawston, T. E., Galloway, W. A., Barnes, M. J., Bunning, R. A., Mercer, E., Reynolds, J. J. and Burgeson, R. E. (1981) 'Metalloproteinases from rabbit bone culture medium degrade types IV and V collagens, laminin and fibronectin', *Biochem J,* 199, (3), pp. 807-11.

Murphy, G., Cockett, M. I., Stephens, P. E., Smith, B. J. and Docherty, A. J. (1987) 'Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes', *Biochem J,* 248, (1), pp. 265-8.

Murphy, G., Knauper, V., Atkinson, S., Butler, G., English, W., Hutton, M., Stracke, J. and Clark, I. (2002) 'Matrix metalloproteinases in arthritic disease', *Arthritis Res,* 4 Suppl 3, pp. S39-49.

Nagase, H. and Woessner, J. F. (1999) 'Matrix Metalloproteinases', *J Biol Chem*, 274, (31), pp. 21491-21494.

Nakahara, H., Mueller, S. C., Nomizu, M., Yamada, Y., Yeh, Y. and Chen, W.-T. (1998) 'Activation of β1 Integrin Signaling Stimulates Tyrosine Phosphorylation of p190RhoGAP and Membrane-protrusive Activities at Invadopodia', *J Biol Chem*, 273, (1), pp. 9-12.

Nakano, S., Mishiro, T., Takahara, S., Yokoi, H., Hamada, D., Yukata, K., Takata, Y., Goto, T., Egawa, H., Yasuoka, S., Furouchi, H., Hirasaka, K., Nikawa, T. and Yasui, N. (2007) 'Distinct expression of mast cell tryptase and protease activated receptor-2 in synovia of rheumatoid arthritis and osteoarthritis', *Clin Rheumatol*, 26, (8), pp. 1284-92.

Narra, K., Mullins, S. R., Lee, H. O., Strzemkowski-Brun, B., Magalong, K., Christiansen, V. J., McKee, P. A., Egleston, B., Cohen, S. J., Weiner, L. M., Meropol, N. J. and Cheng, J. D. (2007) 'Phase II trial of single agent Val-boroPro (Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer', *Cancer Biol Ther*, 6, (11), pp. 1691-9.

Nemunaitis, J., Poole, C., Primrose, J., Rosemurgy, A., Malfetano, J., Brown, P., Berrington, A., Cornish, A., Lynch, K., Rasmussen, H., Kerr, D., Cox, D. and Millar, A. (1998) 'Combined analysis of studies of the effects of the matrix metalloproteinase inhibitor marimastat on serum tumor markers in advanced cancer: selection of a biologically active and tolerable dose for longer-term studies', *Clinical Cancer Research*, 4, (5), pp. 1101-1109.

Neumann, U., Kubota, H., Frei, K., Ganu, V. and Leppert, D. (2004) 'Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme', *Analytical Biochemistry*, 328, (2), pp. 166-173.

Newman, A. P. (1998) 'Articular Cartilage Repair', *The American Journal of Sports Medicine,* 26, (2), pp. 309-324.

Nobuyuki, M., Kazuto, S., Makoto, G., Minoru, S., Motonobu, N., Kazuhiko, I. and Kusuki, N. (1988) 'Augmented interleukin-1 production and hla-dr expression in the synovium of rheumatoid arthritis patients', *Arthritis & Rheumatism,* 31, (4), pp. 480-486.

Noël, A., Santavicca, M., Stoll, I., L'Hoir, C., Staub, A., Murphy, G., Rio, M.-C. and Basset, P. (1995) 'Identification of Structural Determinants Controlling Human and Mouse Stromelysin-3 Proteolytic Activities', *J Biol Chem,* 270, (39), pp. 22866-22872.

Oberst, M. D., Williams, C. A., Dickson, R. B., Johnson, M. D. and Lin, C. Y. (2003) 'The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor', *J Biol Chem,* 278, (29), pp. 26773-9.

Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J.-P., Rio, M.-C. and Basset, P. (1997) 'Expression of Matrix Metalloproteinases during Rat Skin Wound Healing: Evidence that Membrane Type-1 Matrix Metalloproteinase Is a Stromal Activator of Pro-Gelatinase A', *J Cell Biol,* 137, (1), pp. 67-77.

Okada, Y., Nagase, H. and Harris, E. D. (1986) 'A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization', *J Biol Chem,* 261, (30), pp. 14245-14255.

Ospelt, C., Mertens, J. C., Juengel, A., Brentano, F., Maciejewska-Rodriguez, H., Huber, L. C., Hemmatazad, H., Wuest, T., Knuth, A., Gay, R. E., Michel, B. A., Gay, S., Renner, C. and Bauer, S. (2010) 'Inhibition of fibroblast activation protein and dipeptidyl peptidase IV increases cartilage invasion by rheumatoid arthritis synovial fibroblasts', *Arthritis & Rheumatism*.

Ostermann, E., Garin-Chesa, P., Heider, K. H., Kalat, M., Lamche, H., Puri, C., Kerjaschki, D., Rettig, W. J. and Adolf, G. R. (2008) 'Effective immunoconjugate therapy in cancer models targeting a serine protease of tumor fibroblasts', *Clin Cancer Res*, 14, (14), pp. 4584-92.

Ostrowska, E. and Reiser, G. (2008) 'The protease-activated receptor-3 (PAR-3) can signal autonomously to induce interleukin-8 release', *Cell Mol Life Sci*, 65, (6), pp. 970-81.

Palmer, H. S., Kelso, E. B., Lockhart, J. C., Sommerhoff, C. P., Plevin, R., Goh, F. G. and Ferrell, W. R. (2007) 'Protease-activated receptor 2 mediates the proinflammatory effects of synovial mast cells', *Arthritis & Rheumatism*, 56, (11), pp. 3532-40.

Park, J. E., Lenter, M. C., Zimmermann, R. N., Garin-Chesa, P., Old, L. J. and Rettig, W. J. (1999) 'Fibroblast activation protein, a dual specificity serine protease expressed in reactive human tumor stromal fibroblasts', *J Biol Chem*, 274, (51), pp. 36505-12.

Pedrozo, H. A., Schwartz, Z., Gomez, R., Ornoy, A., Xin-Sheng, W., Dallas, S. L., Bonewald, L. F., Dean, D. D. and Boyan, B. D. (1998) 'Growth plate chondrocytes store latent transforming growth factor (TGF)-&bgr;1 in their matrix through latent TGF-&bgr;1 binding protein-1', *Journal of Cellular Physiology*, 177, (2), pp. 343-354.

Pei, D., Majmudar, G. and Weiss, S. J. (1994) 'Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3', *J Biol Chem*, 269, (41), pp. 25849-25855.

Pelletier, J.-P. and Martel-Pelletier, J. (2003) 'Oncostatin M: Foe or friend?', *Arthritis & Rheumatism*, 48, (12), pp. 3301-3303.

Pfander, D., Cramer, T., Weseloh, G., Pullig, O., Schuppan, D., Bauer, M. and Swoboda, B. (1999) 'Hepatocyte growth factor in human osteoarthritic cartilage', *Osteoarthritis and Cartilage,* 7, (6), pp. 548-59.

Pineiro-Sanchez, M. L., Goldstein, L. A., Dodt, J., Howard, L., Yeh, Y. and Chen, W.-T. (1997) 'Identification of the 170-kDa Melanoma Membrane-bound Gelatinase (Seprase) as a Serine Integral Membrane Protease', *J Biol Chem,* 272, (12), pp. 7595-7601.

Pingguan-Murphy, B., Lee, D. A., Bader, D. L. and Knight, M. M. (2005) 'Activation of chondrocytes calcium signalling by dynamic compression is independent of number of cycles', *Arch Biochem Biophys,* 444, (1), pp. 45-51.

Poole, C. A. (1997) 'Review. Articular cartilage chondrons: form, function and failure', *Journal of Anatomy,* 191, (01), pp. 1-13.

Pratley, R. E. and Salsali, A. (2007) 'Inhibition of DPP-4: a new therapeutic approach for the treatment of type 2 diabetes', *Curr Med Res Opin,* 23, (4), pp. 919-31.

Proost, P., De Meester, I., Schols, D., Struyf, S., Lambeir, A. M., Wuyts, A., Opdenakker, G., De Clercq, E., Scharpe, S. and Van Damme, J. (1998) 'Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis and HIV-1-infection', *J Biol Chem,* 273, (13), pp. 7222-7.

Proost, P., Schutyser, E., Menten, P., Struyf, S., Wuyts, A., Opdenakker, G., Detheux, M., Parmentier, M., Durinx, C., Lambeir, A. M., Neyts, J., Liekens, S., Maudgal, P. C., Billiau, A. and Van Damme, J. (2001) 'Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties', *Blood,* 98, (13), pp. 3554-61.

Quesada, V., Ordonez, G. R., Sanchez, L. M., Puente, X. S. and Lopez-Otin, C. (2009) 'The Degradome database: mammalian proteases and diseases of proteolysis', *Nucleic Acids Res*, 37, (Database issue), pp. D239-43.

Qvist, H., Sjostrom, H. and Noren, O. (1998) 'The TATA-less, GC-rich porcine dipeptidylpeptidase IV (DPPIV) promoter shows bidirectional activity', *Biol Chem*, 379, (1), pp. 75-81.

Ramachandran, R., Sadofsky, L. R., Xiao, Y., Botham, A., Cowen, M., Morice, A. H. and Compton, S. J. (2007) 'Inflammatory mediators modulate thrombin and cathepsin-G signaling in human bronchial fibroblasts by inducing expression of proteinase-activated receptor-4', *Am J Physiol Lung Cell Mol Physiol*, 292, (3), pp. L788-98.

Rawlings, N. D. and Barrett, A. J. (1993) 'Evolutionary families of peptidases', *Biochem J*, 290, (1), pp. 205-218.

Reboul, P., Pelletier, J.-P., Tardif, G., Benderdour, M., Ranger, P., Bottaro, D. P. and Martel-Pelletier, J. (2001) 'Hepatocyte growth factor induction of collagenase 3 production in human osteoarthritic cartilage: Involvement of the stress-activated protein kinase/c-Jun N-terminal kinase pathway and a sensitive p38 mitogen-activated protein kinase inhibitor cascade', *Arthritis & Rheumatism*, 44, (1), pp. 73-84.

Reimer, M. K., Holst, J. J. and Ahren, B. (2002) 'Long-term inhibition of dipeptidyl peptidase IV improves glucose tolerance and preserves islet function in mice', *Eur J Endocrinol*, 146, (5), pp. 717-27.

Reinhold, D., Biton, A., Pieper, S., Lendeckel, U., Faust, J., Neubert, K., Bank, U., Tager, M., Ansorge, S. and Brocke, S. (2006) 'Dipeptidyl peptidase IV (DP IV, CD26) and aminopeptidase N (APN, CD13) as regulators of T cell function and

targets of immunotherapy in CNS inflammation', *International Immunopharmacology*, 6, (13-14), pp. 1935-1942.

Rettig, W. J., Garin-Chesa, P., Healey, J. H., Su, S. L., Ozer, H. L., Schwab, M., Albino, A. P. and Old, L. J. (1993) 'Regulation and Heteromeric Structure of the Fibroblast Activation Protein in Normal and Transformed Cells of Mesenchymal and Neuroectodermal Origin', *Cancer Res*, 53, (14), pp. 3327-3335.

Rettig, W. J., Su, S. L., Fortunato, S. R., Scanlan, M. J., Raj, B. K. M., Garin-Chesa, P., Healey, J. H. and Old, L. J. (1994) 'Fibroblast activation protein: Purification, epitope mapping and induction by growth factors', *International Journal of Cancer*, 58, (3), pp. 385-392.

Robak, T., Gladalska, A., Stepien, H. and Robak, E. (1998) 'Serum levels of interleukin-6 type cytokines and soluble interleukin-6 receptor in patients with rheumatoid arthritis', *Mediators Inflamm*, 7, (5), pp. 347-53.

Rockel, J., Bernier, S. and Leask, A. (2009) 'Egr-1 inhibits the expression of extracellular matrix genes in chondrocytes by TNFalpha-induced MEK/ERK signalling', *Arthritis Res Ther*, 11, (1), pp. R8.

Rowan, A. D. (2001) 'Cartilage catabolism in arthritis: factors that influence homeostasis', *Expert Rev Mol Med*, 2001, pp. 1-20.

Rowan, A. D., Koshy, P. J. T., Shingleton, W. D., Degnan, B. A., Heath, J. K., Vernallis, A. B., Spaull, J. R., Life, P. F., Hudson, K. and Cawston, T. E. (2001) 'Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown', *Arthritis & Rheumatism*, 44, (7), pp. 1620-1632.

Saharinen, J., Hyytiäinen, M., Taipale, J. and Keski-Oja, J. (1999) 'Latent transforming growth factor-[beta] binding proteins (LTBPs)--structural extracellular matrix

proteins for targeting TGF-[beta] action', *Cytokine & Growth Factor Reviews*, 10, (2), pp. 99-117.

Salter, D. M., Hughes, D. E., Simpson, R. and Gardner, D. L. (1992) 'Integrin expression by human articular chondrocytes', *Rheumatology*, 31, (4), pp. 231-234.

Schick, C., Pemberton, P. A., Shi, G. P., Kamachi, Y., Cataltepe, S., Bartuski, A. J., Gornstein, E. R., Bromme, D., Chapman, H. A. and Silverman, G. A. (1998) 'Cross-class inhibition of the cysteine proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: a kinetic analysis', *Biochemistry*, 37, (15), pp. 5258-66.

Schmid, T. M. and Linsenmayer, T. F. (1983) 'A short chain (pro)collagen from aged endochondral chondrocytes. Biochemical characterization', *J Biol Chem*, 258, (15), pp. 9504-9509.

Schwarz, H., Villiger, P. M., von Kempis, J. and Lotz, M. (1994) 'Neuropeptide Y is an inducible gene in the human immune system', *J Neuroimmunol*, 51, (1), pp. 53-61.

Schweinitz, A., Dönnecke, D., Ludwig, A., Steinmetzer, P., Schulze, A., Kotthaus, J., Wein, S., Clement, B. and Steinmetzer, T. (2009) 'Incorporation of neutral C-terminal residues in 3-amidinophenylalanine-derived matriptase inhibitors', *Bioorganic & Medicinal Chemistry Letters*, 19, (7), pp. 1960-1965.

Scott, A. M., Wiseman, G., Welt, S., Adjei, A., Lee, F. T., Hopkins, W., Divgi, C. R., Hanson, L. H., Mitchell, P., Gansen, D. N., Larson, S. M., Ingle, J. N., Hoffman, E. W., Tanswell, P., Ritter, G., Cohen, L. S., Bette, P., Arvay, L., Amelsberg, A., Vlock, D., Rettig, W. J. and Old, L. J. (2003) 'A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer', *Clin Cancer Res*, 9, (5), pp. 1639-47.

Sedo, A., Duke-Cohan, J. S., Balaziova, E. and Sedova, L. R. (2005) 'Dipeptidyl peptidase IV activity and/or structure homologs: contributing factors in the pathogenesis of rheumatoid arthritis?', *Arthritis Res Ther,* 7, (6), pp. 253-69.

Shaw, R. J. and Cantley, L. C. (2006) 'Ras, PI(3)K and mTOR signalling controls tumour cell growth', *Nature,* 441, (7092), pp. 424-430.

Shi, Y. E., Torri, J., Yieh, L., Wellstein, A., Lippman, M. E. and Dickson, R. B. (1993) 'Identification and characterization of a novel matrix-degrading protease from hormone-dependent human breast cancer cells', *Cancer Res,* 53, (6), pp. 1409-15.

Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K. and Kitamura, N. (1997) 'Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor', *J Biol Chem,* 272, (10), pp. 6370-6.

Shin, S. Y., Kim, J. H., Baker, A., Lim, Y. and Lee, Y. H. (2010) 'Transcription Factor Egr-1 Is Essential for Maximal Matrix Metalloproteinase-9 Transcription by Tumor Necrosis Factor α', *Molecular Cancer Research,* 8, (4), pp. 507-519.

Sigler, P. B., Blow, D. M., Matthews, B. W. and Henderson, R. (1968) 'Structure of crystalline -chymotrypsin. II. A preliminary report including a hypothesis for the activation mechanism', *J Mol Biol,* 35, (1), pp. 143-64.

Sokolova, E., Grishina, Z., Buhling, F., Welte, T. and Reiser, G. (2005) 'Protease-activated receptor-1 in human lung fibroblasts mediates a negative feedback downregulation via prostaglandin E2', *Am J Physiol Lung Cell Mol Physiol,* 288, (5), pp. L793-802.

Solau-Gervais, E., Zerimech, F., Lemaire, R., Fontaine, C., Huet, G. and Flipo, R. M. (2007) 'Cysteine and serine proteases of synovial tissue in rheumatoid arthritis and osteoarthritis', *Scand J Rheumatol,* 36, (5), pp. 373-7.

Steffensen, B., Wallon, U. M. and Overall, C. M. (1995) 'Extracellular matrix binding properties of recombinant fibronectin type II-like modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen', *J Biol Chem*, 270, (19), pp. 11555-11566.

Steitz, T. A., Henderson, R. and Blow, D. M. (1969) 'Structure of crystalline alpha-chymotrypsin. 3. Crystallographic studies of substrates and inhibitors bound to the active site of alpha-chymotrypsin', *J Mol Biol*, 46, (2), pp. 337-48.

Sternlicht, M. D. and Werb, Z. (2001) 'How matrix metalloproteinases regulate cell behavior', *Annu Rev Cell Dev Biol*, 17, pp. 463-516.

Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G. and Nagase, H. (1990) 'Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin)', *Biochemistry*, 29, (44), pp. 10261-70.

Swingler, T. E., Waters, J. G., Davidson, R. K., Pennington, C. J., Puente, X. S., Darrah, C., Cooper, A., Donell, S. T., Guile, G. R., Wang, W. and Clark, I. M. (2009) 'Degradome expression profiling in human articular cartilage', *Arthritis Res Ther*, 11, (3), pp. R96.

Szabo, R. and Bugge, T. H. (2008) 'Type II transmembrane serine proteases in development and disease', *Int J Biochem Cell Biol*, 40, (6-7), pp. 1297-316.

Tahtis, K., Lee, F. T., Wheatley, J. M., Garin-Chesa, P., Park, J. E., Smyth, F. E., Obata, Y., Stockert, E., Hall, C. M., Old, L. J., Rettig, W. J. and Scott, A. M. (2003) 'Expression and targeting of human fibroblast activation protein in a human skin/severe combined immunodeficient mouse breast cancer xenograft model', *Mol Cancer Ther*, 2, (8), pp. 729-37.

Takada, Y., Ye, X. and Simon, S. (2007) 'The integrins', *Genome Biology*, 8, (5), pp. 215.

Takasaki, K., Iwase, M., Nakajima, T., Ueno, K., Nomoto, Y., Nakanishi, S. and Higo, K. (2004) 'K579, a slow-binding inhibitor of dipeptidyl peptidase IV, is a long-acting hypoglycemic agent', *European Journal of Pharmacology,* 486, (3), pp. 335-342.

Takeuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R. and Craik, C. S. (2000) 'Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates', *J Biol Chem,* 275, (34), pp. 26333-42.

Takeuchi, T., Shuman, M. A. and Craik, C. S. (1999) 'Reverse biochemistry: Use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue', *Proc Natl Acad Sci U S A,* 96, (20), pp. 11054-11061.

Tan, L., Peng, H., Osaki, M., Choy, B. K., Auron, P. E., Sandell, L. J. and Goldring, M. B. (2003) 'Egr-1 Mediates Transcriptional Repression of COL2A1Promoter Activity by Interleukin-1β', *J Biol Chem,* 278, (20), pp. 17688-17700.

Tanaka, S., Murakami, T., Horikawa, H., Sugiura, M., Kawashima, K. and Sugita, T. (1997) 'Suppression of arthritis by the inhibitors of dipeptidyl peptidase IV', *International Journal of Immunopharmacology,* 19, (1), pp. 15-24.

Tanaka, Y., Sekiguchi, F., Hong, H. and Kawabata, A. (2008) 'PAR2 triggers IL-8 release via MEK/ERK and PI3-kinase/Akt pathways in GI epithelial cells', *Biochemical and Biophysical Research Communications,* 377, (2), pp. 622-626.

Tchetverikov, I., Verzijl, N., Huizinga, T. W., TeKoppele, J. M., Hanemaaijer, R. and DeGroot, J. (2003) 'Active MMPs captured by alpha 2 macroglobulin as a marker of disease activity in rheumatoid arthritis', *Clin Exp Rheumatol,* 21, (6), pp. 711-8.

Todorovic, V., Jurukovski, V., Chen, Y., Fontana, L., Dabovic, B. and Rifkin, D. B. (2005) 'Latent TGF-[beta] binding proteins', *The International Journal of Biochemistry & Cell Biology*, 37, (1), pp. 38-41.

Tran, T., Quan, C., Edosada, C. Y., Mayeda, M., Wiesmann, C., Sutherlin, D. and Wolf, B. B. (2007) 'Synthesis and structure-activity relationship of N-acyl-Gly-, N-acyl-Sar- and N-blocked-boroPro inhibitors of FAP, DPP4, and POP', *Bioorganic & Medicinal Chemistry Letters*, 17, (5), pp. 1438-1442.

Tsuji, T., Sugahara, K., Tsuruda, K., Uemura, A., Harasawa, H., Hasegawa, H., Hamaguchi, Y., Tomonaga, M., Yamada, Y. and Kamihira, S. (2004) 'Clinical and oncologic implications in epigenetic down-regulation of CD26/dipeptidyl peptidase IV in adult T-cell leukemia cells', *Int J Hematol*, 80, (3), pp. 254-60.

Tyler, J. A. (1989) 'Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines', *Biochem J*, 260, (2), pp. 543-8.

Uhland, K. (2006) 'Matriptase and its putative role in cancer', *Cell Mol Life Sci*, 63, (24), pp. 2968-78.

Urban, J. P. G. (1994) 'The chondrocyte: a cell under pressure', *Rheumatology*, 33, (10), pp. 901-908.

van der Kraan, P. M., Blaney Davidson, E. N., Blom, A. and van den Berg, W. B. (2009) 'TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: Modulation and integration of signaling pathways through receptor-Smads', *Osteoarthritis and Cartilage*, 17, (12), pp. 1539-1545.

van der Kraan, P. M., Stoop, R., Meijers, T. H., Poole, A. R. and van den Berg, W. B. (2001) 'Expression of type X collagen in young and old C57Bl/6 and Balb/c mice. Relation with articular cartilage degeneration', *Osteoarthritis and Cartilage*, 9, (2), pp. 92-100.

Van Lint, P. and Libert, C. (2006) 'Matrix metalloproteinase-8: Cleavage can be decisive', *Cytokine & Growth Factor Reviews,* 17, (4), pp. 217-223.

Van Wart, H. E. and Birkedal-Hansen, H. (1990) 'The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family', *Proc Natl Acad Sci U S A,* 87, (14), pp. 5578-82.

Velasco, G., Pendás, A. M., Fueyo, A., Knãuper, V., Murphy, G. and López-Otín, C. (1999) 'Cloning and Characterization of Human MMP-23, a New Matrix Metalloproteinase Predominantly Expressed in Reproductive Tissues and Lacking Conserved Domains in Other Family Members', *J Biol Chem,* 274, (8), pp. 4570-4576.

Vincan, E. and Barker, N. (2008) 'The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression', *Clin Exp Metastasis*.

Vincenti, M. and Brinckerhoff, C. (2001) 'Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1beta', *Arthritis Res,* 3, (6), pp. 381 - 388.

Visse, R. and Nagase, H. (2003) 'Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases: Structure, Function, and Biochemistry', *Circ Res,* 92, (8), pp. 827-839.

Vogel, W., Gish, G. D., Alves, F. and Pawson, T. (1997) 'The discoidin domain receptor tyrosine kinases are activated by collagen', *Mol Cell,* 1, (1), pp. 13-23.

Wang, F. L., Connor, J. R., Dodds, R. A., James, I. E., Kumar, S., Zou, C., Lark, M. W., Gowen, M. and Nuttall, M. E. (2000) 'Differential expression of Egr-1 in osteoarthritic compared to normal adult human articular cartilage', *Osteoarthritis and Cartilage,* 8, (3), pp. 161-169.

Wang, H. and Keiser, J. A. (2000) 'Hepatocyte Growth Factor Enhances MMP Activity in Human Endothelial Cells', *Biochemical and Biophysical Research Communications,* 272, (3), pp. 900-905.

Wang, H., Wen, S., Bunnett, N. W., Leduc, R., Hollenberg, M. D. and MacNaughton, W. K. (2008) 'Proteinase-activated receptor-2 induces cyclooxygenase-2 expression through beta-catenin and cyclic AMP-response element-binding protein', *J Biol Chem,* 283, (2), pp. 809-15.

Wang, P. and DeFea, K. A. (2006) 'Protease-activated receptor-2 simultaneously directs beta-arrestin-1-dependent inhibition and Galphaq-dependent activation of phosphatidylinositol 3-kinase', *Biochemistry,* 45, (31), pp. 9374-85.

Welgus, H. G., Jeffrey, J. J. and Eisen, A. Z. (1981) 'The collagen substrate specificity of human skin fibroblast collagenase', *J Biol Chem,* 256, (18), pp. 9511-9515.

Windsor, L. J., Grenett, H., Birkedal-Hansen, B., Bodden, M. K., Engler, J. A. and Birkedal-Hansen, H. (1993) 'Cell type-specific regulation of SL-1 and SL-2 genes. Induction of the SL-2 gene but not the SL-1 gene by human keratinocytes in response to cytokines and phorbolesters', *J Biol Chem,* 268, (23), pp. 17341-7.

Wipff, P.-J. and Hinz, B. (2008) 'Integrins and the activation of latent transforming growth factor [beta]1 - An intimate relationship', *European Journal of Cell Biology,* 87, (8-9), pp. 601-615.

Woessner, J. F. and Nagase, H. (2000) *Matrix Metalloproteinases and TIMPs.* Oxford University Press.

Wolberg, G., Zimmerman, T. P., Hiemstra, K., Winston, M. and Chu, L. C. (1975) 'Adenosine inhibition of lymphocyte-mediated cytolysis: possible role of cyclic adenosine monophosphate', *Science,* 187, (4180), pp. 957-9.

Xiang, Y., Masuko-Hongo, K., Sekine, T., Nakamura, H., Yudoh, K., Nishioka, K. and Kato, T. (2006) 'Expression of proteinase-activated receptors (PAR)-2 in

articular chondrocytes is modulated by IL-1[beta], TNF-[alpha] and TGF-[beta]', *Osteoarthritis and Cartilage,* 14, (11), pp. 1163-1173.

Yazbeck, R., Howarth, G. S. and Abbott, C. A. (2009) 'Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease?', *Trends in Pharmacological Sciences,* 30, (11), pp. 600-607.

Zhang, F., Tom, C. C., Kugler, M. C., Ching, T.-T., Kreidberg, J. A., Wei, Y. and Chapman, H. A. (2003) 'Distinct ligand binding sites in integrin α3β1 regulate matrix adhesion and cell–cell contact', *J Cell Biol,* 163, (1), pp. 177-188.

Zhang, J., Valianou, M. and Cheng, J. D. (2010) 'Identification and characterization of the promoter of fibroblast activation protein', *Front Biosci (Elite Ed),* 2, pp. 1154-63.

Zhang, Q., Hui, W., Litherland, G. J., Barter, M. J., Davidson, R., Darrah, C., Donell, S. T., Clark, I. M., Cawston, T. E., Robinson, J. H., Rowan, A. D. and Young, D. A. (2008) 'Differential Toll-like receptor-dependent collagenase expression in chondrocytes', *Ann Rheum Dis,* 67, (11), pp. 1633-41.

Zhang, X. P., Puzon-McLaughlin, W., Irie, A., Kovach, N., Prokopishyn, N. L., Laferte, S., Takeuchi, K., Tsuji, T. and Takada, Y. (1999) 'α3β1 Adhesion to Laminin-5 and Invasin: Critical and Differential Role of Integrin Residues Clustered at the Boundary between α3 N-Terminal Repeats 2 and 3', *Biochemistry,* 38, (43), pp. 14424-14431.