

**Investigation of the effects of injury upon intracellular
signalling pathways and expression of inflammatory
response genes in articular cartilage**

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ABSTRACT

Damage to joints predisposes to osteoarthritis. The mechanism by which injury to cartilage might lead to net matrix loss and cartilage degeneration remains unknown. Following experimental sharp injury to porcine articular cartilage (dissection from, or scoring of the articular surface), our group has previously shown rapid activation of the 3 mitogen activated protein kinase (MAPK) pathways in cartilage. Activation of ERK, and probably also p38 MAPK, is due to release of fibroblast growth factor (FGF) from the matrix after injury. However, despite a long search, the cause of JNK activation following sharp injury remains unknown. I investigated the extent and regulation of inflammatory signalling after cartilage injury and whether it was sufficient to cause expression of inflammatory response genes.

In this work, I show that a number of intracellular signalling pathways including PI3 kinase and I κ B kinase (IKK) (which leads to activation of NF κ B) are activated by sharp injury to cartilage. The signalling following injury was sufficient to induce a wide range of inflammatory response mRNAs, including pro-inflammatory cytokines such as IL-6, COX-2 and proteinases such as MMP-1 and ADAMTS-4 in a pattern which was not entirely IL-1-like. Pharmacological inhibition experiments suggested that the production by injured cartilage of an inflammatory response gene which could be assayed at the protein level, activin A, was regulated by FGF-mediated pathways (ERK) as well as by NF κ B and tyrosine kinases (src family kinases).

Given these findings, the role of tyrosine kinases in the early response of cartilage to injury was explored. By phosphotyrosine immunoprecipitation and purification from injured cartilage lysates, FAK and its substrate paxillin were identified from silver-stained gels by mass spectrometry. The phosphorylation of these src substrates

accounted for rapidly inducible bands seen on phosphotyrosine western blotting of injured cartilage lysates. However, no evidence of a role for src kinases in the regulation of MAPK/IKK signalling upon injury was found. In contrast, blockade of another tyrosine kinase, the FGF receptor, led to partial inhibition not only of the ERK pathway following sharp injury, but also the other MAPKs and IKK. Whilst activation of the same pathways was also seen following injury to synovium, FGF receptor inhibition had no effect on this signalling. This suggested that FGF may have a pro-inflammatory action following injury *in vivo* which is a particular feature of cartilage.

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Statement of Originality

I confirm that all the experimental work presented in this thesis has been performed by me and is to the best of my knowledge original. Any collaboration, assistance, development of methods or relevant prior experimental findings by others has been expressly acknowledged in the relevant sections. The work has been carried out at the Kennedy Institute of Rheumatology, Imperial College London. The Kennedy Trustees and subsequently the Wellcome Trust funded this work.

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Abbreviations

1D	One dimensional
2D	Two dimensional
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motif
AEBSF	4-(2-aminoethyl) benzene sulphonyl fluoride hydrochloride
AP	Activating protein
APS	Ammonium persulphate
ARE	Adenosine- and uridine-rich elements
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
CD	Cluster of differentiation
cDNA	Complementary DNA
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
DAG	Diacyl glycerol
DAB	Diaminobenzidine tetrahydrochloride
dd	Double-distilled
Diss ⁿ	Dissection
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOK	Downstream of tyrosine kinases
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellularly regulated kinase
ESI	Electrospray ionisation
EST	Expressed sequence tag
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
HEPES	N-2(hydroxyethyl)-1-piperazine N'-2 ethanesulphonic acid
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IGD	Interglobular domain
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase

iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation/immunoprecipitate
IRAK	IL-1 receptor-associated kinase
JNK	c jun N-terminal kinase
kD	Kilodalton
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP	Metacarpophalangeal
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MT-MMP	Membrane bound MMP
<i>m/z</i>	Mass-to-charge ratio
MyD88	Myeloid differentiation factor 88
NCBI	National Centre for Biotechnology Information
NEMO	NFκB Essential Modulator
NFκB	Nuclear factor kappa B
N/I	Non-immune
NIK	NFκB inducing kinase
OA	Osteoarthritis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PI	Phosphoinositide/phosphoinositol
PLC	Phospholipase C
PKC	Protein kinase C
PMSF	Phenyl methyl sulphonyl fluoride
PNPP	Para-nitro phenyl phosphate
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene difluoride
RIP	Receptor interacting kinase
RIPA	Radio-immunoprecipitation assay buffer
r.p.m	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFK	Src family kinase
SH	Src homology
SMAD	Signalling mothers against decapentaplegic
Syk	Spleen tyrosine kinase
TAB	TAK-1 binding protein
TAK1	TGFβ-activated kinase-1
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N'N'N'N'- Tetraethyl methylene diamine

TFA	Trifluoroacetic acid
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TIR	TLR/IL-1 receptor
TLR	Toll-like receptor
T _m	Melting temperature
TNF	Tumour necrosis factor
Tpl-2	Tumour progression locus-2
TRAF	TNF-associated factor
Tyr	Tyrosine residue
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Background

Osteoarthritis (OA) is a common, disabling arthropathy whose pathogenesis is not understood. Injuries to articular cartilage, or to a joint, such as destabilising ligamentous tears, predispose to OA. Understanding the molecular processes involved in the response of cartilage to experimental injury should give insights into cartilage physiology and its degeneration.

Our laboratory has been investigating the cellular responses of porcine articular cartilage to injuries such as scoring its surface, or dissecting the tissue into culture (explantation). Such damage is simple and reproducible, and causes rapid activation of the mitogen-activated protein kinases (MAPK) (Gruber et al., 2004). Explantation of cartilage also causes some degree of degradation of the inhibitor of kappa B ($\text{IkB}\alpha$), implying that there may be activation of nuclear factor kappa B ($\text{NF}\kappa\text{B}$), and there is also evidence of induction of expression of interleukin (IL)-1. The pattern of intracellular signalling pathway activation which follows cartilage injury is very similar to that caused by IL-1. IL-1, as is well known, causes catabolism (breakdown) of the cartilage matrix, so the signalling caused by injury could have a similar effect and therefore lead to degeneration. This might be a direct effect, or secondary to production of IL-1.

Tonia Vincent in our laboratory has shown that the activation of one of the MAPK pathways, the extracellularly regulated kinase (ERK), was due to the release following injury of fibroblast growth factor-2 (FGF-2) from the pericellular matrix (Vincent et al., 2002; Vincent et al., 2007). FGF-2 may be involved in gene expression in cartilage after injury but it would be unlikely to account for the induction of IL-1 gene expression observed by (Gruber et al., 2004). The mechanism of activation of the other MAPK pathways is unknown, as are the extent and significance of activation of

NFκB. Members of our laboratory have searched extensively for a soluble factor released from the cartilage upon injury that was capable of activating the c-jun N-terminal kinase (JNK) MAPK pathway in cultured chondrocytes or cartilage explants, but none has been found (A. Didangelos, Ph.D. thesis, 2008).

The aims of my research project were firstly to investigate whether signalling pathways were activated in response to cartilage injury that would lead to activation of NFκB. Secondly, to investigate the extent and degree of expression of inflammatory response genes following injury and to define pharmacologically which signalling pathways this depended upon. Lastly, I wished to investigate the mechanism by which intracellular signalling was initiated by injury.

To introduce the work, I will describe the anatomy and physiology of articular cartilage and summarise knowledge of the extracellular matrix and its turnover. Current views of the pathogenesis of OA including the role of mechanical factors will be reviewed. Lastly, I will describe the intracellular signalling pathways being studied, and review our knowledge of the effects of injury on cartilage.

1.2 Cartilage structure and function

A synovial joint is comprised of two articulating surfaces, coated with hyaline (articular) cartilage, encased in a capsule which is lined by synovium. The synovium produces synovial fluid which serves as both a surface lubricant and a source of nutrients for the articular cartilage. It is an ultrafiltrate of plasma with additional components secreted by synoviocytes and chondrocytes. Articular cartilage has exceptional mechanical properties (Buckwalter and Mankin, 1998b). It is composed of a strong, resilient extracellular matrix whose major components are type II collagen fibres and the aggregating proteoglycan aggrecan. 75% of the matrix is comprised of water. Of its dry weight, 70% is collagen, 20% is proteoglycan and 10% other matrix

proteins (Eyre, 2002). Articular cartilage is avascular and aneural. There is only one cell type: the chondrocyte.

1.2.1 The chondrocyte

Chondrocytes are derived from the mesenchyme (Goldring et al., 2006). They are about 10 microns in diameter, with a large nucleus to cytoplasm ratio. The chondrocyte is a metabolically active cell which exists in relative hypoxia, depending mainly on anaerobic metabolism (Archer and Francis-West, 2003). It is uncertain whether or not cell division occurs in articular cartilage after an individual reaches skeletal maturity and in the absence of disease. In human articular cartilage, chondrocytes are sparsely distributed, comprising just 1% of its volume. They mostly exist in isolation, but sometimes in twos and threes and are surrounded by specialised extracellular matrix.

1.2.2 The extracellular matrix of cartilage

The extracellular matrix is a system of fibres and soluble polyelectrolytes in which the chondrocytes are embedded.

Collagen: Collagens type II, VI, IX, X and XI are all present in articular cartilage, but the major structural collagen of cartilage is type II. The procollagen molecule is processed during secretion by the chondrocyte. The release of the C-propeptide of type II procollagen (CPII) is measured as a surrogate marker of new synthesis of the molecule (Nelson et al., 1998). The α -helical region of the type II collagen polypeptide has repetitive tri-peptide sequences of gly-pro-X, or gly-X-hyp, X being any amino acid. Because of this unique sequence, the α -helices of the collagen II α 1 chains form a triple helix of three polypeptide chains, which then associate to form collagen fibrils. Bunches of collagen fibrils form fibres, which are responsible for the form and tensile strength of cartilage (Eyre, 2002). The fibres form arcades, which are

predominantly perpendicular throughout the middle zone of cartilage, but curve to lie horizontally across the superficial zone, forming the basic architecture of the cartilage. Little type II collagen is synthesised in adulthood and the matrix is potentially vulnerable to catabolism of this molecule by metalloproteinases (Hermansson et al., 2004).

Proteoglycans: 90% of the proteoglycan mass of cartilage is made up by aggrecan. Each aggrecan molecule consists of a protein core, with three globular domains. This has covalently attached to it numerous sulphated glycosaminoglycan chains (long unbranched chains of repeating disaccharides that contain an amino sugar) of keratan and chondroitin sulphates. Aggrecan molecules aggregate to a backbone of hyaluronan and this interaction is stabilised by link protein. Aggrecan thus forms huge aggregates which fill the interfibrillar space of the matrix (Roughley, 2006). The glycosaminoglycan chains of aggrecan repel each other and are hydrophilic. In cartilage they are underhydrated and exert a swelling pressure which enables the cartilage to resist compression. The repulsive forces of cations mean that the effective pore size of cartilage is low: large proteins such as albumin and smaller negatively charged proteins tend to be excluded from the matrix (Buckwalter and Mankin, 1998b). Proteinases secreted by the chondrocyte are able to cleave aggrecan, leading to its loss from the matrix. Aggrecan is constantly synthesised in adult cartilage to counteract this loss.

Decorin, biglycan, fibromodulin and lumican are small proteoglycans found in the matrix which have functions in its organisation and stabilisation (Roughley, 2006). The glycoprotein cartilage oligomeric matrix protein (COMP) is found mainly, but not exclusively in cartilage, and is important for binding and stabilising collagens (Posey and Hecht, 2008). Fibronectin is a ubiquitous extracellular matrix glycoprotein

also found in cartilage. It can bind integrins, collagen and heparan sulphate proteoglycans.

Pericellular matrix: The region of matrix immediately surrounding the chondrocyte is known as the pericellular matrix. This is structurally and biochemically distinct from the ‘territorial’ extracellular matrix. It is abundant in perlecan (a heparan sulphate proteoglycan) and type VI collagen. Perlecan binds other matrix molecules and growth factors such as FGF-2 (Vincent and Saklatvala, 2006). Cell-matrix interactions are likely to allow the chondrocyte to respond to mechanical forces. Examples of interactions between cell surface molecules and the extracellular matrix include integrins binding to collagen and fibronectin, the tyrosine kinase receptor DDR-2 binding to collagens, and the cellular receptor CD44 binding to hyaluronan (Knudson and Knudson, 2004; Vogel et al., 1997; Xu et al., 2007).

1.3 Cartilage extracellular matrix metabolism

The chondrocytes remodel the matrix by synthesising matrix components and their proteinases: matrix metalloproteinases and aggrecanases.

1.3.1 Proteinases

Metalloproteinases (MMPs): MMPs are zinc-dependent multi-domain endopeptidases which are capable of degrading a wide variety of substrates including all extracellular matrix proteins. There are five main groups: the collagenases, gelatinases, stromelysins, matrilysins and the membrane-associated MT-MMPs (Murphy et al., 2002). MMPs are secreted as latent propeptides and activated by proteolytic cleavage. The type II collagen triple helix can be cleaved by MMPs-1,-8 and -13 at a specific site three quarters along the length of the molecule, generating characteristic one-quarter and three-quarter fragments. The neoepitopes formed by this cleavage can be detected by antibodies (COL2-1/4N1 and COL2-3/4C_{short})

(Billinghurst et al., 1997; Hollander et al., 1994). At physiological temperature this cleavage causes the triple helix to unwind, rendering it susceptible to cleavage by gelatinases such as MMP-9, and stromelysins such as MMP-3. MT1-MMP (MMP-14) also displays collagenolytic activity (Mengshol et al., 2002).

Aggrecanases: Aggrecan contains two major cleavage sites between its first two globular domains (the interglobular domain, or IGD). The first site between residues Asn³⁴¹ and Phe³⁴² is susceptible to all known MMPs. The second site between Glu³⁷³ and Ala³⁷⁴ is susceptible to aggrecanases of the A Disintegrin And Metalloproteinase with ThombosSpondin type 1 Motif (ADAMTS) family, resulting in the C-terminal neoepitope NITEGE and the N-terminal neoepitope ARGSV (Nagase and Kashiwagi, 2003).

Western blotting of synovial fluid and cultured cartilage with antibodies to the various neoepitopes formed by aggrecanolysis has made it clear that the IGD ‘aggrecanase’ cleavage site is functionally the more important (Lohmander et al., 1993; van Meurs et al., 1999). There was a long search to identify the key aggrecanase in cartilage (Sandy et al., 1991). The multidomain ADAMTSs-1,-4,-5,-8,-9,-15,-16 and -18 can all cleave aggrecan at the Glu³⁷³-Ala³⁷⁴ bond, but of these ADAMTS-4 and ADAMTS-5 are the most active aggrecanases under physiological conditions (Fushimi et al., 2008). ADAMTS-5 appears to be the most efficient aggrecanase (Gendron et al., 2007). It has also been implicated as the major aggrecanase in both *in vivo* and *in vitro* murine models of degeneration (Glasson et al., 2005; Stanton et al., 2005). However, in human cartilage, ADAMTS-4 may also be important in aggrecan degradation, because there has been one report that suppression of ADAMTS-4 and ADAMTS-5, either individually or in combination, inhibited the degradation of aggrecan in cytokine-stimulated human cartilage explants (Song et al., 2007). Recently, there has

been a report of novel aggrecanase activity which is up-regulated by retinoic acid in mouse cartilage deficient in ADAMTS-4 and -5. The identity and physiological relevance of this putative aggrecanase is unknown (Rogerson et al., 2008).

1.3.2 Tissue inhibitors of the matrix metalloproteinases (TIMPs)

The net amount of proteolysis in cartilage is dependent on the balance of proteinase and TIMP levels (Murphy et al., 2002). 4 TIMPs regulate the extent of proteolysis: they are synthesised by chondrocytes and are also present in other connective tissues. TIMP-3 inhibits ADAMTS-4 and -5. Its K_i is in the subnanomolar range (Nagase and Kashiwagi, 2003). IL-1-induced cleavage of the porcine aggrecanase site is blocked by TIMP-3 (Gendron et al., 2003). Transforming growth factor (TGF) β and oncostatin M both induce TIMP-3 *in vitro* (Li and Zafarullah, 1998).

1.3.3 Factors regulating turnover of cartilage extracellular matrix

Chondrocytes *in vitro* respond to various growth factors, cytokines and other agents, but the importance of these molecules in *in vivo* matrix metabolism is poorly understood (Hui et al., 2001; Nieminen et al., 2005).

IL-1 and other catabolic factors: IL-1 is a prototypical pro-inflammatory cytokine, locally causing cell activation, leucocyte egress to sites of damage and tissue resorption (see also sections 1.4.6 and 1.5). If it enters the systemic circulation it causes fever and is partly responsible for the acute phase response. It causes chondrocytes to degrade rapidly the proteoglycan of their extracellular matrix by induction of aggrecanases, while collagen degradation occurs more slowly and depends upon MMPs (Kobayashi et al., 2005). IL-1 also inhibits the synthesis of proteoglycan (Bau et al., 2002; Hui et al., 2001; Vincenti and Brinckerhoff, 2002).

Tumour necrosis factor (TNF) α also has IL-1-like effects on cartilage, although is probably less potent than IL-1 (Kobayashi et al., 2005). IL-17 appears to potentiate the effects of IL-1 and TNF α on proteoglycan degradation, although has no effect on its own (Durigova et al., 2008; Van Bezooijen et al., 2002). Oncostatin M enhances the resorptive effects of IL-1 (Barksby et al., 2006). Vitamin A (retinoic acid) also causes degradation of matrix in cultured cartilage explants, either alone, or in combination with IL-1 (Flannery et al., 1999). However, there is little evidence to suggest that any of these agents are normally produced by the chondrocyte and are therefore relevant to physiological matrix turnover (Barksby et al., 2006; Flannery et al., 1999). Other factors present in human cartilage have been reported to up-regulate specific proteinases. Connective tissue growth factor up-regulates ADAMTS-5 expression in synovium (Blaney Davidson et al., 2006) and galectin-3, a soluble lectin, weakly stimulates ADAMTS-5 in OA chondrocytes (Janelle-Montcalm et al., 2007).

Insulin-like growth factor-1 (IGF-1): IGF-1 is present in plasma and is a normal constituent of extracellular fluid. Treatment of cultured articular chondrocytes with IGF-1 causes increased proteoglycan synthesis, as measured by sulphate incorporation (Loeser et al., 2005). IGF-1 is thought to regulate proteoglycan turnover (Luyten et al., 1988). Although active secretion of IGF-1 by cartilage is difficult to demonstrate, insulin-like growth factor binding proteins are expressed by cartilage, and probably act to regulate the autocrine effects of IGFs on cartilage (Sun T. et al., 2008)

Transforming growth factor β : The TGF β superfamily includes the TGF β s, the bone morphogenetic proteins (BMPs) and the activins, and is involved in growth, differentiation and development. There are conflicting reports of the effects of TGF β on cultured chondrocytes: both stimulation (Morales and Roberts, 1988) and

inhibition of proteoglycan synthesis have been documented (Posever et al., 1995; van der Kraan et al., 1992). A recent study showed that cultured bovine chondrocytes treated with TGF β synthesised more glycosaminoglycan and collagen type II than untreated cells (Davies et al., 2008). TGF β might also partially inhibit IL-1 induced aggrecan degradation (Andrews et al., 1989).

Bone morphogenetic proteins: BMPs were first identified by their ability to induce new bone formation. They are important for chondrogenesis and probably in maintenance of cartilage. BMP-2,-4,-6 and -7 are expressed by chondrocytes. BMP-7 (also known as osteogenic protein 1) stimulates cartilage proteoglycan synthesis *in vitro* and potentiates the anabolic effects of IGF-1 (Chubinskaya et al., 2007a; Chubinskaya et al., 2007b). BMP-7 may also counteract the catabolic effect of IL-1 on human chondrocytes (Huch et al., 1997).

Activin A: Activin A is another member of the TGF β superfamily. It is a homodimer of inhibin β A chains. There are 4 different mammalian inhibin β chains. On heterodimerisation with inhibin α chains they form inhibins. Activins are antagonised by inhibins (which compete for cell surface receptors) and by a binding protein follistatin. Activins bind and activate activin receptors (transmembrane serine-threonine kinases on the cell surface related to TGF β receptors) and signal via ALK-4 and smad 2 and 3 signalling (also activated by TGF β).

Activin A is an important regulator of chondrogenesis, osteogenesis and cell proliferation (Hubner et al., 1996). Inhibin β A knockout mice are born with severe craniofacial abnormalities including cleft palate and eyelid defects and die within 24 hours of birth. There is evidence that activin A plays a role in the healing of tissues. For example, its over-expression increases granulation and scar tissue following wounding (Munz et al., 1999). Activin A is expressed by cultured chondrocytes in

response to a number of growth factors and pro-inflammatory cytokines including FGF-2, TGF β and IL-1 α (Hermansson et al., 2004). It is also produced *in vivo* at sites of inflammation (Hubner et al., 1997; Yu et al., 1998).

Work from our laboratory identified inhibin β A by mass spectrometry in medium from cultured human cartilage. No other inhibin or TGF β family members were found by proteomic analysis suggesting that, of this family, activin A was the major member expressed by mature articular cartilage (Hermansson et al., 2004). Consistent with this was the observation that all secreted TGF β -like activity in cartilage cultures appeared to be due to activin A (Alexander et al., 2007). Activin A induced TIMP-1 in chondrocytes, suggesting that it might have an anti-catabolic role in cartilage (Hermansson et al., 2004).

Fibroblast growth factor: FGF-2 is sequestered in the pericellular matrix of articular cartilage, bound to the proteoglycan perlecan (Vincent et al., 2007). FGF signalling is necessary for the function of the chondrocytes of the growth plate: FGF-1 and FGF-2 are both expressed here (Jingushi et al., 1995). Achondroplasia is caused by activating mutations in fibroblast growth factor receptor-3. However, the role of FGFs in normal adult cartilage is not understood.

FGFs are a family of at least 15 structurally related heparin-binding growth factors which primarily exert their effect on cells of mesenchymal and neuroectodermal lineage. FGF receptors (FGFRs), of which there are four, are tyrosine kinases. FGF binding to an FGFR causes receptor dimerisation and auto-phosphorylation on tyrosine residues (Ensoli, 2003). This leads to activation of a number of signalling pathways (see section 1.6 & Fig 1.1).

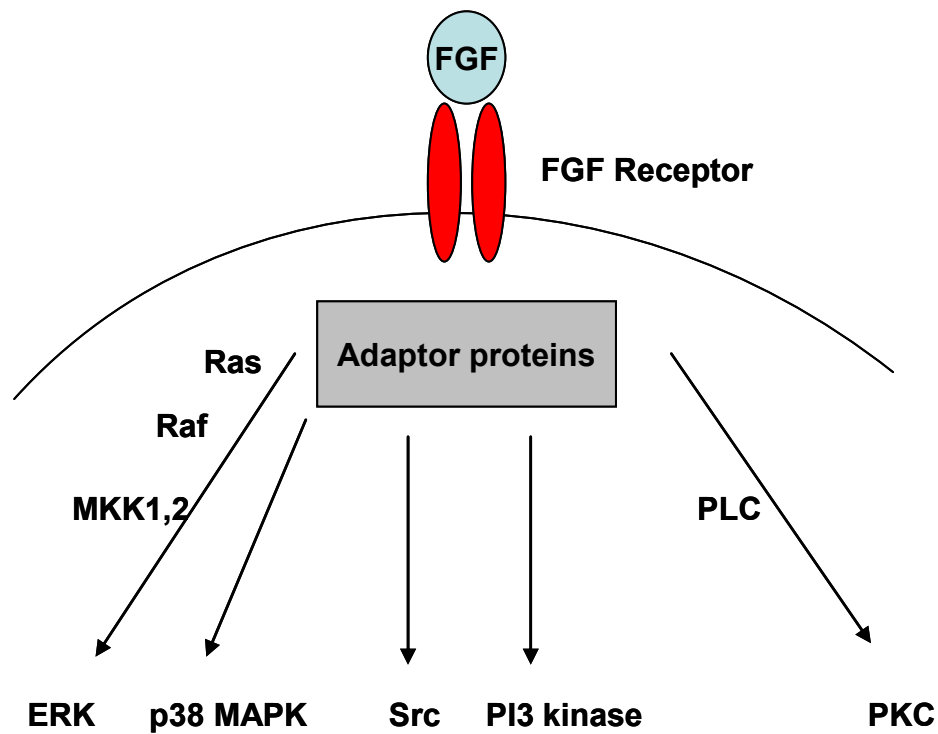


Figure 1.1. FGF-induced signalling in mammalian cells.

Fibroblast growth factor (FGF) binds to a transmembrane receptor causing it to dimerise. Adaptor proteins are recruited causing activation of the MAP kinase (MAPK) pathways extracellularly regulated kinase (ERK) and p38 MAPK and src family kinases (src). The phosphoinositol (PI)3 kinase pathway and protein kinase C (PKC) are also activated.

FGFs have important roles in embryonic growth, specifically in limb and skeletal development. They cause cell survival, proliferation (including angiogenesis) and differentiation, and are implicated in haematopoiesis and tumorigenesis (Ensoli, 2003; Ribatti et al., 2007).

The effects of FGF on cartilage seem complex. It appears to be important in maintaining chondrocyte phenotype *in vitro* (Murakami et al., 2000), and may enhance extracellular matrix formation *in vivo* (Cuevas et al., 1988). FGF-2 induces TIMP-1 in cultured chondrocytes, but also induces metalloproteinases (MMP-1 and -3) (Vincent et al., 2002). In an *in vitro* model of aggrecan breakdown in cartilage explants, pre-incubation with exogenous FGF-2 suppresses IL-1-induced aggrecan neoepitope formation, suggesting that FGF is anti-catabolic in this system (Sawaji et al., 2008). However, another group has shown that FGF-2 inhibits in a dose-dependent manner the anabolic effects of IGF-1 and osteogenic protein 1 on human articular chondrocytes (Loeser et al., 2005).

1.3.4 Mechanotransduction

Low levels of load-bearing appear to be necessary for maintenance of normal cartilage. The thickness of knee cartilage significantly reduces after one year in patients who are not weight-bearing because of spinal cord injury (Vanwanseele et al., 2003). How cartilage senses and responds to mechanical stimuli is not well understood (Millward-Sadler et al., 1999; Vincent et al., 2004).

Integrins: Integrins are a family of extracellular matrix receptors. At least 20 different integrin heterodimers exist, made up of combinations of various α with β subunits. Chondrocytes express a number of these, and much speculation has surrounded whether or not they represent cartilage ‘mechanotransducers’ (Loeser,

2002). $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ can all bind collagen type II and $\alpha_5\beta_1$ is the chondrocyte fibronectin receptor. Each α and β subunit consists of a large extracellular domain, a transmembrane region and a cytoplasmic tail. Integrins do not have intrinsic signalling activity. Their cytoplasmic tails interact with the actin cytoskeleton and various adaptor proteins, including molecules in the focal adhesion complex (Millward-Sadler et al., 1999).

Integrins cause mechanically induced stiffening responses in endothelial cells (Wang et al., 1993). Integrin-dependent cellular responses to mechanical stimuli (which are reduced in the presence of inhibitory integrin peptides) are reported in a number of cultured cells including cardiac myocytes and chondrocytes (Millward-Sadler et al., 1999). The number of α_5 subunits rises in response to mechanical stress applied to bovine cartilage explants (Lucchinetti et al., 2004). However, there is no direct evidence for a role for integrins in the response of intact cartilage to injury.

Other potential mechanisms of mechanotransduction: It is possible that soluble factors such as growth factors transduce biomechanical signals. Even very gentle cyclical loading of cartilage explants causes release of FGF-2 from the cartilage and related intracellular signalling which is prevented by pre-incubation with an FGF receptor inhibitor (Vincent et al., 2004). One group has reported that applying cyclic tensile strain to chondrocytes causes intracellular signalling which suppresses IL-1-induced NF κ B signalling, and is presumably therefore anti-catabolic (Dossunbekova et al., 2007). Intracellular calcium movement can be detected in response to stretch of cultured chondrocytes. Blocking this may inhibit stretch-induced cellular responses (Guilak et al., 1999). Another suggested mediator of mechanical signals is the NMDA receptor (Salter et al., 2004).

Loading of cartilage explants: Rested cartilage explants (dissected discs of articular cartilage) can be exposed to various single or repeated loads using loading rigs. In culture, there is increased sulphate incorporation into cultured cartilage explants after high frequency cyclical loading. However, static or high loads seem to suppress proteoglycan synthesis (Larsson et al., 1991). Loading of cartilage explants appears to induce proteinases in cartilage. One group has shown the up-regulation of MMP-3,-13 and ADAMTS-4 mRNA following cyclical loading, but down-regulation of MMP-1 and ADAMTS-5 (Fitzgerald et al., 2004). However, the net loss of glycosaminoglycan by the cartilage explants seen in this system following injury appears to be due to a passive process, as it is not inhibited by cycloheximide, a protein synthesis inhibitor (DiMicco et al., 2004). Other groups have shown increased immunohistochemical staining of MMP-3 and denatured collagen type II following loading, suggesting increased proteinase activity (Lin et al., 2004; Tew et al., 2000).

1.4 Osteoarthritis

Osteoarthritis (OA) is the clinical and pathological outcome of a range of disorders that results in structural and functional failure of synovial joints (Nuki, 1999). Although osteoarthritis affects various parts of the joint, it has long been thought to initially and primarily affect the articular cartilage (Collins and McElligott, 1960). There is also a view that the primary change may be in the subchondral bone and that cartilage degeneration is secondary to this (Quasnicka et al., 2006).

1.4.1 Clinical aspects of osteoarthritis

OA is the commonest arthropathy and typically affects the knee, hip, hand, spine or feet. OA may be restricted to one joint or area, or be 'generalised'. These different subtypes may have different risk factors and aetiologies (Buckwalter and Mankin, 1998a; Felson et al., 2000). Nearly 5 million people in the UK are estimated to have

radiological evidence of moderate to severe OA of their hands, knees or hips. It is the commonest cause of disability in the UK. 36 million working days are estimated to be lost each year due to OA, which costs £3.197 billion in lost production (Department for Work and Pensions: Analytical Services Division, 2000).

OA causes pain and loss of function of affected joints. Signs include bony swelling, crepitus and restricted range of movement of joints, often with associated deformity. Raised serum C-reactive protein (CRP) and COMP may represent biomarkers for more rapid progression and 'erosive' disease (Posey and Hecht, 2008; Punzi et al., 2005). Plain radiographs remain the gold standard for diagnosis and assessment of progression. Pharmacological therapy for OA is symptomatic and supportive (Conaghan et al., 2008). No widely accepted disease modifying OA drugs (DMOADs) exist. Strategies for development of new therapies broadly involve either boosting anabolic cartilage responses (growth factors such as FGF, modulation of TIMPs) or blocking catabolic molecules (MMP and aggrecanase inhibitors) (Chuma et al., 2004; Fosang et al., 2008; Little et al., 2007; Troeberg et al., 2008). Ultimately, in advanced OA, joint replacement remains the final resort.

1.4.2 The aetiology of osteoarthritis

Many risk factors for development of OA have been identified (Buckwalter and Mankin, 1998a; Felson et al., 2000). Considering all types of osteoarthritis, more women are affected than men, and incidence increases with age. A family history of OA is often present. Genome wide scanning and other approaches have identified candidate gene loci such as the IL-1 gene cluster, the IL-4 receptor, frizzled-related protein-3 and asporin (hip OA) and matrilin-3 (hand OA) (Loughlin, 2005). Such approaches have highlighted the problem of heterogeneity in OA populations and have been largely disappointing in identifying polymorphisms which confer risk to

large groups of patients. Other factors which are probably important include bone density, oestrogen deficiency and vitamin D status (Felson et al., 2000). However, probably the most important risk factors are mechanical – through excessive use (occupations in the past at high risk have included miners, cotton mill workers and shipyard workers) (Hunter et al., 2004), obesity and malalignment (or congenital deformity of the joint) (Buckwalter and Mankin, 1998a). These are arguably all insidious forms of joint injury.

Joint injury: One of the strongest predictors of OA is joint trauma. At least 10% of cases are post-traumatic in origin (Furman et al., 2006). Direct damage to the articular cartilage or indirect damage, via damage to other joint structures, predisposes to OA. Various joint injuries (Gelber et al., 2000), including intra-articular fracture (Furman et al., 2006), anterior cruciate ligament (ACL) rupture (Lohmander et al., 2004), meniscal damage (Sharma et al., 2008) or meniscectomy (Englund and Lohmander, 2004) are all associated with an increase in an individual's risk of developing OA. Interestingly, although ACL rupture appears to be a potent risk factor in animal models, in humans with established OA, its effect appears to be mediated by concurrent meniscal pathology (Amin et al., 2008). There is also a significant correlation between the presence of asymptomatic partial thickness cartilage defects and development of knee OA (Cicuttini et al., 2005). In several studies in young patients without OA, around 30% of defects improve over a two year period on MRI, suggesting that in those individuals, a repair response is at least partially successful (Ding et al., 2007). Factors associated with progression to OA after trauma include obesity, female gender, increasing age, positive family history of OA, malalignment, muscle weakness, smoking, presence of osteophytes and tibial subchondral bone size

(Ding et al., 2007; Ding et al., 2008). The mechanisms by which injury to a joint leads to degeneration are unknown.

Animal models: Certain animals, such as the male STR/ort mouse and the male Dunkin-Hartley guinea pig develop spontaneous osteoarthritis and so have been used to study the disease (Clements et al., 2003). However, as in human OA, the point of disease initiation is difficult to identify in these animals. The relationship between trauma and OA has been exploited in animal models. The Pond-Nuki model (dog ACL section), or medial meniscectomy in dogs, rabbits, rats or mice lead to rapidly progressive cartilage degeneration. A less traumatic murine model involves transection of the anterior attachment of the medial meniscus causing its displacement, with more insidious development of degenerative changes akin to human osteoarthritis (Glasson et al., 2005). Such models provide a useful platform for testing the effects of gene deletions or overexpression on the development and course of the disease. However, potential species differences in matrix catabolism have to be borne in mind.

1.4.3 Histological changes in osteoarthritis

The histological changes of OA are well recognised and progress through a number of stages (Buckwalter and Mankin, 1998a; Collins and McElligott, 1960; Meachim et al., 1965). The earliest change is the loss of integrity of the articular cartilage surface, with superficial fibrillation and loss of sulphated proteoglycan (Collins and McElligott, 1960). Clustering of chondrocytes is associated with increased proteoglycan staining around chondrocytes, suggesting increased anabolic activity. Later, there is more generalised proteoglycan loss, with empty lacunae suggesting cell death. This is associated with the formation of progressively deeper clefts leading to full thickness cartilage defects. In addition, there is subchondral sclerosis of bone,

bone cysts and new bone formation. Synovitis may be present. To what extent changes in bone and synovium are primary or secondary events remains controversial.

1.4.4 Molecular changes in osteoarthritis

The molecular changes in OA are difficult to investigate and poorly understood. Most cartilage available from human patients with OA comes from specimens taken at joint replacement surgery and has advanced degenerative changes. Investigation of early or disease-initiating events in cartilage is therefore difficult except in animal models (see section 1.4.2). There is evidence that chondrocytes in osteoarthritic cartilage produce more type X collagen, and may revert to a 'juvenile' phenotype, re-expressing type IIA procollagen possibly in an attempt at repair (Aigner et al., 1999). There may be increased chondrocyte apoptosis in OA, although the reasons for this are not understood (Hashimoto et al., 1998).

1.4.5 Proteinases and osteoarthritis

Although aggrecan degradation occurs before collagen degradation in *in vitro* models of degeneration, loss of collagen may be more important, because it is not replaced. Changes in mRNA levels of metalloproteinases have been reported in chondrocytes from human osteoarthritic cartilage, although these seem to vary depending on the stage of disease (Aigner et al., 2003; Bau et al., 2002). MMPs-1,-3 and -13, and ADAMTS-4 and -5 have all been implicated in the matrix loss seen in *in vitro* models of cartilage degeneration (Bau et al., 2002; Song et al., 2007; Stanton et al., 2005; Vincenti and Brinckerhoff, 2002). Overexpression of constitutively active MMP-13 in cartilage resulted in OA-like changes, due to collagen type II and proteoglycan degradation (Neuhold et al., 2001). Mice null for ADAMTS-5 showed protection from injury-induced OA, whilst a line null for ADAMTS-4 did not (Glasson et al., 2005). These results suggest that ADAMTS-5 is required for cartilage degeneration in

mice. Alternatively, cartilage developmental abnormalities in such knock-out mice could explain these findings. ADAMTS-4 is probably also important in human cartilage physiology, as discussed earlier in section 1.3.1 (Song et al., 2007). In an ACL section and medial menisectomy rat model with forced mobilisation, microarray analysis at 28 days after surgery showed up-regulation of the proteinases MMP-13 (2.5 fold) and ADAMTS-5 (1.7 fold) (Appleton et al., 2007). The molecular events that might induce such proteinases *in vivo*, initiating the disease process remain unknown.

1.4.6 Other inflammatory response genes and osteoarthritis

The role of inflammatory cytokines in rheumatoid arthritis is well-established, but far less certain in OA. Ever since IL-1 was isolated and shown to induce rapid depletion of proteoglycan in cartilage explant cultures, this molecule has received much attention with respect to its potential role in OA (Saklatvala, 1987). There are two forms of IL-1, α and β , whose effects, including those on cartilage appear to be similar. IL-1 induces transcription of many metalloproteinases and aggrecanases in chondrocyte and cartilage explant cultures (Flannery et al., 1999; Gebauer et al., 2005; Vincenti and Brinckerhoff, 2002). There is evidence that IL-1 is produced in cartilage in animal models of OA (Chambers et al., 1997; Pelletier et al., 1993). Although the conditional expression of IL-1 β in temporomandibular joints of transgenic mice led to degeneration of these joints, another group found that deletion of IL-1 β in mice caused accelerated development of OA following partial medial meniscectomy (Clements et al., 2003; Lai et al., 2006). The grade of osteoarthritis was reported in another study to be inversely proportional to the intensity of IL-1 staining (Towle et al., 1997). IL-1 is mainly produced by macrophages, and whether significant amounts are made by and released from chondrocytes has always been

uncertain. It is of note that although IL-1 activity can be detected in lysates of cultured porcine cartilage, the cytokine is intracellular and does not appear to be secreted (Gruber et al., 2004). An autocrine role for IL-1 in cartilage is unproven.

TNF α is similar to IL-1 in its action on cartilage (Hui et al., 2003; Saklatvala, 1987). It is a pivotal cytokine in inflammatory diseases such as rheumatoid arthritis and psoriatic arthritis, and its blockade ameliorates the disease in a majority of patients. Although its mRNA is reported to be up-regulated in osteoarthritis (Amin, 1999), TNF α does not appear to be released by cartilage, and whether it plays any role in the pathogenesis of OA is also unclear.

There have been reports of increased cyclooxygenase (COX)-2 mRNA and protein in OA cartilage (Amin et al., 1997). COX-2 is inducible and typically rapidly up-regulated at sites of inflammation, forming pro-inflammatory prostanoids by the conversion of arachidonic acid to prostaglandin (PG)H₂. Inducible nitric oxide synthase (iNOS) is also overproduced by chondrocytes isolated from osteoarthritic cartilage (Amin et al., 1995). Induction of all of these inflammatory response genes depends upon inflammatory signalling.

1.5 Inflammatory signalling pathways

Inflammation is the response of tissues to sublethal injury, to infection or to an immune reaction. It is an important effector of both innate and adaptive immune responses (Delves and Roitt, 2000a, b). Inflammation classically causes pain, redness, swelling and warmth of the tissue due to vasodilatation and local mediator production. Blood vessels become more permeable to leucocytes which migrate to the site of damage or infection. The inflammatory response also initiates repair of the tissue via cell proliferation and the formation of granulation tissue. At the molecular level, the

inflammatory response can be thought of as a defined signalling and gene response which is particular to the cell and tissue.

The inflammatory response is mediated by intracellular inflammatory signalling pathways. The core of inflammatory signalling is the three MAP kinase pathways, and NF κ B. Following their activation, these pathways regulate inflammatory response gene expression of cytokines, matrix metalloproteinases, chemokines, adhesion molecules and mediator generating enzymes (e.g. COX-2 and iNOS). This gene regulation is via direct activation of transcription by the pathways and via stabilisation of mRNAs by post-transcriptional regulation (Fig 1.2)(Kracht and Saklatvala, 2002). Inflammatory signalling also has effects on processes as diverse as differentiation and apoptosis. In excess of 100 inflammatory response genes exist, and their control is elaborate, depending on stimulus and cell type.

Inflammatory signalling pathways are typically activated by pro-inflammatory cytokines such as IL-1 and TNF α , or by microbial products such as lipopolysaccharide (LPS) (Kracht and Saklatvala, 2002). The mechanism by which sterile tissue injury activates inflammatory signalling is obscure. There is evidence that these pathways may be activated by endogenous ligands via Toll-like receptors (TLRs). For example, TLR-4 (the LPS recognition receptor) can be activated by heat shock proteins 60 and 70, and fragments of hyaluronic acid or fibronectin (Termeer et al., 2002; Tsan and Gao, 2004). Any of these substances might be released or generated upon tissue injury, or cell death. Supernatants of necrotic cells have been shown to activate dendritic cells in a similar manner to LPS, and therefore probably via the inflammatory signalling pathways (Matzinger, 2002). Her 'danger model' suggests that an alarm signal from distressed or injured cells is needed to activate antigen presenting cells and generate an immune response.

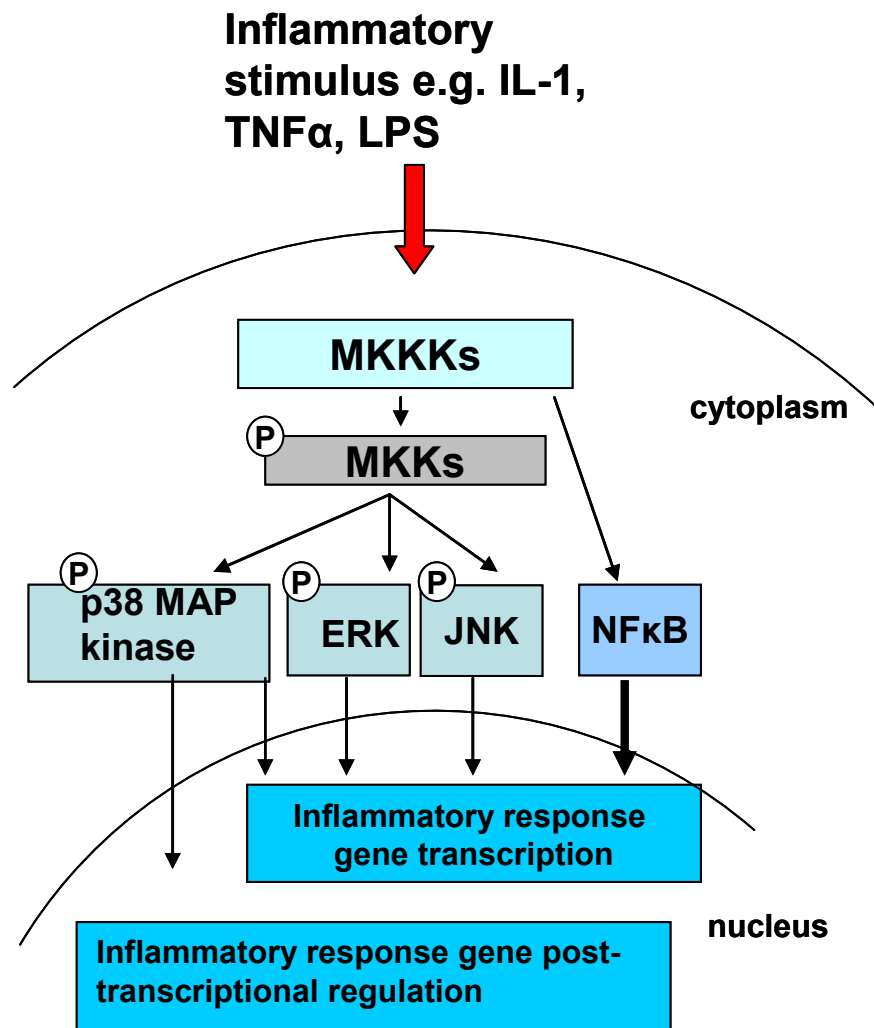


Figure 1.2. Inflammatory signalling in mammalian cells.

Pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF) α or bacterial lipopolysaccharide (LPS) signal via cell surface receptors causing activation of mitogen activated protein kinase kinases (MKKKs), which in turn phosphorylate mitogen activated protein kinase kinases (MKKs). Specific MKKs are responsible for the activation of the MAP kinases (MAPKs) p38 MAP kinase, extracellularly regulated kinase (ERK) and c-jun N terminal kinase (JNK). The MAPKs and nuclear factor kappa B (NF κ B) regulate inflammatory response gene transcription.

1.5.1 The MAP kinases

The MAPKs are defined by their TXY motifs which require dual phosphorylation for activation. There are three types: ERK, JNK and p38 MAPK. There are two closely related genes coding for ERKs 1 and 2 (p44 and p42), there are three JNK genes expressed as two predominant forms, p46 and p54, which arise from alternative splicing, and there are four genes (α , β , γ and δ) for p38 MAPK (Kracht and Saklatvala, 2002). All three types of MAPK are generally strongly activated by inflammatory cytokines or LPS (see Fig 1.2). The ERK pathway is also strongly activated by growth factors including IGF-1, epidermal growth factor (EGF) and FGF (Starkman et al., 2005; Vincent et al., 2002). JNK and p38 MAPK may also be activated by cellular stresses such as hyperosmolarity, UV light and protein synthesis inhibitors, although the underlying mechanisms are poorly understood (Duzgun et al., 2000; Liu et al., 2006; Yujiri et al., 1999). MAPKs are activated by MAPK kinases (MKKs) which in turn are activated by phosphorylation of serine residues by MAPK kinase kinases (MKKKs) (Hagemann and Blank, 2001), (see section 1.5.3 & Fig 1.2).

Important substrates of the MAPKs include downstream protein kinases and transcription factors. The latter include Ets (all three MAPKs) and the activating protein (AP)-1 transcription factors c-jun and activating transcription factor-2 (ATF-2)(JNK). The downstream kinases are MAPK activating protein kinase (MAPKAP-K)-1 and -2, MAPK stimulated kinase (MSK)-1 and -2 and MAPK integrating kinase (MnK) (Kracht and Saklatvala, 2002). The ERK pathway has effects on mitosis and on inflammatory gene transcription. JNK induces and activates early response genes such as the AP-1 binding proteins, and may also be pro-apoptotic (Liu and Lin, 2005; Morton et al., 2004). p38 MAPK regulates gene transcription but is also necessary for the stabilisation of inflammatory response mRNAs. Substrates of the p38 MAPK

pathway such as tristetraprolin which bind to AU rich elements in the 3'untranslated region of inflammatory mRNAs mediate this control (Clark et al., 2003). The MAPKs are inactivated by dual specificity phosphatases (DUSPs), particularly DUSP-1, which is induced by inflammatory signalling and serves as an important negative feedback (Tanoue et al., 2000).

1.5.2 NFκB

NFκB is a primary controller of inflammatory response gene expression (Barnes and Karin, 1997). NFκB signalling is required for inflammatory and immune responses and is also anti-apoptotic. NFκB is typically activated by pro-inflammatory cytokines or microbial products: engagement of all known TLRs leads to NFκB activation (Bonizzi and Karin, 2004; Doyle and O'Neill, 2006). The NFκB proteins are a family of five homologous Rel transcription factors (RelA (p65), RelB, c-rel, p50 and p52). These form hetero-and homo-dimers and are sequestered in the cytoplasm as latent complexes via non-covalent interactions with NFκB inhibitory proteins, the inhibitors of κB (IκBs).

The canonical pathway: The common form of NFκB is a heterodimer of NFκB1 (p50) and RelA (p65). This is inactive in the cytoplasm when bound to the prototypical IκB, IκBα (Fig 1.3). NFκB activation is classically mediated by the phosphorylation of IκBα, which becomes ubiquitinated and is then degraded in the 26S proteasome. The removal of IκBα exposes a nuclear localisation sequence on p50/p65 NFκB which targets it to the nucleus. Here it promotes transcription of inflammatory response genes by binding to NFκB binding sites within their promoters (Bonizzi and Karin, 2004).

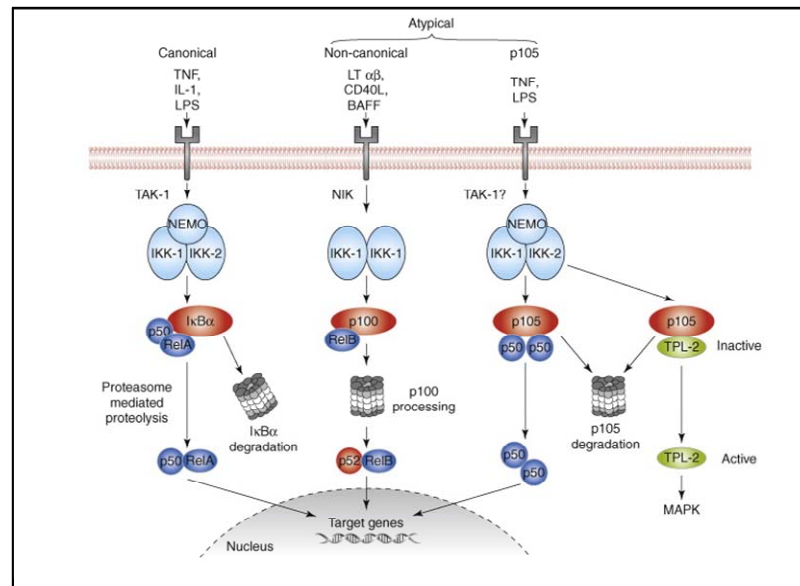


Figure 1.3. Activation of nuclear factor kappa B (NFκB) by canonical and atypical pathways.

The canonical pathway is activated by agonists such as interleukin (IL)-1 and tumour necrosis factor (TNF)α which stimulate IκB kinase (IKK) to phosphorylate Inhibitor of kappa B alpha (IκBα), inducing its degradation. The atypical pathways involve proteolysis of p100 (the non-canonical pathway) and p105 precursor proteins.

Abbreviations: lipopolysaccharide (LPS), lymphotoxin (LT), B cell activating factor (BAFF), TGFβ activating kinase (TAK), NFκB inducing kinase (NIK)

Figure reproduced from Sun & Ley (2008), Trends in Immunology, 29 (10), p472

I κ B kinases: Signal-induced I κ B phosphorylation is mediated by a high molecular weight I κ B kinase (IKK) complex. This tripartite complex consists of α , β and γ subunits. IKK α and β (also known as IKK1 and 2) are related molecules which are active kinases, both capable of independently phosphorylating I κ B (Zandi et al., 1998). In contrast, IKK γ (also known as NF κ B essential modulator [NEMO]) has no catalytic activity, but plays a key regulatory role as an adaptor and is obligatory for NF κ B signalling (Krappmann et al., 2000). Dissociation of NEMO from the IKK complex using a cell permeable peptide spanning the NEMO binding domains of IKK α and β blocks cytokine-induced NF κ B activation (May et al., 2000). Knockout of IKK β is embryonically lethal because of massive liver apoptosis. This is mediated by TNF α , which fails to activate the anti-apoptotic mechanisms dependent upon NF κ B (Bonizzi and Karin, 2004). The canonical pathway, which leads to activation of NF κ B following cytokine (e.g. IL-1, TNF α), LPS or antigen-receptor engagement is considered to be predominantly dependent on IKK β and NEMO (Li et al., 1999). However, a recent report suggests that the presence of IKK α and NEMO alone are sufficient for activation of the canonical pathway by IL-1 (Solt et al., 2007). There is also some evidence that activation of IKK α may contribute to suppression of NF κ B activity by accelerating the turnover of p65 and c-Rel and their detachment from pro-inflammatory gene promoters, thereby exerting an anti-inflammatory effect (Lawrence et al., 2005).

The atypical pathways: Other pathways involving p105 (the NF κ B1 precursor) and p100 (the NF κ B2 precursor) can also lead to NF κ B activation (see Fig 1.3),(Sun and Ley, 2008). The p100 pathway, also called the non-canonical pathway, is triggered by a subset of TNF receptor family members such as lymphotoxin- β receptor, CD40 and B cell-activating factor receptor. Upon ligation, NF κ B inducing kinase (NIK) causes

activation of this pathway. NIK levels are usually low because it is continually degraded following TRAF-3-dependent ubiquitination. Ligand stimulation induces TRAF-3 degradation, allowing the accumulation of newly synthesised NIK and IKK α activation (Wallach and Kovalenko, 2008). Studies in IKK deficient cells have demonstrated that IKK α is the critical kinase for this pathway: it can be activated in the absence of both IKK β and NEMO: (Bonizzi and Karin, 2004). IKK α phosphorylates p100, triggering its polyubiquitination and partial proteolysis. This gives rise to the p52 subunit (NF κ B2) which can homodimerise, or associate with RelB. This dimer translocates to the nucleus and regulates several lymphoid chemokine genes. The major function of this pathway is the development and organisation of lymphoid tissues, and the maturation of B cells (Bonizzi and Karin, 2004).

A further 'atypical' pathway leading to NF κ B activation involves processing of p105 to produce p50 (NF κ B1) following its phosphorylation by IKK α/β . p105 is associated with the MKKK Tpl-2 in the cytoplasm. Proteolysis of p105 releases Tpl-2, which phosphorylates and activates MKKs and subsequently MAPKs (Beinke et al., 2004). p50 subunits dimerise and translocate to the nucleus (Fig 1.3).

1.5.3 Regulation of MAPKs and IKK

MKKs are specific: MKK (MEK)1 and 2 activate ERK, MKK4 and 7 activate JNKs and MKK3 and 6 activate p38 MAPK. MKKKs can activate multiple signalling pathways simultaneously. Some have been shown to activate JNK, p38 and IKKs. These include MEKK1, 2 and 3, Tpl-2 (also known as Cot) and TGF- β activated kinase (TAK)-1 (Kracht and Saklatvala, 2002).

How MKKKs are coupled to receptor signalling complexes in a stimulus-specific manner is not well understood. Most of our knowledge comes from the TLR/IL-1

receptor (TIR) and TNF receptor (TNFR) families (Fig 1.4). In the case of TLR/ IL-1 signalling, the adaptor proteins are MyD88 and Mal, which bind IL-1 receptor associated kinases (IRAKs). Phosphorylated IRAK-1 then binds to TNF receptor associated family (TRAF)-6. TRAF-6 is an E3 ligase and undergoes self-ubiquitination, recruiting TAK-1 via the ubiquitin binding domain of a TAK binding protein, TAB-2, in association with TAB-1. Phosphorylated activated TAK-1 subsequently phosphorylates downstream targets including IKKs and MKKs (Doyle and O'Neill, 2006) (Fig 1.4).

In TNF signalling, the TNFR associates with the TNFR-associated death domain protein (TRADD) which in turn recruits (TRAF)-2 and the receptor interacting protein (RIP)-1 kinase. TRAF-2 and polyubiquitinated RIP-1 recruit the TAK-1-TAB-2 complex and IKK complex, resulting in phosphorylation and activation of IKK, leading to activation of NF κ B and MAPKs (Fig 1.4) (Ea et al., 2006; Hacker and Karin, 2006). MEKK3 also appears to be important for TNF-induced IKK signalling (Blonska et al., 2005).

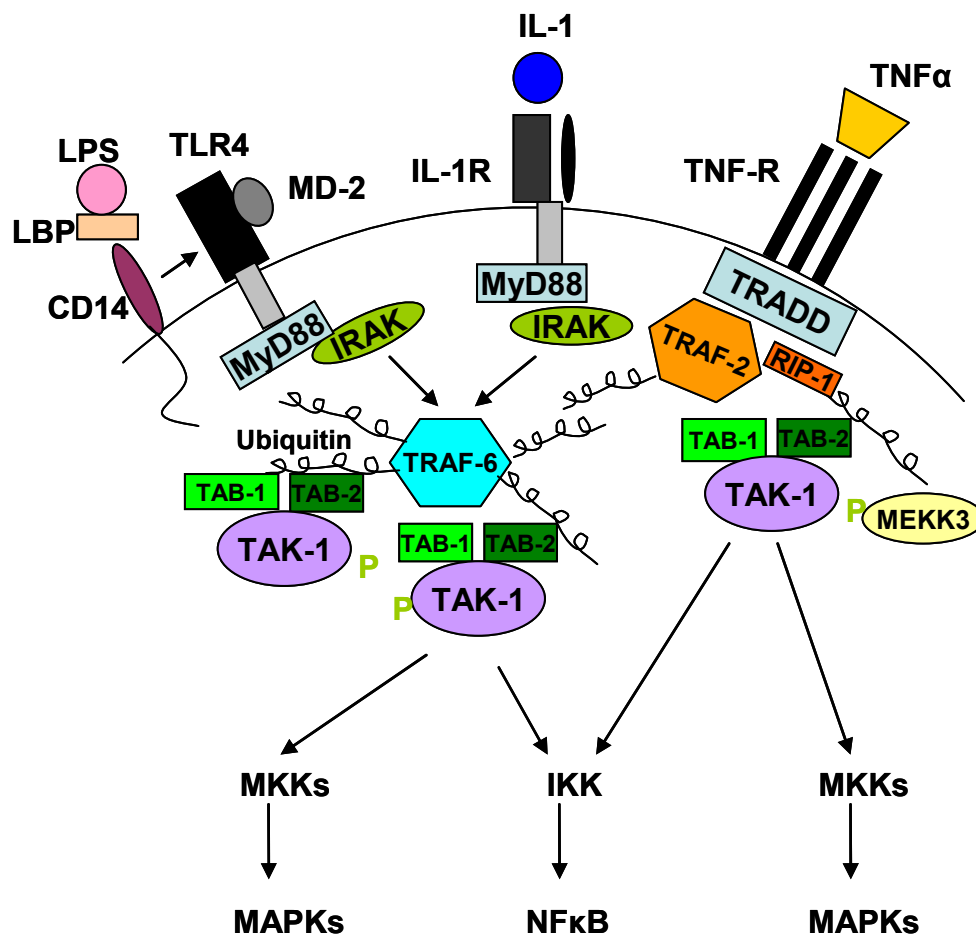


Figure 1.4. The IL-1R/TLR and TNF signalling pathways.

IL-1 or LPS bind to LPS binding protein (LBP) bind to related receptors with a TLR/IL-1R (TIR) intracellular domain. Liganded receptors bind the adaptor protein MyD88, which recruits IL-1 receptor associated kinases (IRAKs). Some TLRs also recruit a related adaptor Mal (not shown). Phosphorylated IRAK-1 then binds to TNF receptor associated family (TRAF)-6 which ubiquitinates itself. TAB-2, which is bound to TGFβ associated kinase (TAK)-1, is an ubiquitin-binding protein. TAK-1 thus oligomerises and is activated by transphosphorylation. Activated TAK-1 phosphorylates and activates IKKs and MKKs. TNF binding to its receptor causes binding of TNFR associated via death domain (TRADD) to the receptor complex. This allows recruitment of TRAF-2 and receptor associated protein (RIP)-1 kinase. The IKK complex is subsequently activated following recruitment of TAK-1 and ubiquitinated NEMO. In addition to RIP-1, MEKK3 also appears to be required for TNF-induced IKK signalling.

1.6 Fibroblast growth factor signalling

As described earlier, FGF-2 is present in the chondrocyte pericellular matrix and is released upon cartilage injury (Vincent et al., 2002; Vincent et al., 2004). It ligates FGF receptors, which are receptor tyrosine kinases, and activates a number of signalling pathways (Fig 1.1).

ERK: Growth factors such as FGF activate the ERK MAPK pathway. This is via activation of the small GTPase Ras, which recruits Raf to the cell membrane. Raf subsequently phosphorylates MEK-1 and -2 (also known as MKK-1 and -2), activating ERK. The activation of Ras is classically via the adaptor protein Grb-2 and the GTP exchange factor SOS interacting with the receptor tyrosine kinase (Ramos, 2008).

Phosphatidylinositol-3 kinase: The phosphatidylinositol (PI)-3 kinase pathway is also activated by growth factors such as FGF and IGF and, to a lesser extent, by inflammatory stimuli such as LPS and TNF α . Activation of PI3 kinase leads to activation of Akt and p70S6 kinase, downstream serine threonine protein kinases involved in cell survival and protein synthesis respectively. In chondrocytes *in vitro*, IGF-I stimulates proteoglycan synthesis via the PI3 kinase pathway (Starkman et al., 2005).

Protein kinase C (PKC): This is the name given to a group of lipid-dependent serine-threonine kinases involved in cell proliferation, differentiation and apoptosis. They include classical (both diacylglycerol [DAG] and calcium-dependent), novel (calcium-dependent) and atypical (neither DAG nor calcium-dependent) enzymes. Growth factors such as FGF activate PKC by activating phospholipase C, which hydrolyses phosphoinositides in the cell membrane. In chondrocytes *in vitro*,

inhibitors of PKC were found to inhibit IL-1 induced aggrecanase transcription and glycosaminoglycan release in one study (LaVallie et al., 2006).

1.6.1 Non receptor protein tyrosine kinases

The non-receptor protein tyrosine kinases (PTKs) include the Src family kinases (SFKs), the Syk family, the focal adhesion kinase (FAK) family (such as Pyk2) and the Tec family kinases (such as Btk). FGF is reported to activate some of these kinases *in vitro*. For example, c-src appears to be necessary for FGF-induced proliferation of cultured fibroblasts (Kilkenny et al., 2003). FAK appears to be required for some FGF-induced responses in myofibroblasts (Greenberg et al., 2006).

During the course of my work, I discovered that protein tyrosine phosphorylation was an early event following cartilage injury, implying activation of tyrosine kinases. PTKs are also implicated in the inflammatory response: following LPS stimulation of monocytes, tyrosine phosphorylation is an early event (Beaty et al., 1994). Over-expression of PTKs has been implicated in oncogenesis. Tyrosine phosphorylation can activate or inactivate protein tyrosine kinases and autophosphorylation can also occur. As well as a tyrosine kinase domain, non receptor PTKs such as SFKs contain a src homology (SH)2 and a SH3 domain (Okutani et al., 2006). These domains are recruited to tyrosine-phosphorylated sites and proline-rich motifs on other molecules respectively. In this way, protein tyrosine kinases can induce binding and localisation of other signalling molecules. PTKs are tightly regulated by protein tyrosine phosphatases (Ostman et al., 2006).

Src family kinases: The src family consists of nine members: c-src, fyn, yes and yrk, which are ubiquitous, and hck, lyn fgr, lck and blk, which are found mainly in cells of haematopoietic lineage (Bromann et al., 2004; Parsons and Parsons, 2004). Much is known about the critical role of SFKs in T cell receptor transduction. Lck and fyn are

the first signalling molecules to be activated downstream of the T cell receptor (Barber et al., 1989). Hck, lyn and fgr are activated by many inflammatory stimuli in monocytes and macrophages (Okutani et al., 2006). Triple mutation of src, yes and fyn in mouse embryonic 'SYF' cells has allowed some study of the roles of SFKs (which have been previously hampered by redundancy between family members) (Klinghoffer et al., 1999). In cells of mesenchymal origin such as fibroblasts, there is expression of c-src, yes, fyn, hck and lyn (Kilkenny et al., 2003).

1.7 The response of articular cartilage to injury

1.7.1 Histological changes after cartilage injury

It was noted in the nineteenth century that damaged articular cartilage does not repair (Redfern, 1851). Further work throughout the twentieth century confirmed that following superficial injury (not breaching bone), the cartilage does not usually repair (Mankin, 1962). A possible exception may be in fetal, or very young animals (Calandruccio and Gilmer, 1962; Namba et al., 1998). Direct cartilage injuries not only fail to heal, but lead to degeneration. Superficial cutting or scarification of the articular surface in rabbit knees leads after six weeks to degeneration characterised by fibrillation, progressive matrix loss and clustering of chondrocytes, similar to the histological changes seen in human OA (Meachim, 1963). Deep injuries, involving bone (which is vascularised) lead to healing of defects by formation of granulation tissue and fibrocartilaginous repair (Key, 1931; Mankin, 1962; Shapiro et al., 1993). This mechanism is exploited in the orthopaedic technique of drilling localised cartilage defects. However, fibrocartilage lacks the mechanical properties of hyaline cartilage and can subsequently degenerate (Key, 1931; Shapiro et al., 1993). It is not clear from these studies if lack of healing and degeneration following cartilage injury

are primarily due to decreased cartilage anabolism, increased catabolism or a combination of the two.

1.7.2 Molecular responses of articular cartilage to injury

To investigate the molecular and cellular responses of cartilage to injury, different *in vitro* methods can be employed. Rested cartilage explants can be exposed to supra-physiological impact loads (Lee et al., 2005). Blunt trephine injuries to cultured cartilage have also been employed (Redman et al., 2004). Our group has studied the effects of scoring or dissection of cartilage on the intact articular surface, and of cutting explants adapted to culture conditions (Gruber et al., 2004; Vincent et al., 2002).

Cell death: Injury to cartilage can lead to chondrocyte death. Whilst low frequency and low energy loads may have anabolic effects on cartilage, supra-physiological loads lead to increased cell death, with a large proportion of this being apoptotic (Chen et al., 2001; Patwari et al., 2004). Trephine injury to cartilage explants causes a band of chondrocyte death (both necrosis and apoptosis) along the cut edge, irrespective of age. The cell death is greater following blunt trauma compared with sharp injury with a scalpel (Redman et al., 2004; Tew et al., 2000). Apoptosis of chondrocytes also occurs in *in vivo* models of acute osteochondral injury and experimentally-induced osteoarthritis (Costouros et al., 2004; Hashimoto et al., 1998). The cause of the apoptosis is not understood. It may be due to soluble factors and be mediated by Fas (Hashimoto et al., 1997; Levin et al., 2001).

Proteinase expression: Few *in vivo* studies examining the molecular responses to direct cartilage injury have been reported. Up-regulation of MMP-3 and ADAMTS-5 was reported after a single injurious load to cultured explants (Lee et al., 2005). Another study showed increased MMP-13 staining and increased MMP activity

(presence of the VDIPEN neoepitope of aggrecan) around partial thickness cartilage defects in pigs (Hembry et al., 2001). That proteinases and other inflammatory response genes are involved in the subsequent degeneration of cartilage after injury seems very likely, but their mechanism of induction remains unclear.

1.7.3 Signalling in articular cartilage after injury

Some years ago, our laboratory noted that dissecting articular cartilage from the joint surface (explantation), or scoring it *in situ* activated the three MAPK pathways ERK, JNK and p38 MAPK. Explantation also caused some degradation of I κ B α , implying the possible activation of NF κ B (Gruber et al., 2004). The activation of NF κ B by cartilage injury has since been shown by confocal microscopy. Translocation to the nucleus of the p65 subunit of NF κ B can be visualised 30 minutes after explantation (Fig 1.5, A. Didangelos, Ph.D. thesis, 2008). How these pathways, typically activated by inflammatory cytokines or microbial products, were activated by injury was unknown.

In the search for an activating factor for the MAPKs, a soluble factor which activated ERK was found to be released from cartilage following explantation or re-cutting of cartilage explants. This was purified from conditioned medium and identified by mass spectrometry as FGF-2 (Vincent et al., 2002). FGF-2 was subsequently shown to be bound to perlecan in the pericellular matrix (Vincent et al., 2007). It was also responsible for ERK activation which followed loading of cartilage explants and alginate-embedded chondrocytes, because the activation was prevented by an FGF receptor inhibitor (Vincent and Saklatvala, 2006; Vincent et al., 2004). p38 MAPK in cultured chondrocytes was also activated by FGF-2 and its activation on explantation may therefore also be FGF-dependent (A. Didangelos, Ph.D. thesis, 2008). However, he found that FGF-2 did not activate JNK in cultured chondrocytes or resting cartilage

explants. Despite an extensive search, there was no evidence of any other soluble factor released following dissection and fine chopping of cartilage which could activate JNK. The activation was not simply due to direct mechanical stress, because if dissected cartilage was rested in culture overnight or longer, then re-cut, JNK activation did not recur, although there was release of FGF-2 and ERK activation. The dissection of cartilage and placing into culture medium allows for some tissue swelling and deformation of the cartilage pieces, which could cause fluid flow and shear of cells within the matrix and subsequent activation of cell signalling. To try to control for such tissue swelling, the intact cartilage of the articular surface was scored *in situ*, or the cartilage was dissected into a culture dish without medium. JNK activation was still seen (A. Didangelos, Ph.D. thesis, 2008). The mechanism of activation of JNK and NF κ B pathways in cartilage following injury remains unknown.

The activation of inflammatory signalling following explantation is sufficient to induce IL-1 α mRNA, and the magnitude of induction is proportional to the amount of cartilage injury (Gruber et al., 2004). Activation of JNK and NF κ B following sharp injury to cartilage is therefore likely to induce other inflammatory response genes including proteinases, and this could initiate catabolism of the extracellular matrix. An understanding of the mechanism of activation of these pathways, and their effect on cartilage is likely to improve our understanding of cartilage biology and processes which may lead to OA.

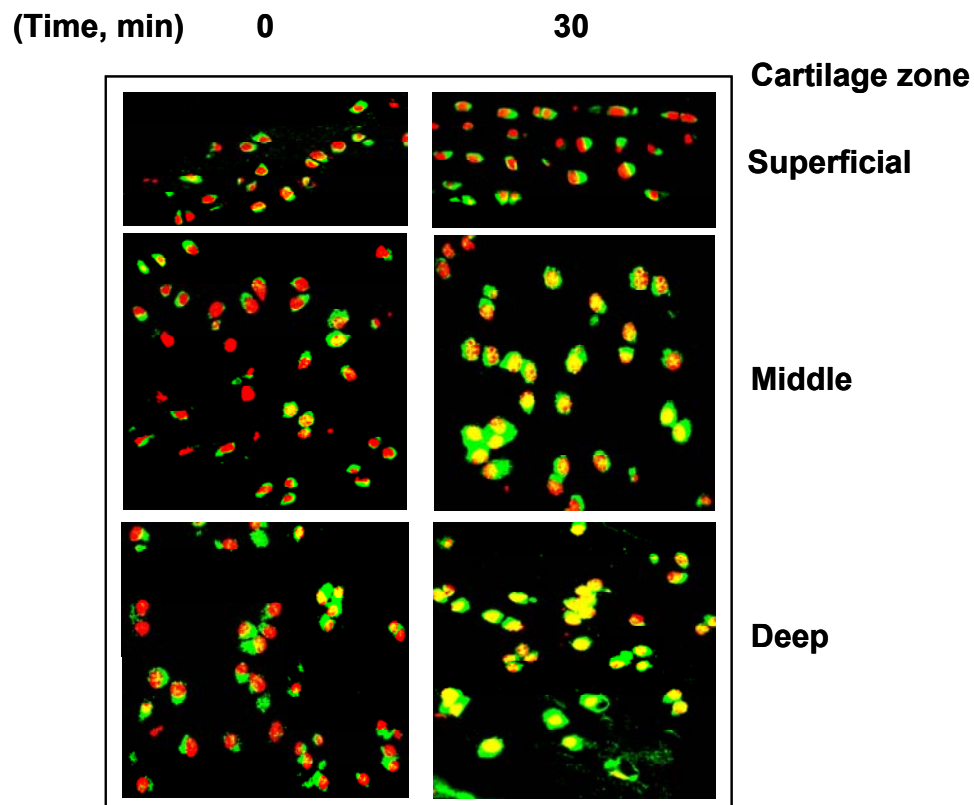


Figure 1.5. Localisation of NF κ B in dissected cartilage explants immediately *ex vivo* (0) and after 30 minutes.

The p65 subunit of NF κ B is stained green, and nuclei red. Yellow signal indicates p65 translocation into the nucleus.

Figure from A. Didangelos, Ph.D. Thesis, 2008.

1.8 Summary, aims and objectives of the project

My hypothesis is that the activation of inflammatory signalling pathways in articular cartilage by injury may lead to subsequent catabolism of the matrix. In this project, the regulation of intracellular signalling pathways and expression of inflammatory response genes in the chondrocyte in response to sharp injury of the intact articular surface will be further explored in experiments utilising porcine joints.

Chapter 3: Inflammatory signalling and gene expression in injured articular cartilage

- Whether canonical NF κ B is activated following dissection of articular cartilage will be investigated by the use of a kinase assay for IKK.
- Whether the signalling is sufficient to induce a range of typical inflammatory response genes including proteinases will be investigated by RT-PCR.
- Expression of two of these inflammatory response genes will be examined by ELISA and western blotting. The signalling necessary for activin A production after cartilage injury will be assessed by the use of pharmacological inhibitors of candidate pathways.

Chapter 4: Investigation of tyrosine phosphorylation in cartilage following injury

- Tyrosine kinases are implicated in the response of cartilage to injury in work presented in Chapter 3. Tyrosine phosphorylation occurring after injury will be further explored by phosphotyrosine western blotting.
- Tyrosine-phosphorylated proteins of interest will be purified by immunoprecipitation and electrophoresis and silver-stainable bands identified by mass spectrometry.

Chapter 5: The role of tyrosine kinases in the response of cartilage to injury

- The tyrosine-phosphorylated proteins identified by mass spectrometry will be verified by western blotting and phospho-western blotting.
- The role of src family kinases in the response of cartilage to injury will be explored by western blotting, immunoprecipitation-kinase assay and pharmacological inhibition experiments.
- The role of the FGF receptor tyrosine kinase in the response of cartilage to injury will be investigated by pharmacological inhibition of the receptor.

Chapter 6: The response of synovium to injury

- Whether the signalling activated by cartilage injury is also activated by injury to another connective tissue, synovium, will be explored by the use of phospho-western blotting.
- Whether inhibition of src and FGF-induced pathways in synovium has similar effects to inhibition of these pathways in cartilage will be explored.
- The localisation of cellular activation in response to tissue injury will be explored by immunolocalisation of phospho-paxillin, a src substrate shown to be activated in both tissues by injury.

2 Materials and Methods

2.1 Reagents

General laboratory reagents were the best available grade from either Sigma-Aldrich (Dorset, UK) or BDH (Dorset, UK) unless otherwise stated. Bovine serum albumin, electrophoresis grade (BSA) was from PAA Laboratories, Pasching, Austria.

2.1.1 General buffers

A 10x stock solution of phosphate buffered saline (PBS) (43mM Na₂HPO₄, 14mM KH₂CO₃, 14mM KCl and 1.37M NaCl at pH 7.3) was from VWR (Lutterworth, UK).

A 1x PBS solution was made by diluting the 10x stock solution with ddH₂O.

A 10x solution of tris-buffered saline (TBS) was prepared (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). A 1x TBS solution was made by diluting the 10x solution with ddH₂O.

2.1.2 Antibodies

Primary and secondary antibodies for western blotting, immunoprecipitation and immunohistochemistry used in experiments are listed in Table 2.1 below. Mouse purified immunoglobulin for non-immune controls in immunoprecipitation experiments was mouse IgG₁ isotype from murine myeloma (M5284, Sigma-Aldrich, Dorset, UK).

Table 2.1. Primary and secondary antibodies.

Antibody specificity	Activity	Antibody type	Source
Phospho-ATF-2 (9221)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
Phospho-p38 MAPK (9211)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
Phospho-ERK (M8159)	Western blotting	Mouse monoclonal	Sigma-Aldrich, Dorset, UK
ERK (sc-154)	Western blotting	Rabbit polyclonal	Santa Cruz Biotechnology, USA
ERK (SAK-2)	Western blotting	Rabbit polyclonal	Laboratory's own
IKK γ (559675)	Immunoprecipitation	Mouse monoclonal	BD biosciences, Bedford, USA
IKK α (556532)	Western blotting	Mouse monoclonal	BD biosciences, Bedford, USA
Phospho-Akt (9271)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
I κ B α (sc-203)	Western blotting	Rabbit polyclonal	Santa Cruz Biotechnology, USA
COX-2 (210-712-1)	Western blotting	Rabbit polyclonal	Alexis biochemicals, Lausen, Switzerland
Phosphotyrosine (4G10) (05-321)	Western blotting	Mouse monoclonal	Upstate, Lake Placid, USA
4G10 agarose conjugate (16-101)	Immunoprecipitation	Mouse monoclonal	Upstate, Lake Placid, USA
4G10 HRP conjugate (16-105)	Western blotting	Mouse monoclonal	Upstate, Lake Placid, USA
FAK (2A7) (05-182)	Immunoprecipitation	Mouse monoclonal	Upstate, Lake Placid, USA
FAK (06-543)	Western blotting	Rabbit polyclonal	Upstate, Lake Placid, USA
Phospho tyr-576 FAK (07-157)	Western blotting	Rabbit polyclonal	Upstate, Lake Placid, USA
Phospho tyr-397 FAK (44-624G)	Western blotting	Rabbit polyclonal	Biosource International, Camarillo, USA
Phospho-paxillin (2541)	Western blotting/IHC	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
Paxillin (5H11) (05-417)	Western blotting	Mouse monoclonal	Upstate, Lake Placid, USA
c-src (D1306)	Western blotting	Rabbit polyclonal	Santa Cruz Biotechnology, USA
c-src (GD11) (05-184)	Western blotting/Immunoprecipitation	Mouse monoclonal	Upstate, Lake Placid, USA
Yes (06-514)	Western blotting	Mouse monoclonal	Upstate, Lake Placid, USA
Fyn (sc-16)	Western blotting	Rabbit polyclonal	Santa Cruz Biotechnology, USA
Hck (06-833)	Western blotting	Rabbit polyclonal	Upstate, Lake Placid, USA
Cortactin (4F11) (05-180)	Western blotting	Mouse monoclonal	Upstate, Lake Placid, USA
Phospho-tyr 215 src (07-791)	Western blotting	Rabbit polyclonal	Upstate, Lake Placid, USA
Phospho-tyr 416 src (2101)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
Phospho-tyr 418 src (07-909)	Western blotting	Rabbit polyclonal	Upstate, Lake Placid, USA
Phospho-EGF receptor (Tyr 845) (2231)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
Phospho-EGF receptor (Tyr 1068) (2236)	Western blotting	Rabbit polyclonal	Cell Signaling Technology, Beverly, USA
EGF receptor (sc-03)	Western blotting	Rabbit polyclonal	Santa Cruz Biotechnology, USA
Tubulin (T9026)	Immunoprecipitation	Mouse monoclonal	Sigma-Aldrich, Dorset, UK
Rabbit IgG HRP (P0217)	Western blotting (secondary antibody)	Swine IgG	DAKO Cytomation, A/S, Denmark
Mouse IgG HRP (P0218)	Western blotting (secondary antibody)	Rabbit IgG	DAKO Cytomation, A/S, Denmark

2.1.3 Cytokines and growth factors

Primary chondrocytes and cartilage explants were stimulated with various factors.

Reagents used are listed in Table 2.2 below.

Table 2.2. Recombinant cytokines and growth factors.

Cytokine/growth factor	Species	Source
Interleukin-1 α	Human	Professor J. Saklatvala
Fibroblast growth factor-2	Human	Peprtech EC, London, UK
Transforming growth factor β	Human	R&D systems, MN, USA

2.1.4 Signalling pathway inhibitors

A number of pharmacological inhibitors of signalling pathways were employed for experiments in chondrocytes and cartilage explants. These are listed in Table 2.3 below. The concentrations used in certain experiments are given in the results sections.

Sodium pervanadate: Sodium orthovanadate and pervanadate are potent tyrosine phosphatase inhibitors. They were therefore both used to up-regulate tyrosine phosphorylations in cell and tissue culture experiments. Pervanadate involves the creation of oxyvanadium ions in various states of oxidation, which probably improves its tissue penetration compared to sodium orthovanadate. Solutions of sodium pervanadate for cell culture were prepared by the addition of 500 μ l of a solution containing 10 μ l of 30% hydrogen peroxide and 870 μ l DMEM to 9.5ml of 10 mM sodium orthovanadate in serum-free DMEM, immediately prior to the experiment.

Table 2.3. Signalling pathway inhibitors.

Inhibitor	Specificity	Source	Vehicle
U0126	MEK inhibitor	Promega, Madison, USA (V1121)	DMSO
PD173074	FGF receptor inhibitor	Gift from GSK	DMSO
SB202190	p38 MAP kinase inhibitor	Calbiochem (559388)	DMSO
LY294002	PI3 kinase inhibitor	Calbiochem (440204)	DMSO
Gö6976	Protein kinase C inhibitor	Calbiochem (365253)	DMSO
Gö6983	Protein kinase C inhibitor	Calbiochem (365251)	DMSO
PP2	Src family kinase inhibitor	Calbiochem (529576)	DMSO
PP1	Src family kinase inhibitor	Biomol International (EI-275)	DMSO
Genistein	Tyrosine kinase inhibitor	Biomol International (EI-147)	DMSO
SP600125	JNK inhibitor	Calbiochem (420119)	DMSO
L-JNKI	JNK inhibitor	Calbiochem (420116)	Water
MG132	Proteasome inhibitor	Biomol International (PI-102)	DMSO
BMS345541	IKK inhibitor	Calbiochem (401480)	DMSO
PF573228	FAK inhibitor	Gift from Pfizer	DMSO
cycloheximide	Protein synthesis inhibitor	Sigma-Aldrich, Dorset, UK (C-6255)	Absolute ethanol
Sodium orthovanadate	Tyrosine phosphatase inhibitor	Sigma-Aldrich, Dorset, UK (S6508)	Water

2.2 Cell and tissue culture

Dulbecco's Modified Eagle's Medium (DMEM) was obtained as a 1x endotoxin-free solution from PAA Laboratories, Pasching, Austria). This was supplemented with 25nM sterile HEPES buffer (PAA Laboratories, Pasching, Austria). For cell culture, medium was also supplemented with 10% Foetal Calf Serum (FCS) and penicillin-streptomycin (100U/ml) (PAA Laboratories, Pasching, Austria). Aliquots were stored at -20°C and diluted into medium at appropriate concentrations. All tissue culture plastics were from Beckton-Dickinson (Franklin Lakes, USA).

Porcine trotters and knees were obtained from a local abattoir within 12h of slaughter. Before use, porcine trotters were soaked in 1% virkon solution (Dupont Relyon Prevention Solutions, Sudbury, UK). Most of the experiments presented in this thesis were performed on cartilage dissected from the articular surface of the fused 2nd-3rd porcine metacarpophalangeal (MCP) joint (Fig 2.1). After skinning the trotter, the MCP joint was opened carefully in a biological safety cabinet, using a size 10 disposable scalpel (Fig 2.1A). Cartilage was then dissected from the articular surface (Fig 2.1B), using a size 15 disposable scalpel (all scalpels were sterile, and from Swann Morton, Sheffield, UK). Cartilage was used mainly as explants. However, some experiments used porcine chondrocytes, which were prepared from this cartilage (see below).

Synovium was dissected from the anterior surface of the opened porcine metacarpophalangeal joint.

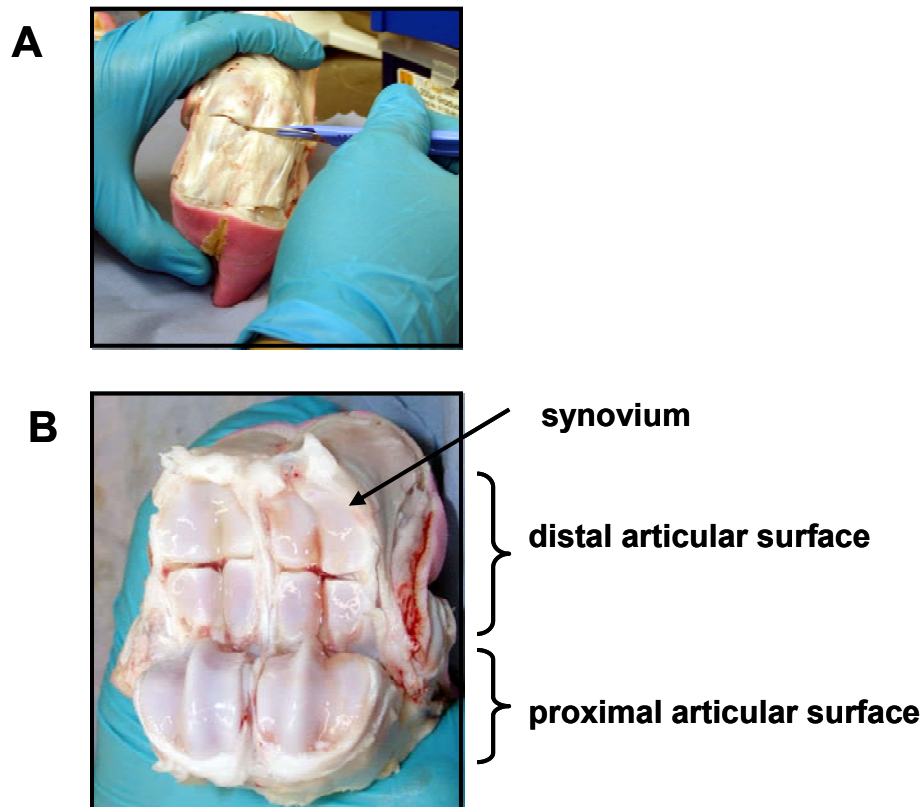


Figure 2.1. The porcine compound metacarpophalangeal joint.

A. Porcine trotters were immersed in 1% Virkon for 20min, then skinned, and carefully opened using a size 10 scalpel.

B. ~0.5g cartilage was dissected from the articular surfaces, using a size 15 scalpel (see also Fig 2.2). 0.1g synovium was trimmed from the cut edge of the anterior side of the joint.

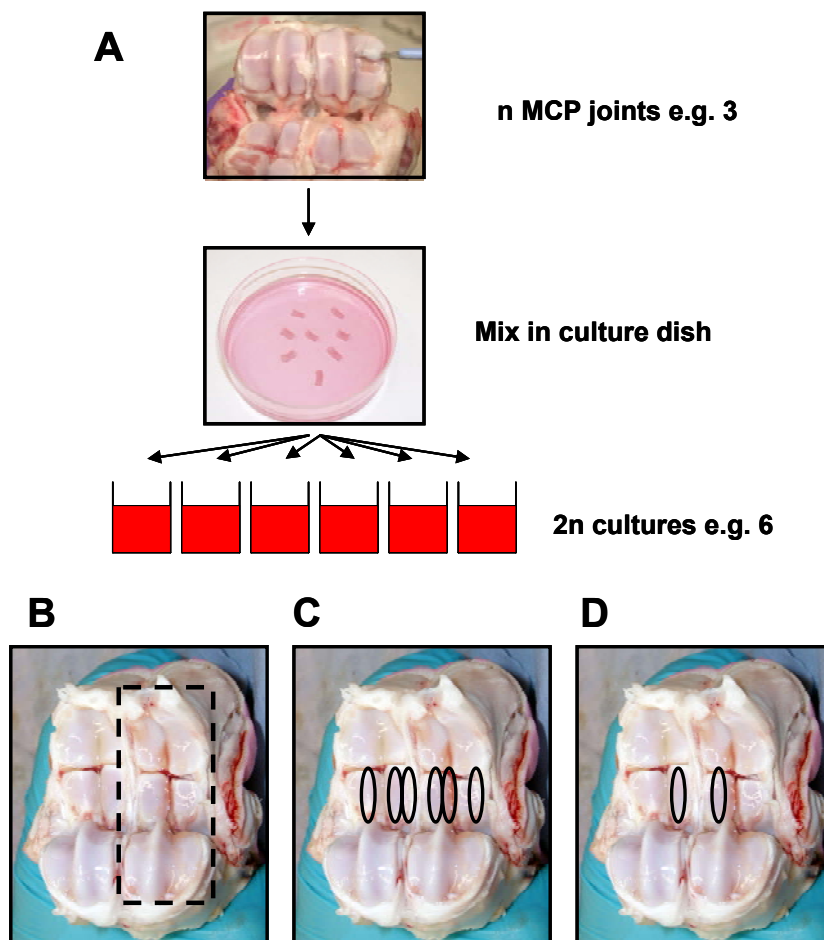


Figure 2.2. Experimental design for cartilage experiments.

A. Rested cartilage explants. All cartilage from n MCPJs was dissected into a culture dish containing serum-free medium and washed, prior to transferring equal numbers of cartilage explants to 2n bijous to rest for 24 hours prior to treatment with stimulus.

B.-D. Explant time courses. The metacarpophalangeal joint surface is shown. **B.** For long experiments (hours) e.g. for activin A ELISA Ch. 3, half of the distal and proximal surfaces were dissected per point. 3-4 biological replicates per point from different joints were used. **C.** For medium time course experiments (10 minutes +) e.g. signalling time courses Ch. 3, 6 pieces of cartilage were dissected from the distal surface (marked with ovals). Lysates from 2 different joints were later pooled for each point prior to electrophoresis. **D.** For short time course experiments (15 seconds +) e.g. phosphotyrosine signalling Ch. 4, 2 pieces of cartilage were dissected from the distal surface (marked with ovals) and placed in culture medium for the appropriate time prior to snap freezing. This procedure was repeated 3 times and cartilage from each short culture then lysed together for each point (total of 6 pieces).

2.2.1 Primary porcine chondrocytes

Freshly dissected cartilage explants were digested with collagenase IA from *Clostridium histolyticum* (Sigma-Aldrich, Dorset, UK) (1mg/ml) in 10% FCS-containing DMEM for 4-5h at 37°C. The digest was passed through a cell strainer (BD Falcon, BD Biosciences, Bedford, USA), then centrifuged at 1,800 r.p.m for 5min. Pellets were washed twice with serum-free DMEM to remove collagenase, and then re-suspended in 10% FCS containing DMEM. Cells were counted using a standard haemocytometer, and plated on 12 well plates at a density of 2 million cells per well (90% confluent). Cells were then incubated at 37°C /5% CO₂ for 48h prior to use. Before use, cells were serum-starved for 3h.

2.2.2 Cartilage explants

Approximately 0.5g cartilage (half of the cartilage from a porcine trotter from the distal and proximal surfaces) was dissected and cultured for each point for most experiments. For some experiments, approximately 0.2g cartilage (6 pieces) from the distal articular surface only was used (see Fig 2.2). Unless stated cartilage explants were cultured in 2-4ml serum-free medium.

Rested cartilage explants: Porcine trotters were skinned, opened in a tissue culture hood, and cartilage dissected using aseptic technique into serum-free medium containing antibiotics and cut into pieces about 3 x 3mm in size. Explants were then washed three times in serum-free DMEM without antibiotics and cultured for 24 or 48h at 37°C, 5% CO₂ prior to use (Fig 2.2A).

Explant time course experiments: Porcine trotters were soaked in 1% Virkon then rinsed briefly in 70% ethanol. Trotters were skinned, MCP joints opened, then closed and equilibrated at 37°C, 5% CO₂ for 4h prior to explantation. Freshly dissected

cartilage explants were cultured in serum-free, antibiotic free DMEM at 37°C, 5% CO₂ for increasing times prior to snap freezing or lysis. For '0' time points, the joint was opened and the surface rapidly washed with 250ml 1x PBS, prior to immediate dissection of the cartilage into a tube on dry ice (Fig 2.2B).

Scoring of the articular surface: Trotters were skinned, MCP joints opened, then closed and equilibrated at 37°C, 5% CO₂ for 4h. Joints were then opened in the tissue culture hood, and the distal articular surface was scored to a depth of 1-2mm using a sterile blade (#15, Swann Morton, Sheffield, UK), at 1mm intervals in both coronal and sagittal planes. The joint was then closed and returned to the incubator for the appropriate time, prior to washing the surface rapidly in ice cold 1x PBS and immediate dissection of the cartilage into a tube on dry ice.

Injection of whole joint experiments: Porcine trotters were soaked in 1% Virkon then rinsed briefly in 70% ethanol. Trotters were equilibrated at 37°C, 5% CO₂ for 4h. After 3h, 3ml serum-free DMEM +/- appropriate concentrations of inhibitor or vehicle were injected via a dorsal approach into the MCP joint with a 25G needle (BD, Drogheda, Ireland). Following this, trotters were skinned, opened and cartilage dissected immediately into serum-free medium +/- inhibitor and cultured for the appropriate time.

Freeze-thawed cartilage: In some experiments, the generation of freeze-thawed cartilage was necessary. Explants were snap frozen in liquid nitrogen, defrosted in a waterbath for 5min at 37°C and these steps were repeated twice more.

Conditioned medium: Conditioned medium was generated by dissecting 0.5g cartilage in 1ml serum-free medium. The explants were then chopped into 1 x 1mm

pieces with a scalpel, prior to culture for the indicated time and removal of the medium.

2.3 Preparation of cell and tissue lysates

Unless otherwise stated, monolayer chondrocytes and cartilage explants were lysed using a dissociative lysis buffer (RIPA). Lysis buffer was supplemented with fresh 2mM DTT. For serine phospho-western blotting, the buffer was also supplemented with proteinase inhibitor cocktail, microcystin and fresh β glycerophosphate. For tyrosine phospho-western blotting, the buffer was supplemented in addition with fresh sodium fluoride and sodium orthovanadate (see Table 2.4).

2x RIPA buffer

100mM Tris/HCl pH 7.4
300mM sodium chloride
20mM sodium pyrophosphate
20mM sodium Fluoride
2mM EDTA
200 μ M β glycerophosphate
2% sodium orthovanadate
0.2% sodium deoxycholate
1% NP-40/ Igepal
0.4% SDS

2.3.1 Chondrocytes

350µl of lysis buffer was added to each well and agitated at 4°C for 20min. Wells were then scraped and lysates aspirated and clarified by centrifugation at 13,000 r.p.m for 20min.

2.3.2 Cartilage Explants

700µl of lysis buffer was added to ~0.5g tissue. Samples were vigorously shaken at 4°C for 2h. Lysates were aspirated and clarified at 13,000 r.p.m for 20min.

2.3.3 Synovium

400µl of lysis buffer was added to ~0.2g tissue. Samples were vigorously shaken at 4°C for 1.5h. Lysates were aspirated and clarified at 13,000 r.p.m for 20min.

2.3.4 Protein determination by the Bradford Assay

This assay was used to assess protein concentration in cellular lysates (the presence of matrix as well as cellular proteins meant that a meaningful assessment of cellular protein content in tissue lysates was not possible using this method). 1µl sample was mixed with 99µl 1x PBS and 900µl Bradford reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) H₃PO₄). The A₅₉₅ of the sample was determined using a digital spectrophotometer (Cecil Instruments, Cambridge, UK). Protein concentration was estimated by comparison of the A₅₉₅ of the sample to a standard curve from known concentrations of BSA.

Table 2.4. Protease and phosphatase inhibitors used during lysis.

Reagent	Dilution	Target	Source
100x Protease inhibitor cocktail, containing:	1: 100		Sigma-Aldrich, Dorset, UK
AEBSF 104 μ M		Serine proteases	
Aprotinin 80 μ M		Serine proteases	
Leupeptin 2.1mM		Serine/cystine proteases	
Bestatin 3.6 mM		aminopeptidases	
Pepstatin A 1.5mM		Aspartic proteases	
E-64 1.4mM		Thiol/cysteine protease, chymase & trypsin	
Phenyl methyl sulphonyl fluoride (PMSF) 200mM	1:200	Serine proteases	Sigma-Aldrich, Dorset, UK
Microcystin 1 mg/ml	1:500	Serine proteases	Alexis, Lausen, Switzerland
Para-nitrophenyl phosphate (PNPP) 1M	1:500	General phosphatase inhibitor	Calbiochem, La Jolla, USA
β glycerophosphate 1M	1:500	General phosphatase inhibitor	Sigma-Aldrich, Dorset, UK
Sodium fluoride 200 mM	1:100	Tyrosine phosphatases	Sigma-Aldrich, Dorset, UK
Sodium orthovanadate 100 mM	1:100	Tyrosine phosphatases	Sigma-Aldrich, Dorset, UK
Sodium iodoacetate in methanol 200mM	1:200	Tyrosine phosphatases	Sigma-Aldrich, Dorset, UK
EDTA		Metal chelator	Sigma-Aldrich, Dorset, UK

2.4 SDS-PAGE

2.4.1 Buffers and reagents

Separating buffer	380mM Tris/HCl pH 8.8 8% or 11% (w/v) acrylamide 0.1% (w/v) SDS 0.07% (w/v) ammonium persulphate 0.07% (v/v) TEMED
Stacking buffer	125mM Tris/HCl pH 6.8 5% (w/v) acrylamide 0.1% SDS 0.1% (w/v) ammonium persulphate 0.1% (v/v) TEMED
4x SDS sample buffer	0.4M Tris/HCl pH 6.8 0.4% (w/v) SDS 40% (v/v) glycerol 1% (v/v) β -mercaptoethanol or DTT 0.02% (w/v) bromophenol blue
Running buffer	25mM Tris base 190mM glycine 0.1% (w/v) SDS

2.4.2 Preparation of samples

Chondrocytes: 250µl of clarified lysate was mixed with 100µl 4x SDS sample buffer, vortexed, boiled for 7min and sonicated for 10min prior to electrophoresis.

Cartilage explants: 500µl of clarified lysate was mixed with 200µl of 4x SDS sample buffer, vortexed, boiled for 7min and sonicated for 10min prior to electrophoresis.

Synovium: 300µl of clarified lysate was mixed with 150µl of 4x SDS sample buffer, vortexed, boiled for 7min and sonicated for 10min prior to electrophoresis.

2.4.3 Electrophoresis

Proteins from cell or tissue lysates were separated by 1.5mm gel electrophoresis (Laemmli, 1970) using Cambridge electrophoresis apparatus (Cambridge Electrophoresis Ltd, Cambridge, UK). 8% SDS-acrylamide gels were run for western blotting of phospho-tyrosines and 11% gels for all other purposes, using Tris/glycine/SDS running buffer (National Diagnostics, USA). Usually 180µl of each sample was loaded into one of 15 pre-cast wells of the stacking gel. Pre-stained molecular weight markers were run alongside the samples to allow approximate sizing of proteins (SeeBlue markers, Invitrogen, UK). Gels were run for 5h at 38mA or overnight at 10 mA.

Alternatively, particularly where multiple bands were being analysed, such as analysis of 4G10 immunoprecipitates and for silver staining, a pre-cast mini-gel system was employed. The NuPAGE Bis-Tris discontinuous pre-cast 1.5mm polyacrylamide gels were from Novex (Invitrogen, UK). The gels have a 4% acrylamide stacking gel and a 4-12% acrylamide gradient separating gel. 40µl of the cell/tissue lysate was loaded into the wells of the stacking gel. The gels were then run at 100V for 2h, using NuPAGE MOPS-SDS running buffer, in the bespoke XCell SureLock Mini-Cell

electrophoresis apparatus (both by Novex, Invitrogen, UK). After separation of the proteins, gels were either silver stained or western blotting was carried out.

2.5 Western blotting

Transfer buffer	25mM Tris base
	192mM Glycine
	20% (v/v) methanol
Blocking solution	5% dried milk (w/v)
	1x TBS, or 1x PBS
	0.1% Tween (v/v)

Western blotting was carried out to identify proteins from cellular or tissue lysates or immunoprecipitates. Proteins from SDS-PAGE were transferred on to pre-hydrated PVDF membranes (Millipore, Bedford, USA) in a Trans-blot chamber (Biorad, Hercules, USA) at 100V for 70min at 4°C. Membranes were stained with Coomassie Brilliant Blue stain to determine efficiency of transfer and equal protein loading, then de-stained with 50% methanol. Membranes were gently agitated in blocking solution for 1.5h, and then incubated with the primary antibody for 3h at room temperature or overnight at 4°C (see Table 2.1). Antibodies were diluted as per manufacturer's guidelines in blocking solution. Phospho-antibodies were generally diluted in 5% BSA in TBS. The membrane was then washed 3 times in PBS or TBS-tween and incubated with secondary horseradish peroxidase-conjugated antibody (1:2000) for 1h at room temperature. The membrane was washed a further 3 times in PBS/TBS-tween and signal developed by enhanced chemiluminescence (ECL, Amersham, UK). Protein bands were visualised by autoradiography (Fuji Super RX x-ray film, Fuji Photo Film, Dusseldorf, Germany). Films were developed using a Curix 60 film processor from AGFA-GEVAERT Ltd, Brentford, UK.

4G10 western blotting: 4G10 western blotting was optimal when the following steps were incorporated. Proteins from SDS-PAGE were transferred on to a pre-hydrated

nitrocellulose membrane (NitroBind, Osmonics, USA) at 100V for 90min. Membranes were then washed briefly in TBS and blocked overnight in a solution of 5% BSA in TBS-tween. The membrane was incubated with primary antibody at a concentration of 1:20,000 (and latterly an antibody-HRP conjugate was used). Both the primary and secondary antibodies were in 5% BSA in TBS-tween. After the secondary antibody incubation, the membrane was washed extensively with TBS-0.1% tween for 1.5h prior to chemiluminescence.

Stripping proteins from membranes: To re-probe membranes with a further antibody where necessary, membranes were washed in PBS, then incubated with 1x stripping buffer (Re-Blot Plus Strong Solution, Chemicon, Temecula, USA) for 10min at RT, prior to washing again for 5min in PBS, prior to blocking and subsequent western blotting analysis.

2.6 Immunoprecipitation kinase assays

[γ -³²P]Adenosine 5-triphosphate (ATP) was 'Easy Tides' [γ -³²P]ATP from Perkin-Elmer, Boston, USA.

2.6.1 In vitro IKK assay

To assay the activity of IKK α/β within chondrocyte or cartilage lysates, the tripartite IKK complex was immunoprecipitated from the lysates using an antibody to IKK γ , and the ability of this complex to phosphorylate the substrate I κ B α assessed using a kinase assay. This protocol has been adapted from a protocol kindly provided by M. Bebien, Kennedy Institute of Rheumatology.

Buffers for IKK kinase assay**1x Non-dissociative kinase assay lysis buffer**

50mM Tris/HCl pH 7.5

250mM NaCl

3mM EDTA

3mM EGTA

1% Triton X-100

0.5% Igepal

10% glycerol

1x Immunoprecipitation buffer

As lysis buffer, but 400mM NaCl

10x kinase wash buffer

200mM Hepes/HCl pH 7.5

100mM MgCl₂

Reaction mix	μl
10x kinase wash buffer	2.5
ATP 200μM	2.5
β glycerophosphate 1M	0.6
PNPP 1M	0.3
DTT 200mM	0.3
GST-IκBα	0.35 (i.e. 1-2 μg per reaction)
ddH ₂ O	18
[γ- ³² P]ATP	0.4 (at activity date)
Total	25

Non-dissociative lysis buffer, immunoprecipitation buffer and 1x kinase wash buffer were supplemented immediately prior to use with proteinase inhibitor cocktail, DTT, microcystin, PNPP, β glycerophosphate, sodium orthovanadate and PMSF at the appropriate concentration (see Table 2.4), and the solutions kept on ice until use.

Chondrocytes: For a 12 well plate, 120 μ l per well of non-dissociative kinase assay lysis buffer was added per well, and the plate agitated on ice for 20min. The wells were then scraped with a cell scraper (Corning Inc, NY, USA) and the lysate aspirated and transferred to a 1.5ml Eppendorf tube. Lysates were clarified at 13,000 r.p.m at 4°C for 20min, and the supernatant stored at -80°C until later use, or kept on ice if used immediately. Bradford assay was carried out on the lysates. 40 μ g of protein per sample was added to 400 μ l immunoprecipitation buffer in a 1.5ml Eppendorf tube.

Cartilage: ~0.5g cartilage was lysed in 700 μ l lysis non-dissociative kinase assay buffer. Alternatively, 6 pieces of cartilage were lysed in 400 μ l lysis buffer. Samples were vigorously shaken at 4°C for 2h. Lysates were aspirated and clarified at 13,000 r.p.m at 4°C for 20min. The supernatant was stored at -80°C until later use, or kept on ice. 150 μ l lysate was added to 250 μ l immunoprecipitation buffer in a 1.5ml Eppendorf tube.

Immunoprecipitation: For either chondrocyte or cartilage lysates in immunoprecipitation buffer, 1 μ l IKK γ antibody (BD-Pharmingen, Bedford, USA) and 25 μ l of a 50:50 mix of re-suspended 1:1 slurry of protein G agarose and Protein A agarose in PBS (both from Sigma, Dorset, UK) were added to each sample, and incubated overnight, rotating at 4°C.

Kinase assay: The following day, samples were centrifuged at 13,000 r.p.m for 1min and the supernatant discarded. Immunoprecipitates on ice were washed twice in 600 μ l

immunoprecipitation buffer and once in 600 μ l 1x kinase wash buffer (after the addition of each wash, samples were shaken for 6sec, then centrifuged at 13,000 r.p.m for 1min and the supernatant aspirated).

The reaction mix for n+1 samples was prepared on ice (see above). Prior to addition of [γ -³²P]ATP, the mix was vortexed and pulsed in a centrifuge. Using a gel loading tip, all remaining kinase wash buffer was aspirated from the immunoprecipitates. The [γ -³²P]ATP was then added to the reaction mix and the mixture pipetted repeatedly to mix thoroughly. 25 μ l reaction mix was added to each immunoprecipitate, prior to incubation at 30°C for 30min. The kinase reaction was terminated by the addition of 10 μ l 4x SDS sample buffer and boiling for 10min. Samples were then centrifuged at 13,000 r.p.m for 1min, and the whole supernatant (35 μ l) loaded on to a 11% polyacrylamide gel. Following electrophoresis, proteins were transferred on to a PVDF membrane at 100V for 1.5h. Autoradiography of the membrane was carried out by exposing overnight to x-ray film. Subsequent quantification of phosphorylation of the substrate was carried out, using a phosphorimager (see section 2.6.4). Western blotting of the membrane with antibodies to IKK α (BD-pharmingen, Bedford, USA) was then carried out to ensure equivalent immunoprecipitation of the IKK complex between samples.

2.6.2 In vitro c-src kinase assay

The c-src kinase assay performed in these studies assessed activity of c-src both by way of its auto-kinase activity (the ability to phosphorylate itself) and its ability to phosphorylate a substrate, enolase. The protocols for these experiments have been adapted from protocols kindly provided by M. Smolinska-Bylanska and T. Page, Kennedy Institute of Rheumatology.

Buffers for c-src kinase assay

2x kinase buffer	μl
ddH ₂ O	900
1M Hepes pH 7.5	40
0.1M MnCl ₂	40
0.1M DTT	20
Total	1000
 Reaction mix	 μl
2x kinase buffer	5
100 μM ATP	0.1
Denatured enolase	1
ddH ₂ O	2.9
[γ- ³² P]ATP	1 (at activity date)
Total	10

Immunoprecipitation: Cartilage lysates were prepared by lysis of 0.5g cartilage in 700μl RIPA buffer supplemented with proteinase inhibitor cocktail, DTT, β glycerophosphate, sodium orthovanadate, sodium iodoacetate, microcystin and sodium fluoride. Samples were clarified by centrifugation at 13,000 r.p.m for 20min. Lysates were pre-cleared by addition of 40μl re-suspended 1:1 slurry of Protein G agarose in PBS, with rotation at 4°C for 45min. The supernatant was recovered following centrifugation at 4°C for 1min. c-src was immunoprecipitated from the

lysates by the addition of 4 μ l GD11 c-src antibodies (Upstate) and 30 μ l protein G agarose bead slurry to the whole supernatant (approximately 600 μ l), and rotation at 4°C for 1h. Following centrifugation for 1min, the supernatant was discarded and beads were washed four times in RIPA buffer and once in 1x TBS (both supplemented with proteinase inhibitor cocktail, sodium orthovanadate, sodium fluoride, β glycerophosphate and DTT).

Preparation of enolase: 100 μ g (28.6 μ l) rabbit muscle enolase (EO379, Sigma Aldrich, Dorset, UK) was centrifuged at 13,000 r.p.m for 5min at 4°C. Supernatant was discarded using a gel loading tip and 10 μ l of 1mM DTT/50mM Hepes pH 7.5 was added. The enolase suspension was incubated on ice for 60min, and then 10 μ l glycerol was added. Just prior to the kinase assay, 20 μ l of 100mM acetic acid was added to the enolase suspension, and the mixture incubated at 30°C for 5min to denature the enolase.

Kinase assay: Using a gel loading tip, all remaining kinase wash buffer was aspirated from the immunoprecipitates. 10 μ l 1x kinase buffer was added to each sample. The reaction mix for n+1 samples was prepared on ice (see above). Prior to addition of [γ -³²P]ATP, the mix was vortexed and pulsed in a centrifuge. The [γ -³²P]ATP was then added to the reaction mix and the mixture pipetted repeatedly to mix thoroughly. 10 μ l of reaction mix was added to each sample. Samples were vortexed gently and incubated at 30°C for 10min. The kinase reaction was terminated by addition of 15 μ l 4x SDS sample buffer and boiling for 10min. Samples were then centrifuged at 13,000 r.p.m for 1min, and the whole supernatant (35 μ l) loaded on to a 4-12% minigel. Following electrophoresis, the gel was dried on to paper using a gel dryer (model 583 Geldryer, Biorad, Hercules, USA). Activity of the kinase was assessed by autoradiography of the gel, which showed two bands, one relating to phosphorylated

c-src and one relating to phosphorylated enolase. Subsequent quantification of autophosphorylation and phosphorylation of the substrate was carried out, using a phosphorimager (see section 2.6.4).

2.6.3 Autoradiography

Dried gels or membranes were subjected to autoradiography using x-ray film (Hyperfilm ECL, Amersham biosciences or Fuji Super RX x-ray film, Fuji Photo film, Dusseldorf, Germany). After a few hours, or overnight, the films were developed with a Curix 60 film processor from AGFA-GEVAERT Ltd, Brentford, UK.

2.6.4 Quantification of phosphorylation

Protein phosphorylation data was acquired with a phosphorimager (FLA5100, Fujifilm). Phosphorimager data was quantified and analysed using Advanced Image Data Analyser Software (AIDA version 4.10, Raytest, Straubenhardt, Germany).

2.7 Reverse Transcriptase-PCR from cartilage

To investigate whether inflammatory response gene mRNA was induced in cartilage, total RNA was extracted from cartilage explants, using techniques adapted from published protocols (Martin et al., 2001).

2.7.1 RNA extraction from cartilage explants

RNA was isolated from ~0.2g freshly dissected cartilage explants (approximately 20 pieces of cartilage chopped into 3 x 3mm squares, cultured in a 15ml polypropylene tube). Culture medium was removed from each sample and 3ml Tri Reagent (Helena Biosciences, Sunderland, UK) was added. Samples were kept on ice whilst homogenised using a Polytron PT1600 rotor-stator homogenizer (Kinematica AG, Littau-Luzern, Switzerland) for 1min, and the homogenate clarified by centrifugation at 4°C for 10min. 2ml supernatant was aspirated and allowed to stand at room temperature for 5min to allow complete dissociation of nucleoprotein complexes. 200µl chloroform was then added per 1ml supernatant and the samples vortexed for 15sec prior to resting at room temperature for 5min. Samples were then centrifuged at 4°C for 15min. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, a white interphase (containing DNA), and a colourless RNA-containing upper aqueous phase. This upper phase was carefully aspirated and mixed with the same volume of 70% ethanol, prior to binding to a QIAmp spin column and washing once with RW1 buffer and twice with RPE buffer, as per manufacturer's protocol (QIAmp RNA Blood Mini kit, Qiagen, Chatsworth, USA). Following the second wash with RPE buffer, the spin column was transferred into a fresh collection tube and centrifuged again at 13,000 r.p.m for 3min. RNA was eluted in 30µl of RNAase free water. UV absorbance was measured at 260nm, and samples were stored at -80°C until use.

2.7.2 Reverse Transcription of RNA (First strand synthesis)

Reverse transcriptase (RT)-polymerase chain reaction (PCR) was carried out. 1µg RNA in a total volume of 10µl RNAase free water was incubated at 65°C for 10min with 1µl of random hexamers and 1µl 10mM dNTP mix (Invitrogen). Samples were then kept on ice for a further 5min. RT mix (see below) contained an engineered version of M-MLV reverse transcriptase, Superscript II (Invitrogen, UK) and the RNAase inhibitor RNAGuard (Amersham biosciences, Little Chalfont, UK). 8µl RT mix was added to the denatured RNA on ice, and the reverse transcription reaction carried out at 30°C for 10min, 42°C for 50min and 70°C for 15min. cDNA was then stored at -20°C until use.

RT mix	per sample	µl
5x First-Strand buffer		4
0.1M DTT		1
SuperScript II		2
RNAGuard		1

2.7.3 PCR

PCR of cDNA for inflammatory response genes was then carried out.

Primers: Some primers were from published studies using porcine/bovine material, and these are indicated below. Others where possible were designed from Genbank porcine sequences, using Vector NTI (Invitrogen, UK); (see Table 2.5). Where insufficient porcine sequence was available, bovine, ovine and human sequences were aligned using Vector NTI and primers designed from consensus sequences. Primers for ADAMTS-4, ADAMTS-5 and GAPDH were designed by Y. Sawaji in our group. Primers were for a variety of inflammatory response genes: proteinases (MMP-1,-13 and -3, ADAMTS-4 and -5), TIMP-1 (known to be regulated by FGF-2), COX-2,

iNOS (inflammatory mediators), cytokines (IL-1,-6 and -8) and growth factors important to cartilage metabolism (activin A).

22µl RNAase free water and 1µl each of sense primer, anti-sense primer and cDNA were added to PuReTaq Ready-to-go PCR Beads (Amersham Biosciences, Little Chalfont, UK) and PCR carried out using a T3 thermocycler (Biometra, Göttingen, Germany). The beads contained 2.5 units of Taq DNA polymerase, 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl₂, 200µM dATP, dCTP, dGTP, dTTP and stabilisers including BSA. PCR included three distinct stages; denaturation, primer annealing and primer extension. The denaturation for each PCR reaction was an initial incubation at 95°C for 5min, then 95°C for 30sec per cycle. The annealing stage allows hybridisation of primers to complementary target sites on the DNA molecule. The annealing temperature for individual PCRs was estimated by calculation of the melting temperatures (T_m) for each primer within the pair (using a T_m calculator, Vector NTI). Initial experiments to optimise the reaction, using temperatures at or above the estimated T_m were carried out. Annealing temperatures for each PCR are shown in Table 2.5. The final primer extension stage was 72°C for 45sec per cycle. Following the specified number of cycles, there was a final phase of incubation at 72°C for 5min. GAPDH was used as a housekeeping control, to confirm equal amounts of mRNA between samples. Samples were mixed with 6x bromophenol blue buffer and electrophoresed on a 1.2% agarose-TAE gel, and stained with either SYBR green (Molecular Probes Inc, USA) or 0.5µg/ml ethidium bromide. A 1Kb Plus DNA ladder (Invitrogen, UK) was electrophoresed alongside to allow approximate sizing of PCR products. Products were visualised with ultraviolet light (254nm) and recorded using a camera and transilluminator (UVP inc, San Gabriel, USA).

IL-1 α stimulated cartilage was used as a positive control for these experiments, as it induces all these inflammatory response genes in chondrocytes (Gebauer et al., 2005). For each primer pair, a cycle number within the linear amplification phase was selected, which optimally detected regulation of the PCR product by IL-1 (see Table 2.5).

50x TAE Buffer

2M Tris/HCl pH 8.5

50mM EDTA

5.7% (v/v) Glacial acetic acid

Bromophenol Blue buffer

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in H₂O

Table 2.5. Primers used for RT-PCR.

(See following page)

a (Goodstone and Hardingham, 2002)

b (Schubert et al., 2001)

c (Lo et al., 2003)

d (Kanaki et al., 2002)

e (Li et al., 1997)

Target	Accession number	Orientat ⁿ	Primer sequence 5'-3'	Product size	Annealing temperature °C	Cycle number
GAPDH	NM002046	sense	CATGGAGAAGGCTGGGGCTC	671	58	22
		antisense	ATGAGGTCCACCACCCTGTT			
COX-2	U68486	sense	GCTCTTCCTCCTGTGCCTGATGAC	449	58	22
		antisense	CATCATCAGACCAGGCACCAGAC			
iNOS ^a	U59390	sense	TTCGAGCACATCTGCAGG	302	53	25
		antisense	GTCAGGAGGGATTTTCGAA			
IL-1 α	X60167	sense	GAGGCAGTGAAATTTGACATGG	245	58	34
		antisense	GGCAATGAACAACCTTTGGATGGG			
IL-1 β	X56972	sense	GCTGAAGGCTCTCCACCTCC	330	63	40
		antisense	TCCCAGGAAGACGGGCTTT			
IL-6	X68723	sense	GAACCCAGCTATGAACTCCCTCT	331	58	27
		antisense	GATTGAACCCAGATTGGAAGC			
IL-8	AB057440	sense	GGCTCCCAAGAATTTCTCAGTA	318	58	33
		antisense	CAGCAGCCTAGGGTTGCAAG			
MMP-1 ^b	AF267156	sense	CCGTTTGTTCTTACTCCAGGGAA	416	58	29
		antisense	GGGTACATCAGAGCCCCAATGTC			
MMP-13 ^c	AY091604	sense	GTGACAGGCAGACTTGATGA	331	58	29
		antisense	AAGCGTGAGCCAACAGACCA			
MMP-3 ^d	AF201725	sense	GGAGTTCCTGATGTTGGTTACTTC	266	63	30
		antisense	CAAAACTTTTCCAGGTCCGTCAAA			
ADAMTS-4	AF148213	sense	ACCACTTTGACACAGCCATTCTG	691	63	35
		antisense	ACCCCCACAGGTCCGAGAGCAG			
ADAMTS-5	NM007038	sense	TGTGCTGTGATTGAAGACGAT	672	61	32
		antisense	GACTGCAGGAGCGGTGAGTGG			
TIMP-1	AF156029	sense	GCGTTATGAGATCAAGATGACC	212	53	40
		antisense	CTGTTCCAGGGAGCCACAA			
Inhibin β A ^e	NM214028	sense	GACATCCGGACTGCCTGCGAGCAG	315	65	30

2.8 ELISA

An ELISA kit specific for quantification of Activin A was used (Duoset, R&D systems, Minneapolis, USA). The manufacturer's protocol for the kit was followed. Supernatants from cell or explant culture were clarified by centrifugation and stored at -20°C until use. 96 well ELISA plates (NUNC, Roskilde, Denmark) were coated with 1µg/ml capture antibody diluted in PBS. 100µl of the capture antibody solution was added to each well and incubated overnight at room temperature. The following day, wells were aspirated and washed 3 times with wash buffer (1x PBS, 0.05% tween). Wells were blocked with 1% BSA in PBS for 2h, and then washed again in wash buffer. Activin A standards were prepared by 2 fold serial dilution in DMEM from 8ng/ml to 0.125ng/ml. Where necessary, samples were diluted in DMEM so that the activin A concentration in the sample was within the linear range for the assay. 100µl of either standards or samples were added to wells. 100µl 6M urea in PBS was then immediately added to each well and mixed with the standards and samples. After 2h incubation at room temperature, wells were aspirated and washed 3 times with wash buffer. 100µl 0.5µg/ml biotinylated detection antibody diluted in 1% BSA in PBS was added to each well and left for a further 2h at room temperature, with gentle agitation. Wells were aspirated and washed 3 times with wash buffer. 100µl of streptavidin solution diluted to 1:200 in 1x PBS was added to the wells for 40min. The aspiration/wash step was repeated and 100µl substrate solution (1:1 mixture of hydrogen peroxide and tetramethylbenzidine, KPL, Gaithersburg, USA) was added to each well for 20min incubation with mild agitation. The reaction was stopped by adding 50µl of 2M sulphuric acid. Plates were read immediately using a spectrophotometric plate reader (Multiskan Biochromatic, Labsystems, Finland) set to

a wavelength of 450nm, and analysed using Ascent software, Thermo Fisher Scientific, Waltham, USA.

2.9 Purification of tyrosine-phosphorylated proteins from cartilage lysates

For all procedures involving preparation of samples for mass spectrometry, the utmost care was taken to avoid contamination. All reagents were of ultra-pure analytical grade, and H₂O was double distilled (ddH₂O), free from protein and microbial products. Cleaned and baked glassware was used where possible. Surfaces and all plasticware were cleaned with 50% methanol, 0.1% TFA prior to use.

2.9.1 Standard 4G10 immunoprecipitation and elution of phosphorylated proteins

This protocol for purification of tyrosine phosphorylated proteins from lysates was adapted from methods described by M. Peirce, Kennedy Institute of Rheumatology (Peirce et al., 2005). The methods were optimised and validated for tissue lysates (See Ch 4, section 4.5.2) and are summarised in Figure 2.3. 600µl clarified RIPA lysate was added to a 1.5ml Eppendorf and incubated with 20µl of a re-suspended 1:1 slurry of Protein A agarose beads in PBS (Sigma-aldrich, Dorset, UK), rotating at 4°C for 30min-1h. The supernant was then recovered by centrifugation at 13,000 r.p.m for 1min at 4°C. The pre-cleared lysate was then incubated with 10µl protein A agarose beads, covalently linked to 4G10, in PBS (Upstate, USA), rotating overnight at 4°C. The next day, beads were precipitated by centrifugation at 13,000 r.p.m for 1min at 4°C and the supernatant was discarded. The beads were washed 5 times with 600µl RIPA buffer (including proteinase and phosphatase inhibitors [see Table 2.4]). After the addition of each wash, samples were shaken for 6sec, then centrifuged at 13,000 r.p.m for 1min and the supernatant aspirated. After the last wash, all remaining RIPA buffer was aspirated from the beads with a gel loading tip. A solution of 0.2M phenyl

phosphate in RIPA was freshly prepared. The beads were incubated with 20 μ l of phenyl phosphate, shaking gently for 2h at 4°C. The tube was pulsed briefly in the centrifuge. Using a cut-off 200 μ l pipette tip, the phenyl phosphate-bead slurry was carefully transferred to a 0.2 μ m cellulose acetate spin column (Spin-X, Corning Costar Inc., NY, USA). (Several eluates from like samples were pooled in one column). Columns were then centrifuged at 8,000 r.p.m for 2min. The recovered eluate was then either concentrated further (see below, section 2.9.2), or boiled immediately with 20 μ l SDS sample buffer for 7min, sonicated for 10min and boiled again for 2min, prior to electrophoresis for either western blotting or silver staining.

In some experiments, Cibacron Blue Sepharose beads were used (Blue Sepharose 6 Fast Flow, GE healthcare, New Jersey, USA).

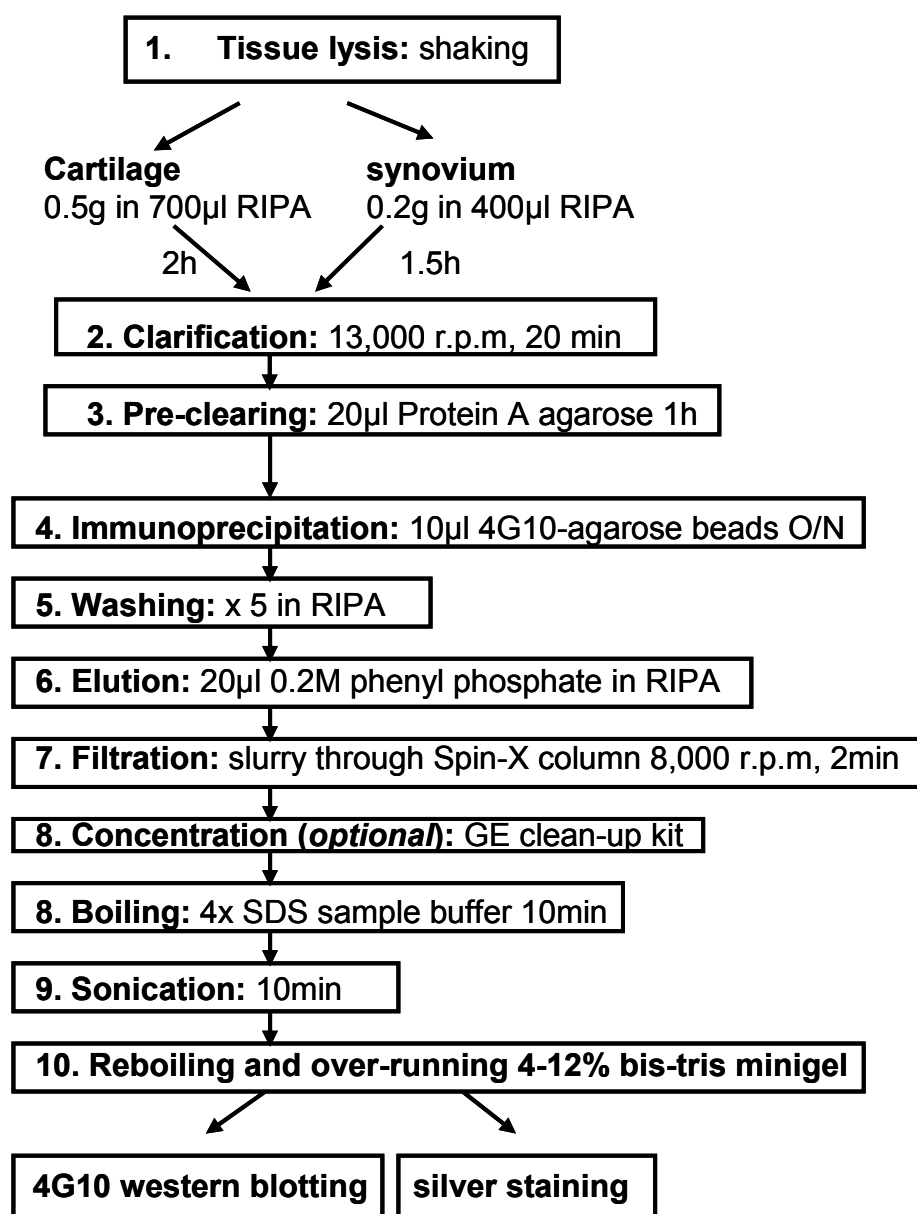


Figure 2.3. Standard 4G10 immunoprecipitation protocol.

A summary is shown of the optimised methods used for immunoprecipitation of tyrosine-phosphorylated proteins from tissue lysates, and their selective elution prior to electrophoresis.

2.9.2 Concentration of proteins

Acetone precipitation: To purify and concentrate proteins, acetone precipitation was employed. Acetone was cooled to -20°C in advance of the procedure. 4 times the volume of cold acetone was added to the protein solution. The contents were then vortexed for 10sec and the solution incubated for 60min at -20°C. Following this, the sample was centrifuged at 13,000 r.p.m for 10min at 4°C. The supernatant was then removed using a gel loading tip, taking care not to disturb the protein pellet. 4x SDS sample buffer was added to the pellet and the sample vortexed, boiled and sonicated to assist in solubilising the pellet prior to electrophoresis.

SDS PAGE clean-up kit: This kit (GE healthcare, New Jersey, USA) involved a two-stage modified acetone precipitation protocol, and included buffers to assist re-solubilisation of the protein pellet. The kit was used to concentrate phenyl phosphate eluates following 4G10 immunoprecipitation, prior to electrophoresis and subsequent identification of proteins by mass spectrometry. The manufacturer's protocol was followed. For samples under 100µl, 300µl precipitant and 300µl co-precipitant solutions were added. For samples over 100µl (during scale-up) 3 volumes of precipitant and co-precipitant were added. The samples were centrifuged at 13,000 r.p.m for 5min and then the supernatant removed carefully from the pellet, which was then re-suspended in H₂O with extensive vortexing. 1ml wash buffer (acetone, pre-cooled to -20°C) and 5µl wash additive were added and the sample vortexed until the pellet was completely dispersed. The samples were then incubated at -20°C for at least 1h, with vortexing every 10min for the first 30min. The samples were then centrifuged at 13,000 r.p.m for 5min at 4°C. The supernatant was carefully discarded and the pellet air dried for 2min. The pellet was re-suspended in two buffers, with incubation at room temperature for 5min, and sonication for 10min to assist re-

solubilisation. Finally, 4x SDS sample buffer was added and the sample vortexed, incubated at room temperature for 5min, then boiled for 10min, prior to electrophoresis.

2.9.3 Staining of protein bands

Some gels were stained to visualise protein bands, either by Coomassie Brilliant Blue staining or silver staining.

Coomassie Brilliant Blue staining: The gel was incubated in Coomassie Brilliant Blue G solution for 1h at room temperature. The gel was then washed in repeated changes of de-stain solution (50% (v/v) methanol) for 1h, and then left overnight in de-stain solution. The next morning the gel was washed with ddH₂O, and left in ddH₂O until further analysis.

Coomassie Brilliant Blue Stain

50% (v/v) methanol

20% (v/v) acetic acid

0.1% (w/v) Coomassie Brilliant Blue G

Silver staining: For silver staining of gels, the EMBL protocol was employed, which was compatible with detection of proteins in the low femtomole range and identification by mass spectrometry (Mortz et al., 2001; Shevchenko et al., 1996). The gel was fixed in a solution containing 50% methanol and 5% acetic acid for 20min, and then washed in a 50% methanol solution for 10min. The gel was then placed in ddH₂O overnight at 4°C. The next morning, the gel was equilibrated to room temperature whilst fresh sensitiser, silver and developing solutions were made. The gel was incubated in the sensitiser solution (0.02% (w/v) sodium thiosulphate) for 1min, washed for 2min in 4 changes of ddH₂O and then incubated at 4°C for 20min

with pre-cooled 0.1% (w/v) silver nitrate solution. The gel was then washed with ddH₂O for 1min, and the gel chamber changed. It was then washed in two changes of ddH₂O for a further minute. The gel was placed in the developing solution of 2% (w/v) sodium carbonate and 0.04% (v/v) formalin, the latter added immediately before use. To reduce background staining, the developing solution was changed twice within the first 2min of incubation, and then subsequently once the colour of the developing solution started to change to yellow. The developing reaction was stopped with 5% acetic acid solution, once the staining of the protein bands of interest was sufficient. The gel was then washed with ddH₂O and stored at 4°C until further analysis.

2.9.4 Protein identification by mass spectrometry

Background to the technique: Protein bands from gel electrophoresis were identified by mass spectrometry in collaboration with Dr. Robin Wait, Proteomics Unit, Kennedy Institute of Rheumatology. Mass spectrometry measures the relative molecular masses of ionised molecules and is reliant on the principle that ions of different masses can be resolved according to their response to electric (and magnetic) fields. Various methods can be used to ionise sample molecules and transfer them to the gas phase prior to mass analysis. In electrospray ionisation, the sample solution is passed through a needle at high electrical potential which is positioned near a small orifice leading into the mass spectrometer vacuum system. The solution is thus dispersed into extremely fine, highly charged droplets which contain protonated sample molecules. Evaporation of the solvent reduces the size of the droplets, increasing the charge density to the point where electrostatic repulsion causes droplet fission, which is followed by further solvent evaporation and fission, until only gas phase, protonated sample molecules remain. These are accelerated towards the orifice

plate of the spectrometer, which is maintained at an opposite electrical potential. Thus the sample molecules enter the vacuum system of the spectrometer for mass analysis.

Electrospray is a so called soft ionisation technique, which means that little excess energy is imparted, so that predominantly protonated molecules (possibly of varying charge states) are produced. This enables determination of the mass of the intact sample, but does not in itself provide further structural information (such as amino acid sequence in the cases of peptides). It is therefore necessary to force the molecule to fragment after ionisation, usually by collision with a neutral gas such as argon or nitrogen. In the case of peptides, fragmentation occurs predominantly at the peptide bonds, resulting in a series of truncated peptide cations, differing by the masses of whole amino acids, which enables the sequence to be determined. These types of experiments are performed using tandem MS (MS-MS), in which two mass analysers are connected in series, with a region containing the collision gas between them. The first analyser then selects a precursor peptide ion (possibly present in a complex mixture) which is fragmented in the collision region, and the second mass analyser measures the masses of resulting fragments. Time-of-flight (Tof) analysis means that ions are boosted to the same kinetic energy, and the time taken for the ion to reach the detector measured.

The peptide sequence can then be determined either by manual interpretation of the spectra, or more usually now, by computerised comparison of the fragment masses observed to the pattern of fragments calculated from a database of proteins with known sequence, which also enables a statistical assessment of the likelihood of a correct identification. For successful identification, the target sequence has to be present in the database used. For organisms where the complete genome sequence is available this is usually the case. However my studies used porcine material, and

unfortunately sequencing of the porcine genome is currently incomplete (though full expressed sequence tag (EST) coverage is now available), so protein identification sometimes required the presence in the database of the corresponding homologues from other mammalian species.

In-gel digestion: Silver-stained protein bands were manually cut out of the gels for digestion with trypsin, as previously described (Shevchenko et al., 1996). Methods were adapted so that excised pieces of gel could be digested in specialised plates, carried out using a robotic digestion system (Investigator ProGest, Genomic Solutions, Huntingdon, UK) (Wait et al., 2001). The gel pieces were destained with a mixture of potassium ferricyanide and sodium thiosulphate washed with 50mM ammonium hydrogen carbonate buffer and dehydrated with acetonitrile, prior to reduction with DTT and blocking of SH groups with iodoacetamide. Gel pieces were then digested with modified trypsin (10µl at 6.5ng/ml in 25mM ammonium hydrogen carbonate) for 8h at 37°C, and peptides recovered by sequential extraction with 25mM ammonium carbonate, 5% formic acid and acetonitrile. The pooled extracts were lyophilized and then dissolved in 0.1% formic acid prior to electrospray.

Electrospray mass spectrometry: Tandem mass spectra were recorded with a quadrupole/orthogonal acceleration time-of-flight (q-Tof) mass spectrometer (Waters, Manchester, UK) interfaced to a Waters CapLC capillary chromatograph. Samples were dissolved in 0.1% (v/v) formic acid in ddH₂O, injected onto a Pepmap C18 column (LC Packings, Amsterdam, Netherlands) and eluted into the electrospray source with an acetonitrile/ 0.1% formic acid gradient. The capillary voltage was set to 3,500V and data-dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey mass range of 400-1400. Precursor ions were fragmented by collision with argon and the collision voltage was varied between

18-45V depending on the charge and mass of the precursor, using an empirically determined lookup table for each charge state. Proteins were identified by correlation of un-interpreted tandem mass spectra with entries in SwissProt/TREMBL, using ProteinLynx Global Server (Version 1.1, Micromass). No taxonomic, mass or pI constraints were applied. Two missed cleavages per peptide were allowed, and the fragment ion tolerance window was set to 100p.p.m. Carbamidomethylation of cysteine was set as a fixed modification, but other potential modifications were not considered in the first pass search. All matching database hits were manually reviewed, and if the reported score (ProteinLynx Global Server) was less than 100, additional searches were performed against the NCBI database using MASCOT, which utilises a robust probabilistic scoring algorithm. Measured parent and fragment masses were typically within 0.03Da of their theoretical values.

2.10 Immunohistochemistry

The phosphorylation of paxillin in synovium was assessed using immunohistochemical methods involving an avidin-biotin cascade technique. This was carried out in collaboration with D. Essex, senior histopathology technician, Kennedy Institute. Synovial samples were fixed in formalin for a minimum of 24h. Samples were then paraffin-embedded, and 4µm sections cut and placed on slides. Sections were incubated at 60°C for 1h, and then de-waxed in xylene for 2x 2min, rehydrated in 2 changes of absolute alcohol, and then washed in H₂O. Sections were placed in citrate buffer (0.01M, pH 6.0) at room temperature, then warmed to 99°C and boiled for 10min (to aid in unmasking antigen), before cooling for 30min. Sections were washed in TBS, and then placed in 3% hydrogen peroxide in 30% methanol for 10min at room temperature, to block endogenous peroxidase activity. Sections were washed again in H₂O, then blocked in 10% normal goat serum for

10min at room temperature. Sections were then incubated in rabbit anti-phosphopaxillin antibody (1:50 dilution) for 1h at room temperature. Sections were washed twice in TBS, then incubated with secondary antibody (goat anti-rabbit 1:400) for 35min at room temperature. After further washing in TBS, slides were incubated in 1 in 100 dilution of Avidin Biotin Complex (ABC) for 35min at room temperature. 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Peterborough, UK) was used as a substrate for peroxidase. The DAB-hydrogen peroxide mixture was prepared immediately prior to use. The sections were incubated in this for 5min, then counterstained with Haematoxylin for 1min, differentiated in 0.5% acid alcohol and then washed extensively in running water. Sections were dehydrated in alcohol, cleared with xylene and mounted in Pertex. DAB formed a brown reaction product at the site of the antigen, which if present, was visible by light microscopy. Sections were visualized with a Leitz Dialux 22EB light microscope and representative images recorded using Spot software, Diagnostic Instruments Inc., Michigan, USA.

2.11 Statistics

Unpaired *t*-tests were employed to compare samples, with a P value <0.05 considered significant. Data was analysed using Graphpad Prism and Microsoft Excel software, 2003.

3 Inflammatory signalling and gene expression in injured articular cartilage

3.1 Introduction

Our laboratory studies wounding of cartilage as a simple, reproducible, scaleable model of injury. It is essential to study injury of intact cartilage on the articular surface because its response differs from that of cartilage adapted to culture. For example, re-cutting cultured explants does not re-activate JNK. We have not attempted injurious loading, because it is technically difficult to deliver to an intact articular surface.

Although it had been shown that the three MAPK pathways were activated rapidly upon explantation, and that there was also some degradation of I κ B α , activation of IKKs had not been investigated (Gruber et al., 2004). In this chapter, I further examined activation of intracellular signalling mechanisms in cartilage by injury, and in particular IKK. I also investigated to what extent the signalling induced genes characteristic of an inflammatory response and, by the use of pharmacological inhibitors, the importance of individual pathways for expression of two such genes.

3.2 The MAPKs are rapidly activated by explantation of articular cartilage

The published experiments showing activation of p38 MAPK and JNK following explantation employed immunoprecipitation kinase assays (Gruber et al., 2004). Since that time, the laboratory has used antibodies to the phosphorylated forms of these kinases to assess their activation state by western blotting of cartilage lysates. These western blotting assays use less tissue than the kinase assays which make them preferable. In the case of phospho-JNK, only one antibody (mouse monoclonal #9255, Cell Signaling Technology, Beverly, USA) had proved successful with cartilage samples, but different batches behaved variably. An additional problem encountered with cartilage extracts was that the porcine IgG heavy chain (present in the tissue)

stained with the secondary antibody and obscured the larger JNK isoform. JNK phosphorylates the AP-1 transcription factor ATF-2. ATF-2 may also be a substrate for p38 MAPK (Morton et al., 2004). There is evidence in other tissues that there are negative regulators of ATF-2 such as Kruppel-like factor 2 (KLF-2) (Fledderus et al., 2007). ATF-2 is abundant in cartilage and can be detected with antibodies to phosphorylated ATF-2. Prior studies by members of our group using these antibodies have shown that IL-1 induction of phospho-ATF-2 was not inhibited by a p38 inhibitor or the MEK inhibitor U0126, and that FGF-2, which strongly activates ERK, did not induce phosphorylation of ATF-2 (A. Didangelos, Ph.D. thesis, 2008). This suggested that in cartilage ATF-2 may be an indicator of JNK activation. Figure 3.1 shows that when cartilage was explanted into medium and left for 10min there was induction of ATF-2 phosphorylation, compared with cartilage dissected immediately from the articular surface into lysis buffer (0min). However, if cartilage was explanted immediately into a solution of p38 MAPK inhibitor SB202190 and cultured for 10min, there was no inhibition of phospho-ATF-2 (Fig 3.1). (It appeared that phosphorylation of ATF-2 was enhanced). This experiment suggested that ATF-2 was not being phosphorylated by p38 MAPK upon cartilage dissection. Given that the phospho-ATF-2 antibody was sensitive and reliable in western blotting of porcine cartilage lysates, I have used this as an indicator of JNK activation.

To investigate how rapidly the MAPK pathways were activated by explantation, cartilage pieces (about 0.2g of tissue) were dissected from the surface of the porcine compound MCP joint into serum-free medium and cultured for the increasing times indicated prior to lysis in ice cold RIPA buffer (Fig 3.2). (Trotters were equilibrated at 37°C prior to the experiment).

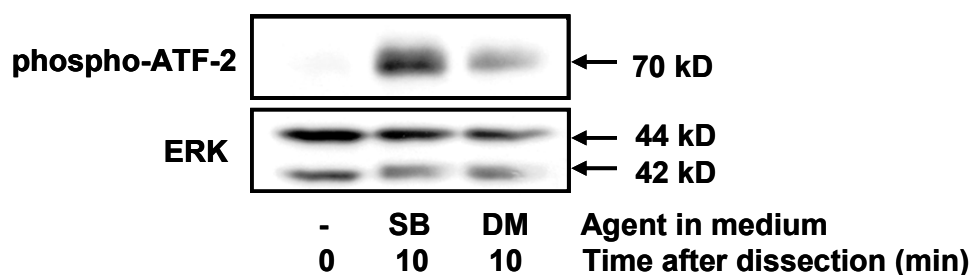


Figure 3.1. The p38 inhibitor SB202190 does not reduce the phosphorylation of ATF-2 which is caused by explantation of articular cartilage.

Trotters were equilibrated at 37°C for 4h. ~0.2g cartilage was dissected either immediately into ice cold lysis buffer (-,0), or into medium containing 10µM SB202190 (SB) and cultured for 10min, or dissected into medium containing 0.1%DMSO alone (DM) and cultured for 10min prior to lysis. Cartilage lysates were assayed for phospho-ATF-2 by western blotting. The membrane was stripped and re-probed with ERK to ensure equal cellular protein loading. This is representative of 2 experiments.

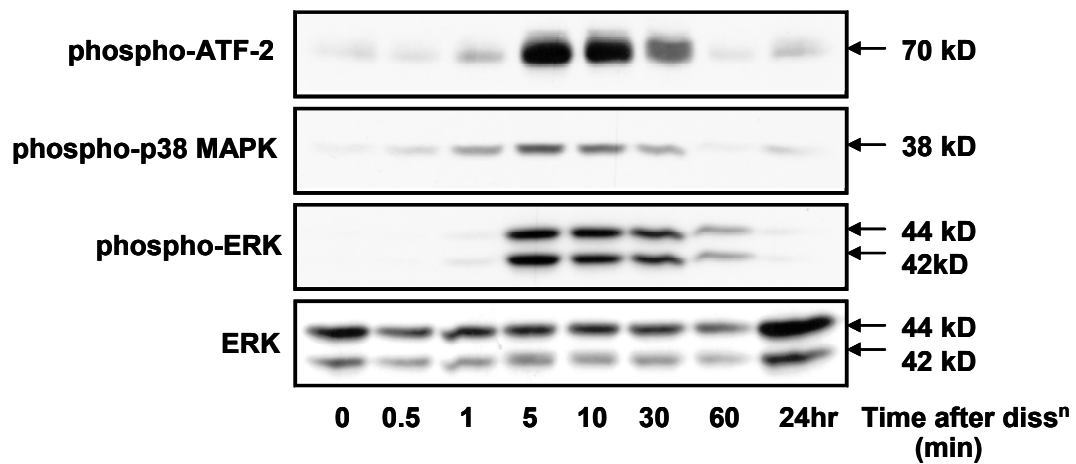


Figure 3.2. The mitogen activated protein kinases are activated following explantation of articular cartilage.

Trotters were equilibrated at 37°C prior to dissecting ~0.2g cartilage from separate joints for each point immediately into lysis buffer (0) or into serum-free medium and cultured for increasing times indicated prior to lysis. Cartilage lysates were assayed for phospho-ATF-2, phospho-p38 and phospho-ERK by western blotting. The membrane was stripped and re-probed for ERK to ensure equal cellular protein loading.

As a control, cartilage was dissected immediately from the articular surface into lysis buffer, to detect the baseline phosphorylation of kinases in the tissue (0min). Increased phosphorylation of ERK, p38 MAPK and ATF-2 could be seen within one minute of dissection (Fig 3.2). Activation of ATF-2 and p38 was maximal by 5-10min and had returned almost to baseline by 60min (Fig 3.2). This was comparable with the response of cartilage to IL-1 (not shown). The activation of ERK was more sustained, which was in-keeping with previous results (Vincent et al., 2002). To check for equal loading of cellular protein, the membrane was stripped and re-probed with an antibody to ERK 1 and 2 proteins.

3.3 *IKK activity is increased following injury to articular cartilage*

To test the possibility that the canonical NF κ B pathway was activated by cartilage explantation, an immunoprecipitation kinase assay for IKK was employed (see Materials & Methods, section 2.6.1). Initial experiments were performed on primary porcine chondrocytes. Confluent monolayers were serum starved for 2h and then stimulated with IL-1 α , a known activator of IKK, for the times indicated prior to lysis in non-dissociative kinase assay lysis buffer. The chondrocyte lysates were incubated overnight with agarose beads coated with antibody to IKK γ , and the ability of the immunoprecipitated IKK heterotrimeric complex (α,β,γ) to phosphorylate the substrate GST-I κ B α was assessed by incubation with [γ -³²P]ATP. The phosphorylation reaction was terminated by boiling the reaction mixture with SDS sample buffer prior to electrophoresis of the supernatant. Proteins were transferred on to a PVDF membrane which was exposed to film overnight, and also quantified using a phosphorimager. IKK activity in lysates from IL-1 stimulated chondrocytes was increased at 5min and reached a peak at 30-60min (Fig 3.3A).

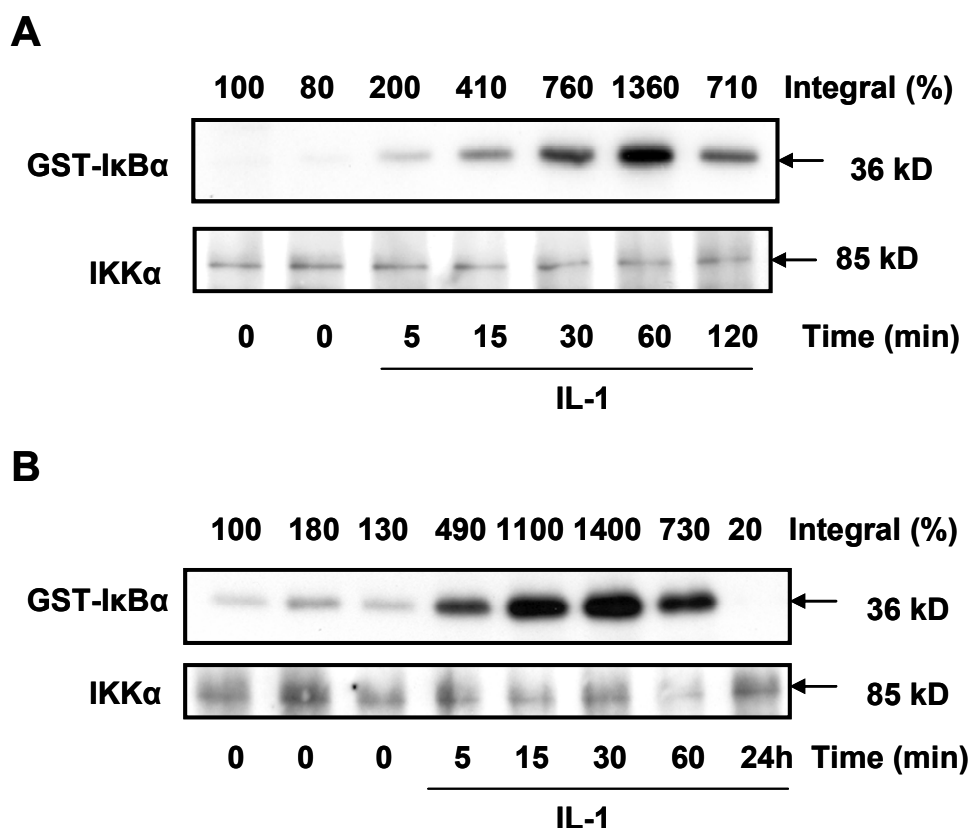


Figure 3.3. IL-1 α induces IKK activity in (A) isolated chondrocytes and (B) cartilage explants.

A. Porcine primary chondrocytes (2 million cells per point) were serum starved for 3h, then stimulated for increasing times indicated with serum-free medium containing 10ng/ml IL-1 α (IL-1) or the medium changed (0). The medium was then aspirated and 120 μ l kinase assay lysis buffer added per well. IKK was immunoprecipitated from the chondrocyte lysates and IKK activity assayed on the substrate GST-I κ B α .

B. Porcine cartilage explants were rested for 24h in serum-free medium, then stimulated with serum-free medium containing 100ng/ml IL-1 α for the times indicated. The medium was then aspirated and the explants lysed in 600 μ l kinase assay lysis buffer for 2h. IKK was immunoprecipitated from the lysates and IKK activity assayed on the substrate GST-I κ B α .

For both experiments, quantitation of phosphorylation of the substrate is shown as a % of a 0 measurement. The membrane was stripped and re-probed for IKK α to ensure equal immunoprecipitation of the IKK complex from samples.

Some baseline IKK activity at 0min was evident (cells serum starved and not stimulated by IL-1). The membranes were probed for IKK α , another member of the complex, to ensure equal loading of the lanes. A similar assay was carried out using rested cartilage explants.

Approximately 0.5g cartilage was dissected and rested in serum-free medium for 24h prior to stimulation with IL-1 α for increasing times prior to lysis and immunoprecipitation kinase assay. IL-1 stimulation caused over 10 fold increase in the ability of the precipitates to phosphorylate I κ B α , again peaking at 30min (Fig 3.3B). 24h after stimulation, IKK activity was undetectable (below that in unstimulated explants, 0min).

To assess whether there was increased IKK activity after explantation of articular cartilage, porcine trotters were equilibrated at 37°C prior to ~0.2g cartilage from each of 2 MCP joints being dissected into serum-free medium and left for increasing times prior to lysis and assay. To detect baseline activity, cartilage was dissected immediately from the articular surface into a tube on dry ice, prior to lysis. Some IKK activity was detected in these *ex vivo* samples. In response to explantation, there was increased IKK activity that was maximal at 5min (Fig 3.4). This peak of IKK activity was earlier than the peak seen following IL-1 stimulation of either cartilage explants or isolated chondrocytes (Fig 3.3A&B). In three separate experiments there was on average an 8 fold increase in IKK activity at 5 min after explantation compared with *ex vivo* cartilage (Fig 3.4B). By 30min, there was a 3 fold increase over baseline activity.

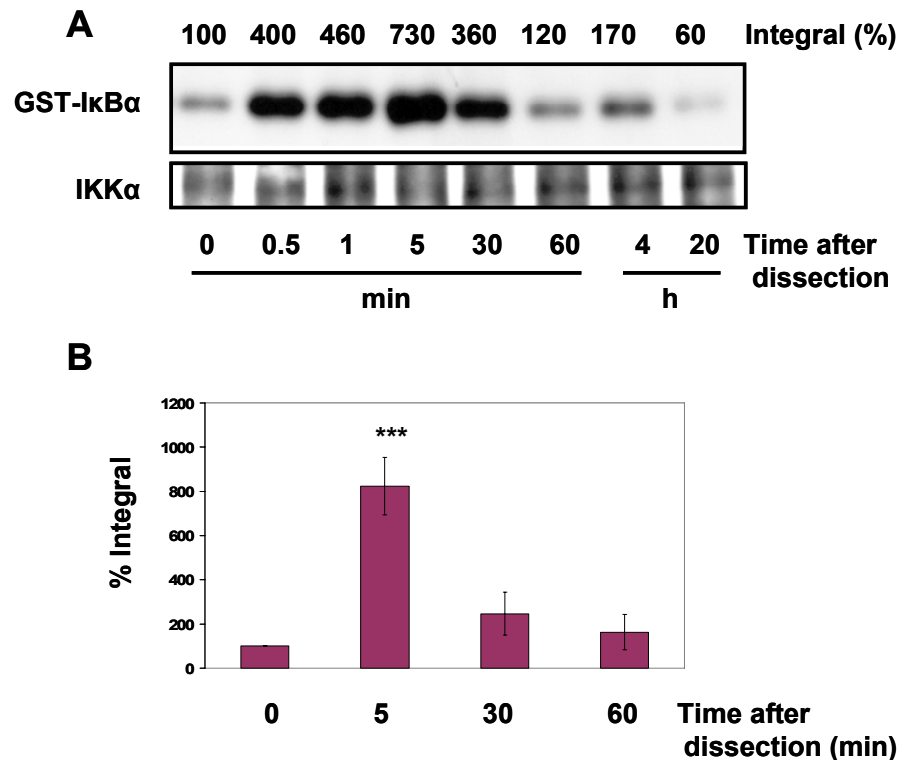


Figure 3.4. Explantation of cartilage rapidly induces IKK activity.

A. Trotters were equilibrated at 37°C prior to dissection of ~0.2g cartilage from each of two MCP joints either immediately into lysis buffer (0) or into serum-free medium and culture for increasing times indicated prior to lysis. IKK was immunoprecipitated from the cartilage lysates and IKK activity assayed on the substrate GST-IκBα. Autoradiography of the membrane is shown (upper panel). Quantitation of phosphorylation of the substrate is shown as a % of the 0 measurement. The membrane was stripped and re-probed for IKKα to ensure equal immunoprecipitation of the IKK complex from samples.

B. The results from 3 similar experiments were pooled. The measurement of IKK activity from 0 points from individual experiments was normalised to 100%, and quantitation of IKK activity of other samples expressed as a % of this. The bars show the mean % integral and the error bar shows the standard deviation of these measurements. ***=P=0.0006 vs. 0min, by unpaired t-test.

The activation of IKK in these experiments could have been due to an effect of culture medium on the cartilage. To investigate this possibility, the response of the intact articular surface to scoring was investigated. Trotters were equilibrated at 37°C prior to opening the joint and scoring with a scalpel (see Materials & Methods for details, section 2.2.2). Cartilage was dissected either immediately after scoring into lysis buffer or after 5 min. As a control, cartilage which had not been scored was dissected into medium and cultured for 5min. The lysates were assayed for IKK activity. The IKK α western blot suggested that the scored lane was somewhat overloaded compared with the control, and so quantitation of these bands was performed. Normalising for IKK α , a 4 fold increase was seen in response to scoring (Fig 3.5).

It was possible that the activation of IKK following cartilage injury was due to release of a soluble mediator, perhaps from cells upon damage, or from the matrix. If this was the case, re-cutting rested cartilage, or stimulating rested explants with conditioned medium from finely cut fresh cartilage should cause re-activation of the pathway. Cartilage explants were rested for 48h in serum-free medium prior to stimulation with IL-1, or cutting the cartilage finely, and culturing for a further 30min (the peak of IKK activity following IL-1 stimulation), or stimulating the cartilage for 30min with medium conditioned for 30min by finely chopped cartilage. I also tested the effect of FGF-2, since it is known to be released from cartilage on cutting, although it would not be expected to activate NF κ B. None of these stimuli other than IL-1 increased IKK activity in the explants (Fig 3.6).

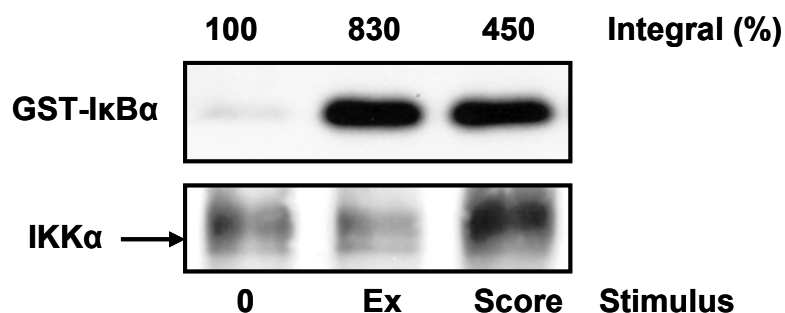


Figure 3.5. Scoring the intact articular surface activates IKK.

Trotters were equilibrated at 37°C prior to: fine scoring with a scalpel of the articular surface and immediate dissection of the cartilage immediately into lysis buffer (0), or dissection of the cartilage into serum-free medium and culture for 5min prior to lysis (Ex), or fine scoring of the articular surface and incubation at 37°C for a further 5min, prior to rapid dissection of the scored cartilage into lysis buffer (Score). IKK was immunoprecipitated from the cartilage lysates and IKK activity assayed on the substrate GST-IκBα. Autoradiography is shown. Quantitation of phosphorylation of the substrate is shown as a % of the 0 measurement, normalised for IKKα staining. The membrane was stripped and re-probed for IKKα to ensure equal immunoprecipitation of the IKK complex from samples. This is representative of 2 experiments.

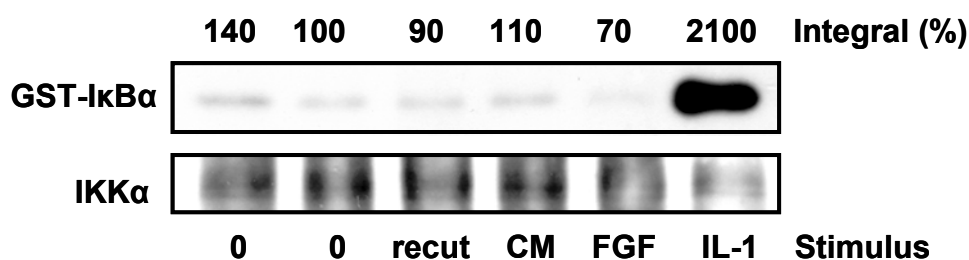


Figure 3.6. Re-cutting cartilage, treatment with conditioned medium or treatment with FGF-2 does not re-activate IKK in cartilage explants.

Cartilage explants (~0.5g per point) were rested for 48h in serum-free medium and then treated in the following ways: cut into 2 x 2mm squares and cultured for 30min (recut), incubated with medium which had been conditioned with freshly re-cut explants for 30min (CM), incubated with 50ng/ml FGF-2 for 30min (FGF) or incubated with 100ng/ml IL-1 α for 30min (IL-1) prior to lysis, or immediately placed in lysis buffer (0). IKK was immunoprecipitated from the explant lysates and IKK activity assayed on the substrate GST-I κ B α . Quantitation of phosphorylation of the substrate is shown as a % of a 0 measurement. The membrane was stripped and re-probed for IKK α to ensure equal immunoprecipitation of the IKK complex from samples. This is representative of 2 experiments.

3.4 PI3 kinase is activated by explantation of cartilage

It was possible that other signalling pathways known to be activated by growth factors or cytokines might be activated by cartilage explantation. Phosphorylation of Akt, a downstream substrate of PI3 kinase, is conventionally used as a read-out for activation of the PI3 kinase pathway. Cartilage explants were rested for 48h prior to stimulation with FGF-2 for increasing times prior to lysis, or left unstimulated. Phosphorylation of Akt was strongly induced within 10min of stimulation with FGF-2 (Fig 3.7). IL-1 α was also used as a stimulus since there is little literature on the actions of inflammatory stimuli on this pathway. IL-1 was a weaker inducer of phosphorylation of Akt than the growth factor. The membrane was stripped and re-probed with antibodies to phosphorylated ERK. ERK phosphorylation was also seen with both stimuli, and again, the activation was stronger with FGF. To check for equal loading of cellular protein, the membranes were stripped and re-probed for total ERK. The effect of explantation was then tested. Figure 3.8 shows there was strong increase of phosphorylation of Akt by 5min which was sustained for 1h, but had declined to basal level after 20h.

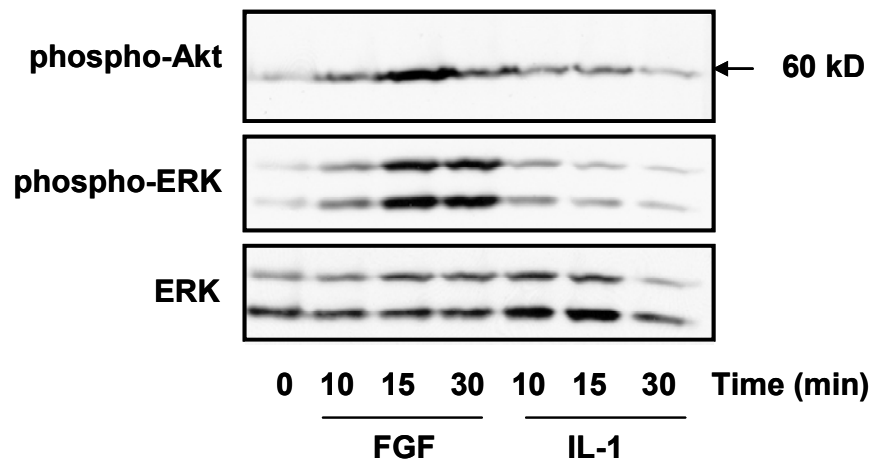


Figure 3.7. Akt is phosphorylated in response to stimulation of cartilage explants with either FGF-2 or IL-1 α .

~0.5g cartilage explants were rested in serum-containing medium for 24h, and then serum starved for a further 24h prior to immediate lysis (0) or stimulation with 250ng/ml FGF-2 (FGF) or 100ng/ml IL-1 α (IL-1) for increasing times prior to lysis. Lysates were assayed for phospho-Akt by western blotting. The membrane was stripped and re-probed for phosphorylated and total ERK.

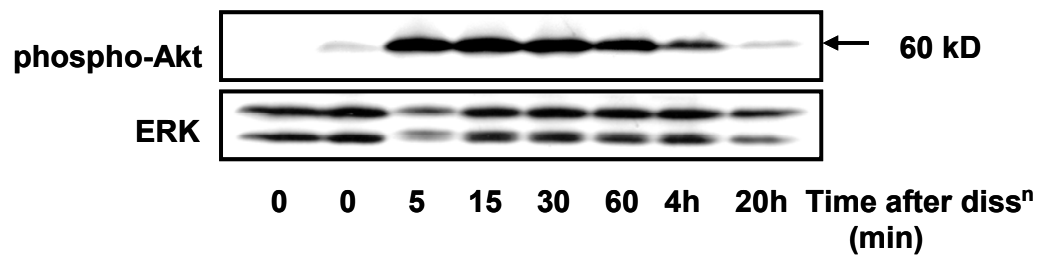


Figure 3.8. PI3 kinase is activated following explantation of cartilage.

~0.5g cartilage was dissected either directly into lysis buffer (0) or into serum-free medium and cultured for increasing times indicated prior to lysis. Cartilage lysates were assayed for phospho-Akt by western blotting. The membrane was stripped and re-probed for ERK to ensure equal cellular protein loading.

3.5 Inflammatory response gene induction in articular cartilage

It was apparent that various signalling pathways were activated by articular cartilage injury, some that were probably due at least in part to release of FGF-2 (ERK, p38 MAPK and perhaps PI3 kinase) and some possibly FGF-independent (JNK, NF κ B). I next investigated to what extent such signalling induced expression of inflammatory response genes.

A panel of 12 inflammatory response genes was selected. This included typical inflammatory response genes such as COX-2 and iNOS, cytokines (IL-1, IL-6), chemokines (IL-8) and proteinases relevant to cartilage physiology (ADAMTS-4,-5, MMP-1,-3 and -13). TIMP-1 was included as it was known to be induced by FGF stimulation of chondrocytes (Vincent et al., 2002). Activin A was included because the laboratory had previously identified it on proteomic analysis of cultured human cartilage. It is induced in cell culture by inflammatory cytokines and growth factors (Hubner and Werner, 1996). The PCR identifies the mRNA for inhibin β A, the subunit of activin A.

In order to establish the method for extracting RNA from cartilage, preliminary experiments were carried out on IL-1 stimulated tissue. GAPDH was selected as a house-keeping gene. In some experiments, GAPDH appeared to be regulated by IL-1 or FGF, particularly after prolonged stimulation e.g. 20h. Because of this, 4h or shorter time points were used where possible. Since these experiments were performed, there has been increased discussion of this issue in the literature. Alternative house-keeping genes were not tested, as GAPDH was felt to be adequate for the purpose of looking for strong regulation of genes. As expected, mRNAs of all of these genes were up-regulated in cartilage after 4h of IL-1 treatment (Fig 3.9).

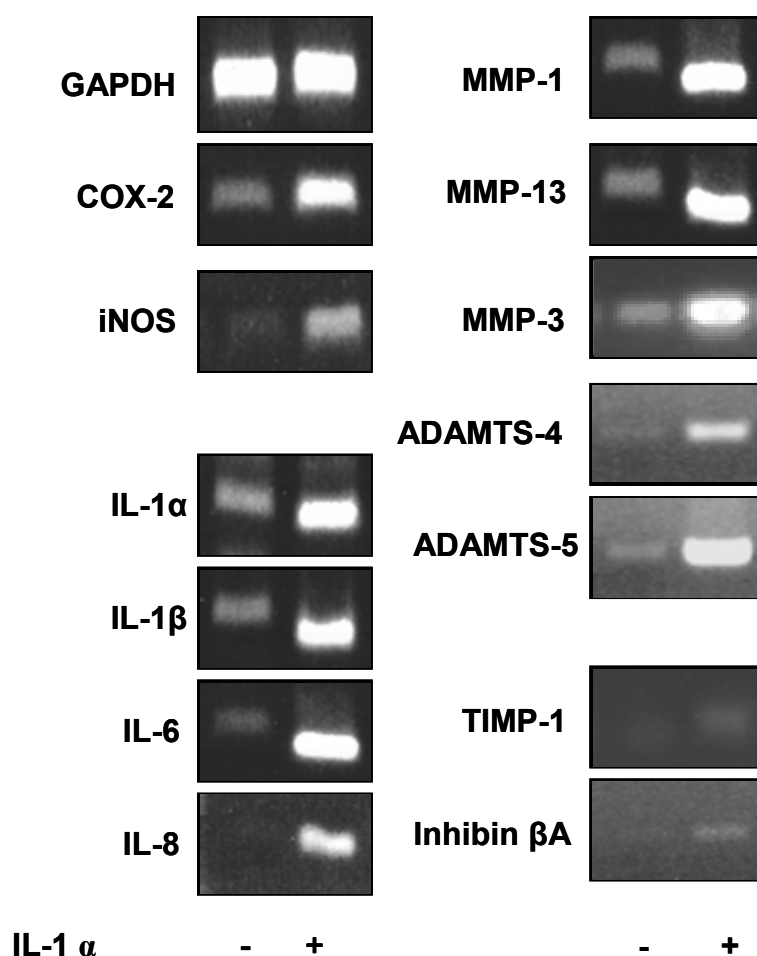


Figure 3.9. IL-1 induces inflammatory response gene mRNAs in rested cartilage explants.

Porcine articular cartilage was dissected into serum-free culture medium, washed 3 times and rested for 24h. Explants were then stimulated for 4h with culture medium alone (-) or culture medium containing 20ng/ml IL-1 α (+). Cartilage was immediately homogenised in ice cold TRI reagent prior to RNA extraction and RT-PCR for various inflammatory response genes. PCR products were electrophoresed on a 1.2% agarose gel and stained with SYBR Green. GAPDH is shown as a house-keeping control. The results shown are representative of 3 similar experiments.

Abbreviations: Glyceraldehyde 3 phosphate dehydrogenase (GAPDH); interleukin (IL); cyclooxygenase (COX)-2; matrix metalloproteinase (MMP); A Disintegrin And Metalloproteinase with ThombosPondin Motif (ADAMTS); inducible nitric oxide synthase (iNOS); Tissue Inhibitor of Metalloproteinases (TIMP)

The optimum cycle number to detect a difference between control and IL-1 stimulated explants for all PCRs was recorded. This meant that future RT-PCR for inflammatory response genes could be semi-quantitative. The SYBR Green dye used in these initial experiments caused differences in the migration of PCR products. This is a well-recognised phenomenon when pre-staining cDNA with SYBR Green. Higher DNA concentrations (over 50ng/band) cause a significant alteration of mobility (Miller et al., 1999). To prevent this, ethidium bromide was added to gels in further experiments.

3.6 Inflammatory response genes are regulated by explantation of articular cartilage

Experiments were then performed to find out which of these genes were induced by injury. Cartilage was dissected either directly into ice cold TRI reagent for RNA extraction, or cultured in serum-free culture medium for 4 or 20h. Some cartilage was cultured for 4h in medium containing IL-1 as a positive control. GAPDH again served as the house-keeping gene. 8 out of the 12 mRNAs were induced at 4h after explantation (Fig 3.10A, Expt 1). These were COX-2, IL-1 α , IL-6, the three MMPs, ADAMTS-4 and inhibin β A. Induction of these genes was stronger when the cartilage was explanted into IL-1 (n.b. the strength of the GAPDH band suggests the IL-1-treated samples may be underloaded). Some genes (iNOS, IL-8, ADAMTS-5) which were not induced at 4h by explantation alone, were induced when IL-1 was present. Additional data from a separate experiment is shown in support of some of these findings (Fig 3.10A, Expt 2).

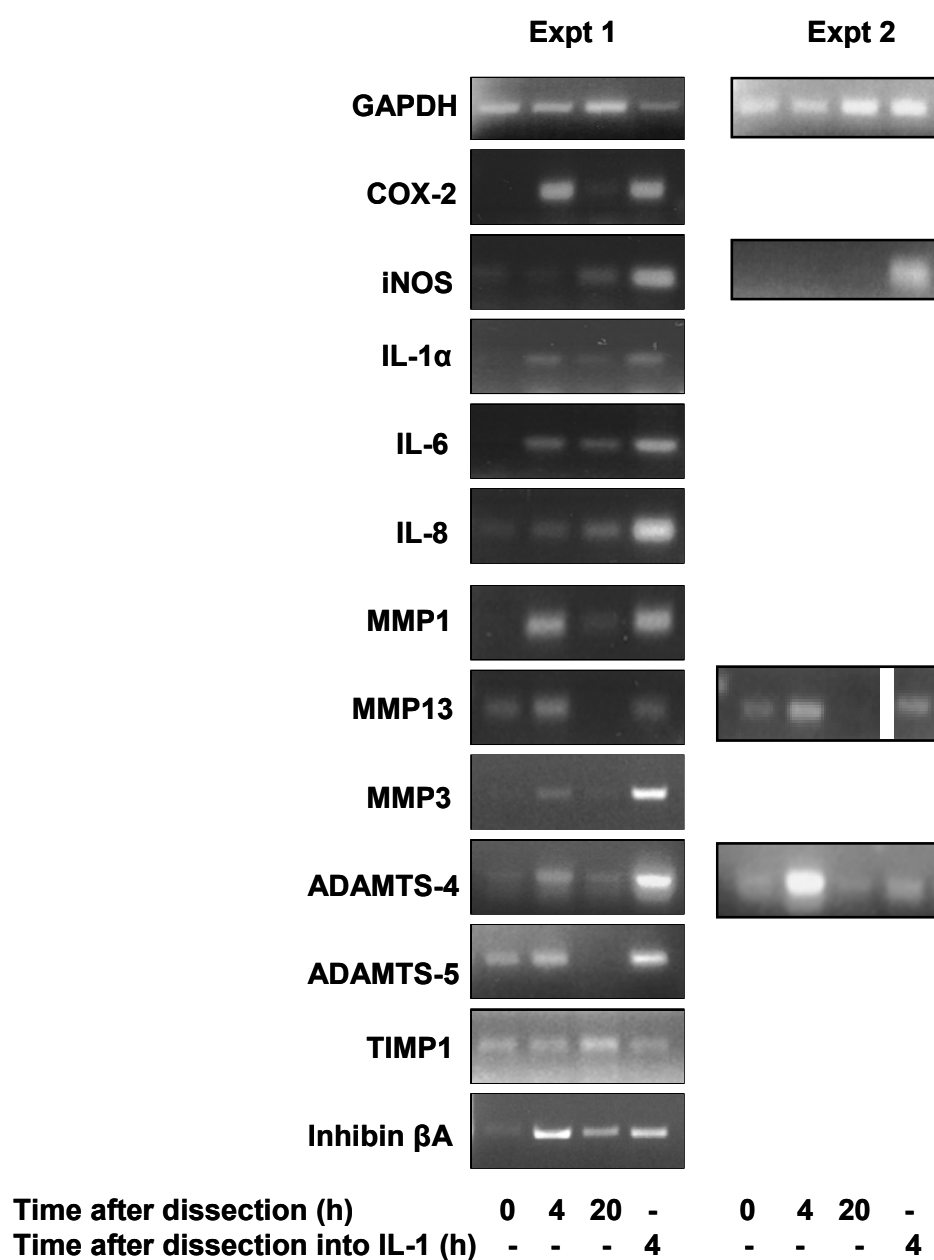


Figure 3.10A. Inflammatory response gene induction is seen 4 hours after explantation of articular cartilage.

Porcine articular cartilage was dissected directly into ice cold TRI reagent and homogenised prior to RNA extraction (0) or cultured in serum-free medium for either 4 or 20h +/- 10 ng/ml IL-1α prior to homogenisation and RNA extraction. RT-PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. GAPDH is shown as a house-keeping gene control. The results shown are representative of 4 separate experiments. Results from 2 separate experiments are shown.

It was possible that there was induction of iNOS, IL-8, ADAMTS-5 or TIMP-1 before 4h. The RNA extraction was therefore carried out at earlier times (Fig 3.10B). GAPDH in this experiment is apparently regulated by IL-1. IL-8 mRNA was not induced at any time. There was only convincing induction of COX-2, MMPs and ADAMTS-4 at 2h after explantation.

3.7 FGF-2 induces some of the inflammatory response genes seen following cartilage injury

The induction of inflammatory response mRNAs on explantation could perhaps be due in part to the release of FGF-2. Five of the mRNAs were reproducibly induced by FGF-2 stimulation of cultured explants: COX-2, MMP-1, MMP-3, TIMP-1 and inhibin β A (Fig 3.11). The induction of these mRNAs by FGF-2 was in keeping with published data (Hermansson et al., 2004; Vincent et al., 2002). These experiments do support the hypothesis that the signalling following cartilage injury is sufficient to induce a range of inflammatory response genes, including proteinases, in cartilage. To what extent the induction is due to FGF-dependent and FGF-independent signalling pathways is unclear.

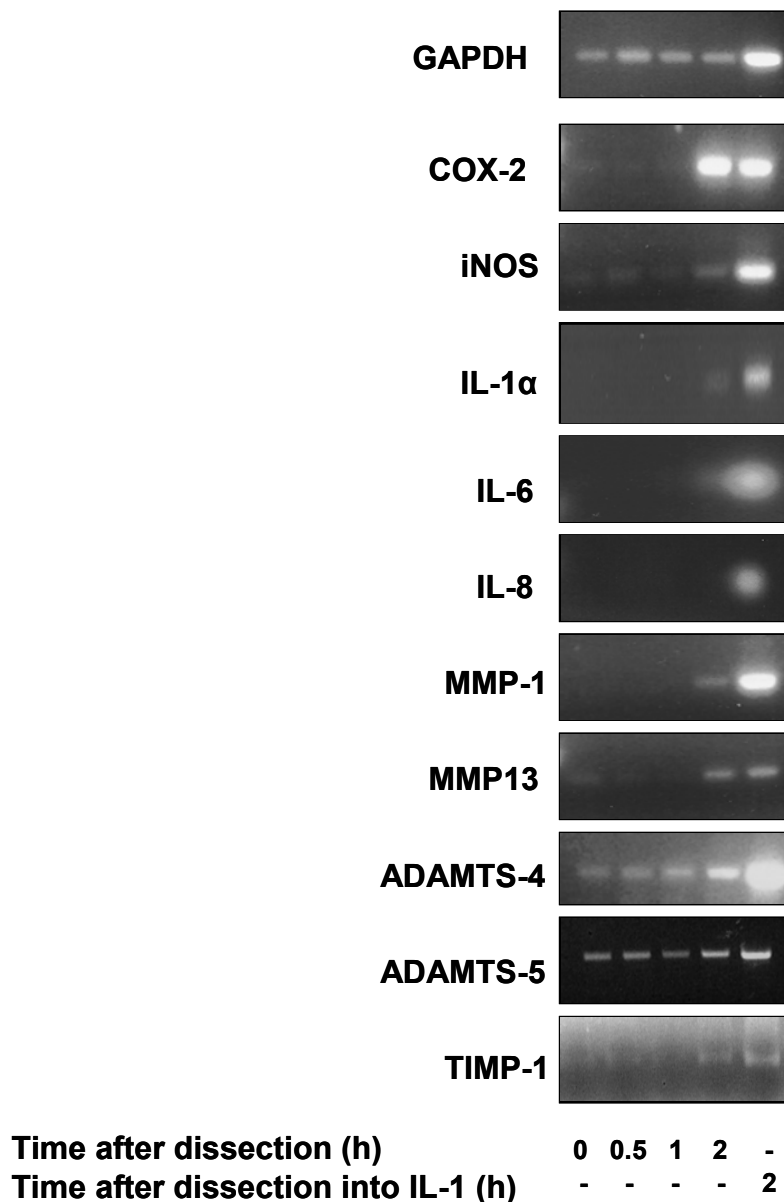


Figure 3.10B. Inflammatory response gene induction is seen 2 hours after explantation of articular cartilage.

Porcine articular cartilage was dissected directly into ice cold TRI reagent and homogenised prior to RNA extraction (0 h) or cultured in serum-free culture medium for either 0.5, 1 or 2h +/- 10ng/ml IL-1α prior to homogenisation and RNA extraction. RT-PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. GAPDH is shown as a house-keeping gene control. The results shown are representative of 2 similar experiments.

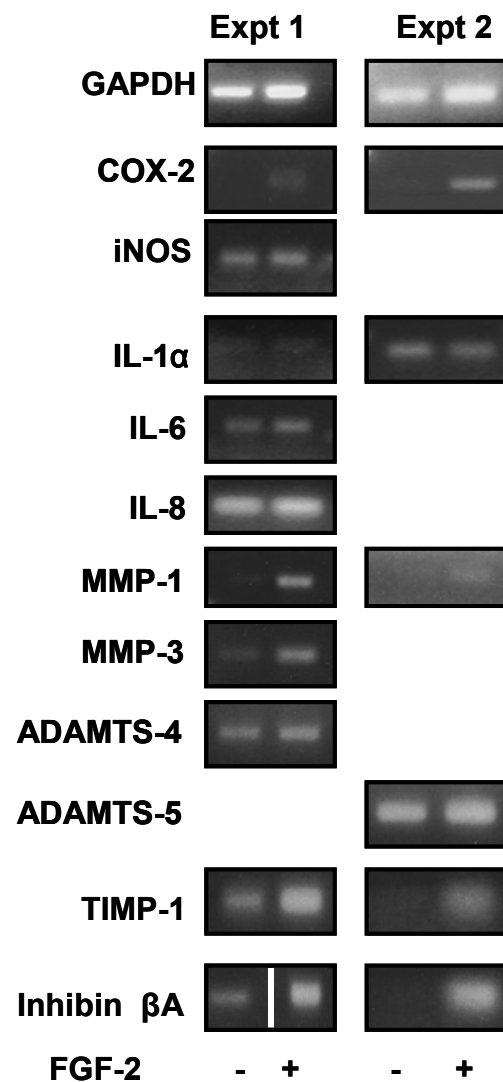


Figure 3.11. FGF-2 induces some inflammatory response mRNAs in rested cartilage explants.

Porcine articular cartilage was dissected into serum-free culture medium, washed 3 times and rested for 24h. Explants were then stimulated for 4h with culture medium alone (-), or medium containing 20ng/ml FGF-2 (+). Cartilage was immediately homogenised in ice cold TRI reagent prior to RNA extraction and RT-PCR for various inflammatory response genes. PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. GAPDH is shown as a house-keeping control. Results from 2 separate experiments are shown.

3.8 Measurement of activin A and COX-2 protein production in cartilage following injury

Explantation of cartilage was sufficient to induce mRNA of a number of inflammatory response genes. It was important to establish whether there was increased protein expression for any of these genes. There were no quantitative assays available for porcine MMPs or IL-6. I decided to study activin A since we had previously measured the protein by ELISA in cartilage cultures (S. Alexander, Ph.D. thesis, 2005). It was possible that the release of activin A by articular cartilage was partly by passive leaching from the tissue of previously synthesised protein. If this was the case, its production would not be affected by inhibition of protein synthesis, or by killing the cells. However, the production of activin A following explantation was largely prevented by either dissecting the cartilage into the protein synthesis inhibitor cycloheximide, or by freeze-thawing the cartilage before culture (Fig 3.12). Thus the production of activin A was due to synthesis during culture. This and the observation that the secretion of activin A by cartilage following injury was partially FGF-dependent, had been previously made within our group (S. Alexander, Ph.D. thesis, 2005).

COX-2 protein production was also investigated. It was seen at 4h following explantation by western blotting cartilage lysates. Its expression was strong at 8 and 16h, and was maintained at 24h (Fig 3.13).

The signalling following explantation was therefore sufficient to induce expression of inflammatory response genes at the level of both mRNA and protein. I wanted to use pharmacological inhibitors to find out which signalling pathways mediated the expression of particular genes. To do this, I needed to study a strongly regulated gene whose protein could be measured accurately. Activin A fulfilled these criteria.

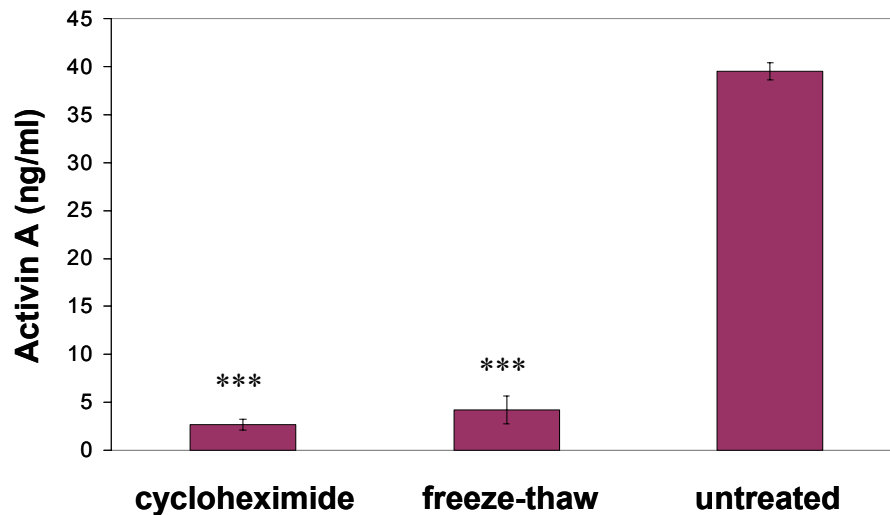


Figure 3.12. Secretion of activin A by dissected cartilage is an active cellular process.

Porcine cartilage was dissected and cultured for 16h in 1g/ml serum-free culture medium. Some explants were cultured with 10 μ g/ml cycloheximide, some were frozen and thawed 3 times before culture, and some were left untreated. Activin A in the medium was measured by ELISA. Bars show the mean $\pm$ S.D. of triplicate cultures. ***=P<0.0001 vs. untreated cultures, by unpaired *t*-test.

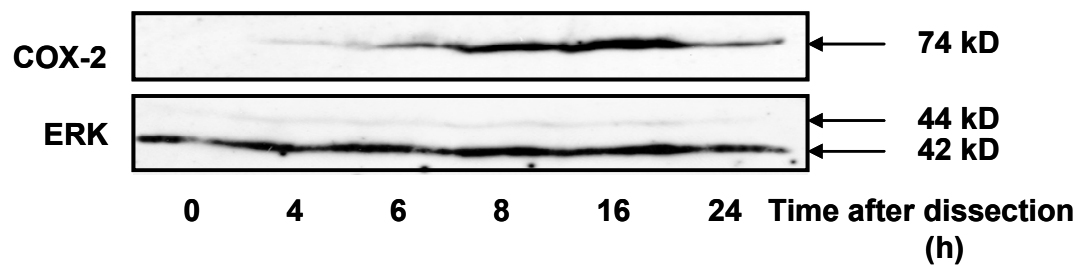


Figure 3.13. COX-2 protein is seen 4 hours after dissection of cartilage from the articular surface.

Porcine articular cartilage was either dissected immediately into ice cold lysis buffer (0) or into serum-free medium and cultured for the times specified (h) prior to lysis. Cartilage lysates were assayed for COX-2 by western blotting. The blot was stripped and re-probed for ERK to ensure equal loading of cellular protein. This is representative of 3 similar experiments.

3.9 Testing the efficacy of pharmacological inhibitors of signalling pathways

Pharmacological inhibitors of signalling pathways were selected, many of which are well-characterised and have been widely used (see Materials & Methods, Table 2.3). However, the inhibitors of JNK and IKK were less well-characterised and were first tested in cell-based assays.

3.9.1 JNK inhibitors

The anthrapyrazolone compound SP600125 is reported to be a selective JNK inhibitor (Bennett et al., 2001). Cultured porcine chondrocytes were incubated with increasing concentrations of this inhibitor, and then stimulated with IL-1 α . The cell lysates were assayed for phospho-ATF-2 by western blotting. As previously discussed, phosphorylation of ATF-2 is an indicator of JNK activity in this system (Fig 3.1). The vehicle DMSO, when added to the culture medium did not cause phosphorylation of ATF-2. Increasing concentrations of SP600125 from 1-100 μ M did not inhibit IL-1 induced phosphorylation of ATF-2 in chondrocytes, and even appeared to enhance phosphorylation (Fig 3.14A).

L-JNKI is a cell permeable peptide which is reported to inhibit the phosphorylation of c-jun by JNK in neuronal cells (Borsello et al., 2003). This compound was tested and also failed to inhibit IL-1-induced ATF-2 phosphorylation in chondrocytes (Fig 3.14B). It was decided to abandon use of putative JNK inhibitors, since no validated convincing inhibitor for use in cell experiments is available.

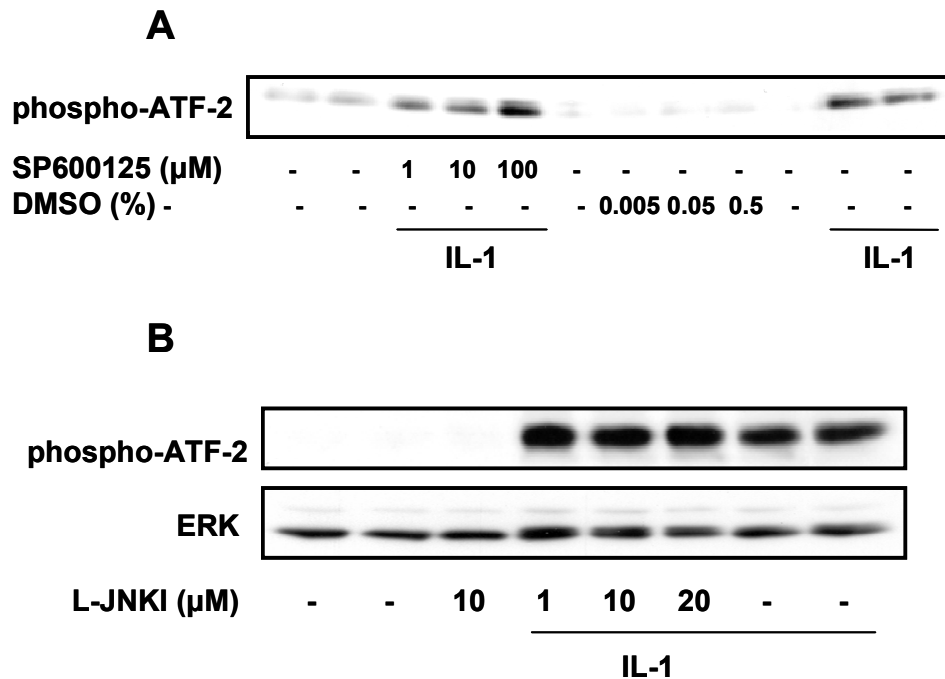


Figure 3.14. The JNK inhibitors (A) SP600125 and (B) L-JNKI are not effective when tested on IL-1 stimulated porcine primary chondrocytes.

For both **A.** & **B.**, porcine primary chondrocytes (2 million cells per well) were serum starved for 3h, then pre-incubated for 30min with medium alone (-) or medium containing increasing concentrations of either **A.** SP600125 or **B.** L-JNKI, or equivalent concentrations of the vehicle DMSO. Chondrocytes were then stimulated for 20min with serum-free medium alone (first 2 lanes), medium +/- fresh inhibitor plus IL-1α 10ng/ml (IL-1) or medium +/- fresh DMSO. The medium was then aspirated and 250μl dissociative lysis buffer added per well. Chondrocyte lysates were assayed for phosphorylated ATF-2 by western blotting. For **B.**, the membrane was stripped and re-probed for ERK to ensure equal loading.

Both **A.** and **B.** are each representative of 2 experiments.

3.9.2 NF κ B and PI3 kinase inhibitors

BMS345541 (4-(2'-aminoethyl)amino-1,8-dimethylimidazo[1,2-a]quinoxaline) is an inhibitor of IKK (Burke et al., 2003). Its effects were tested on primary porcine chondrocytes stimulated with 20ng/ml IL-1, and the lysates were western blotted for I κ B α . IL-1 caused degradation of I κ B α as predicted (Fig 3.15A). This degradation was partially inhibited by 10 μ M BMS345541. The membrane was stripped and re-probed with ERK to ensure equivalent cellular protein loading. Quantitation of the western indicated a 30% reduction in IL-1-induced I κ B α degradation in response to 10 μ M of this inhibitor. A similar effect was seen in a separate experiment. Whether this effect of BMS345541 is sufficient to affect subsequent translocation and activation of NF κ B was not assessed. An alternative inhibitor of NF κ B signalling is MG132. This small peptide aldehyde proteasome inhibitor prevents proteasomal degradation of phosphorylated, ubiquitinated I κ B α and thus inhibits NF κ B activation (Jiang et al., 2004). It has been widely used for this purpose, and was therefore employed in addition to BMS345541 in subsequent experiments.

LY294002 is a widely used inhibitor of PI3 kinase. Its effectiveness at inhibiting phosphorylation of Akt was tested in chondrocytes. LY294002 prevented FGF-induced phosphorylation of Akt in chondrocytes at 1 and 10 μ M (Fig 3.15B).

3.9.3 Inhibitors of MEK and of the FGF receptor tyrosine kinase

U0126 is an inhibitor of activation of MEK, the kinase kinase of the ERK pathway, and is well-characterised and specific for this kinase when tested *in vitro* (Davies et al., 2000).

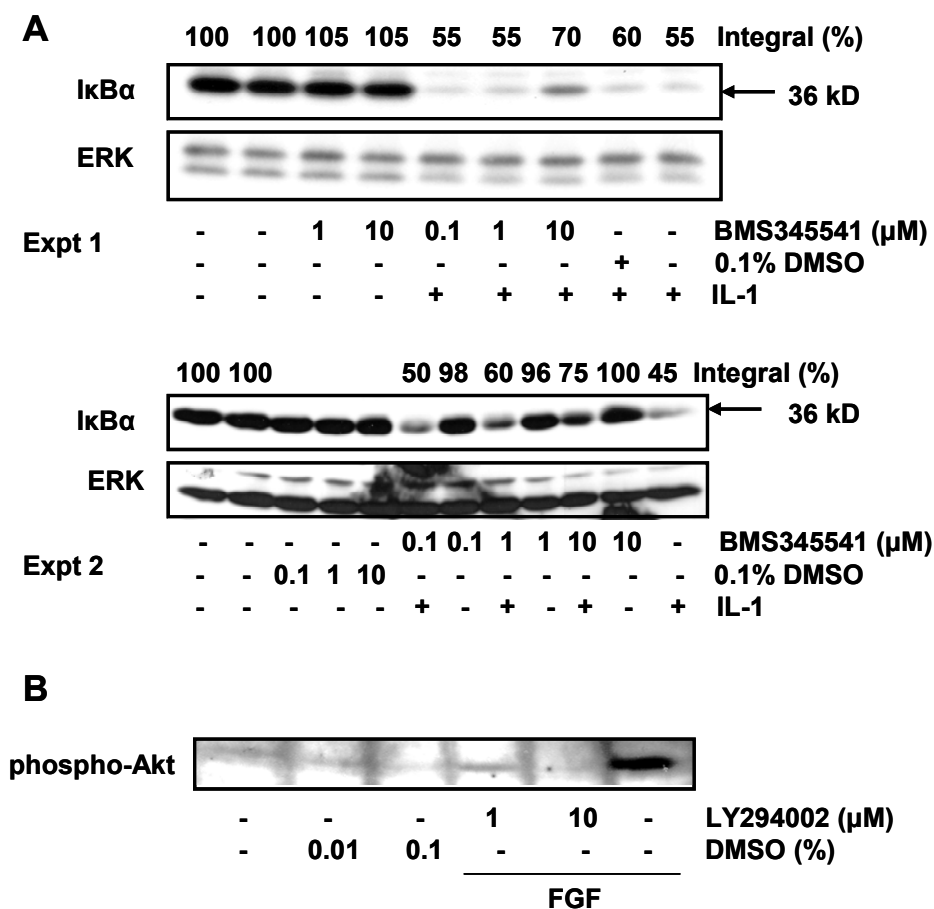


Figure 3.15. Testing inhibitors of (A) IKK and (B) PI3 kinase pathway on primary chondrocytes.

A. Porcine primary chondrocytes (2 million cells per point) were serum starved for 3h, then pre-incubated for 30min with medium alone (-) or medium containing increasing concentrations of the diluent DMSO or the IKK inhibitor BMS345541. Chondrocytes were then stimulated for 20min with serum-free medium alone, the inhibitor alone, or medium +/- DMSO or fresh inhibitor containing 20ng/ml IL-1 α . The medium was then aspirated and 250 μ l lysis buffer added per well. Chondrocyte lysates were assayed for I κ B α by western blotting. 2 different experiments are shown. Quantitation of the blots are shown as a % of a 0 measurement. The membranes were stripped and re-probed for ERK.

B. Porcine primary chondrocytes (2 million cells per point) were serum starved for 3h, then pre-incubated for 30min with medium alone (-) or medium containing the vehicle DMSO, or medium containing the PI3 kinase inhibitor LY294002 1 μ M or LY294002 10 μ M. Chondrocytes were then stimulated for 30min with serum-free medium alone +/- DMSO (lanes 1-3), or medium +/- fresh inhibitor plus FGF-2 20ng/ml (FGF). The medium was then aspirated and 250 μ l dissociative lysis buffer added per well. Chondrocyte lysates were assayed for phosphorylated Akt by western blotting.

PD173074 (also known as SB402451) is in the pyrido[2,3-d]pyrimidine class of tyrosine kinase inhibitors (Hamby et al., 1997). It is an ATP-competitive inhibitor of FGF receptor 1, and also has a weaker effect on the vascular endothelial growth factor receptor. It poorly inhibits other tyrosine kinases such as srcs, and receptors of IGF, EGF and PDGF, and other protein kinases such as protein kinase C and MEK (Mohammadi et al., 1998). It was initially obtained from GlaxoSmithKline, who had shown that it inhibited FGF induced ERK activation with an IC_{50} of about 10nM (Skaper et al., 2000). Both inhibitors had been tested previously by our group on porcine chondrocytes. PD173074 inhibited FGF-2 induced ERK activation in chondrocytes. It also inhibited PDGF induced ERK activation at a similar IC_{50} , but did not inhibit activation of ERK by IGF-1 (T. Vincent, Ph.D. thesis, 2002). PD173074 at a concentration of 50nM was also shown to prevent the re-activation of ERK by re-cutting of cartilage explants (Vincent et al., 2002). Effectiveness of PD173074 and U0126 at inhibiting ERK activation following cartilage explantation is shown later in Figure 3.18.

3.10 Activin A production in chondrocyte and explant culture

I wanted to investigate firstly, which of the FGF-induced pathways was involved in the expression of activin A following explantation, and secondly, whether inflammatory signalling was also involved (see Ch 1, Figs 1.1 & 1.2).

Cultured primary chondrocyte monolayers should provide the simplest way to test the effects of signalling pathway inhibitors. Chondrocytes were therefore isolated and rested for 48h, prior to serum starvation and stimulation with FGF-2 for 18h. I also tested the effects of TGF β and IL-1 α for purposes of comparison. Activin A levels were measured by ELISA in the medium. FGF-2 stimulation of chondrocytes caused

only a doubling of activin A production compared with control (Fig 3.16A). This was far less marked than the response to IL-1 α , or the response to explantation (Fig 3.12).

It was possible that cartilage explants would behave differently to cultured chondrocytes. Rested cartilage explants were stimulated with FGF-2, TGF β or IL-1 α , or left untreated. Untreated cartilage explants produced moderate amounts of activin A and none of the agents significantly increased this (Fig 3.16B). The rested explant cultures responded differently to IL-1 compared with chondrocyte monolayers with respect to activin A production, despite the IKK response to IL-1 in chondrocytes and explants being similar (Fig 3.3). This was surprising and perhaps suggested that IL-1 might activate different pathways in chondrocytes and explants, and that more than one pathway may be required for the response. Re-cutting is known to cause FGF-2 release and ERK activation. Re-cutting of rested explants caused an increase in activin A production (Fig 3.17). However, the magnitude of the response was not as great as that seen in freshly dissected tissue. This may be related to the fact that the signalling response of cultured cartilage and the intact articular surface differ: JNK is known to be refractory to re-cutting, for example (A. Didangelos, Ph.D. thesis, 2008) and I have also shown that IKK is not re-activated by re-cutting (Fig 3.6). Given that I could not reproduce the strength of induction of activin A caused by injury using defined stimuli or re-cutting in culture, I returned to study the effects of pharmacological inhibitors on the production of activin A following explantation, as in Figure 3.12.

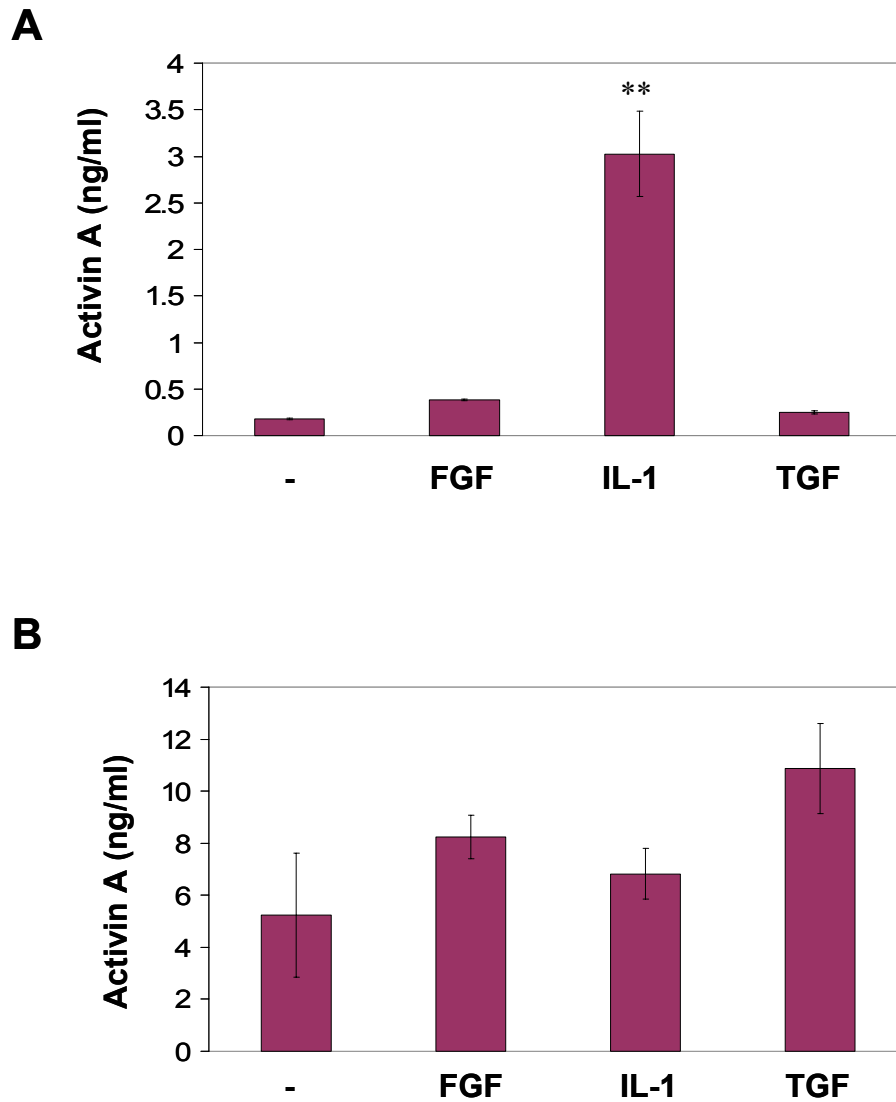


Figure 3.16. Secretion of activin A by (A) cultured primary chondrocytes and (B) cartilage explants in response to FGF-2, IL-1 α and TGF β .

A. Porcine primary chondrocytes (2 million cells per point) were serum starved for 5h and then stimulated with serum-free medium containing 20ng/ml FGF-2, 10ng/ml IL-1 α or 20ng/ml TGF β , or medium alone (-) for 18h. The medium was then aspirated, clarified and assayed for activin A by ELISA.

B. ~0.5g porcine cartilage explants were rested in serum-containing medium for 24h, and then serum starved for a further 24h. Explants were then stimulated with 1ml serum-free medium/g tissue containing FGF-2 250ng/ml, IL-1 α 100ng/ml or TGF β 100ng/ml, or medium alone (-) for 16h. The medium was then aspirated, clarified and assayed for activin A by ELISA.

Bars show mean $\pm$ S.D. from triplicate cultures. **= $P=0.0004$ vs. untreated chondrocytes (-), by unpaired *t*-test. Both **A.** and **B.** are representative of 2 experiments.

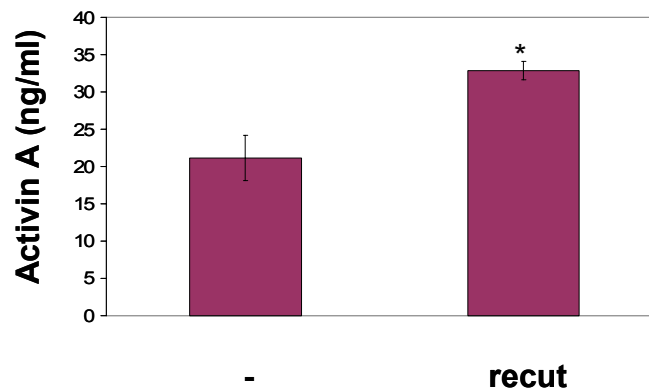


Figure 3.17. Activin A release following the re-cutting of cartilage explants.

~0.5g cartilage explants were rested for 24h in serum-free culture medium prior to re-cutting the cartilage into 2 x 2mm pieces (recut), or leaving the cartilage untreated (-). Cartilage was then cultured for 17h prior to clarification of medium and assay for activin A by ELISA. Bars show mean $\pm$ S.D. of triplicate cultures. $*=P<0.04$ vs. untreated cultures, by unpaired *t*-test. These results are representative of 2 separate experiments.

Given that activation of inflammatory signalling caused by explantation is very rapid, it was suspected that to achieve maximal inhibition of signalling with pharmacologic agents in tissues it would be better, rather than simply dissecting the cartilage into a solution containing the inhibitor, also to inject the inhibitor into the joint, so that it could diffuse through the cartilage before injury occurred. To test the effectiveness of PD173074 and U0126 in inhibiting the activation of ERK following explantation, the inhibitors were injected into porcine MCP joints. After 2h the joints were opened and articular cartilage dissected either immediately into lysis buffer, or into medium with, or without, the inhibitor and cultured for 30min prior to lysis. The lysates were examined for phosphorylated ERK by western blotting. The membrane was stripped and re-probed for total ERK. As predicted, ERK was strongly activated by explantation (Fig 3.18). This activation was markedly reduced by either the FGF receptor inhibitor or the MEK inhibitor. There was some residual ERK activation remaining in the presence of the FGF receptor inhibitor and this was reproducible (Fig 3.18, left hand panel). This could have been because the inhibitor was not fully effective, or that there were FGF-independent signalling pathways also activating ERK.

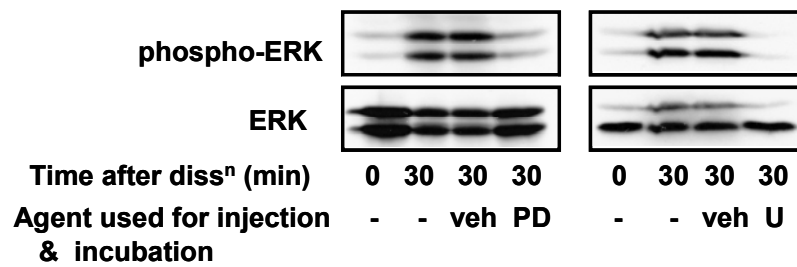


Figure 3.18. Efficient blockade of explantation-induced ERK activation by intra-articular injection of inhibitors.

Porcine MCP joints were initially injected with 3ml serum-free culture medium containing vehicle (0.1% DMSO) (veh), 250nM FGF receptor inhibitor PD173074 (PD, left hand panel), 20μM MEK inhibitor U0126 (U, right hand panel), or left untreated (-). Following equilibration at 37°C for 2h, ~0.5g cartilage was dissected either immediately into ice cold lysis buffer (0) or cultured for 30min in serum-free medium +/- respective inhibitor/vehicle prior to lysis. Phosphorylated ERK was assayed by western blotting. The membrane was stripped and re-probed for ERK to ensure equal loading. These results are representative of 2 similar experiments.

3.11 The production of activin A on explantation is FGF- and ERK-dependent

Partial inhibition of activin A production after explantation had been seen previously when the cartilage was dissected into medium containing PD173074 (S. Alexander, Ph.D. thesis, 2005). It was possible that the inhibition was incomplete because the inhibitor did not have adequate time to diffuse into the cartilage to prevent rapid cell activation by FGF. In view of the strong inhibition of ERK activation achieved by injection of the inhibitor in the previous section, the experiment was carried out using this procedure. Prior injection gave significantly better inhibition of activin A production (around 80%), compared with dissection of cartilage directly into inhibitor without prior injection (Fig 3.19). In contrast, injection of medium alone into the joint (the vehicle) did not significantly perturb the production of activin A by the cartilage. Therefore, in all further experiments, intact porcine MCP joints were first injected with medium containing the inhibitors prior to dissection into medium containing the inhibitor.

To test whether MAPKs were necessary for activin A production after explantation, porcine trotters were injected with the FGF receptor inhibitor PD173074, the MEK inhibitor U0126 or the p38 MAPK inhibitor SB202190 and equilibrated at 37°C, prior to dissection and culture in medium containing the respective inhibitors. As controls, cycloheximide or 0.1% DMSO (the vehicle) were injected. Activin A was measured in the medium after 16h (the time of its maximal production following injury).

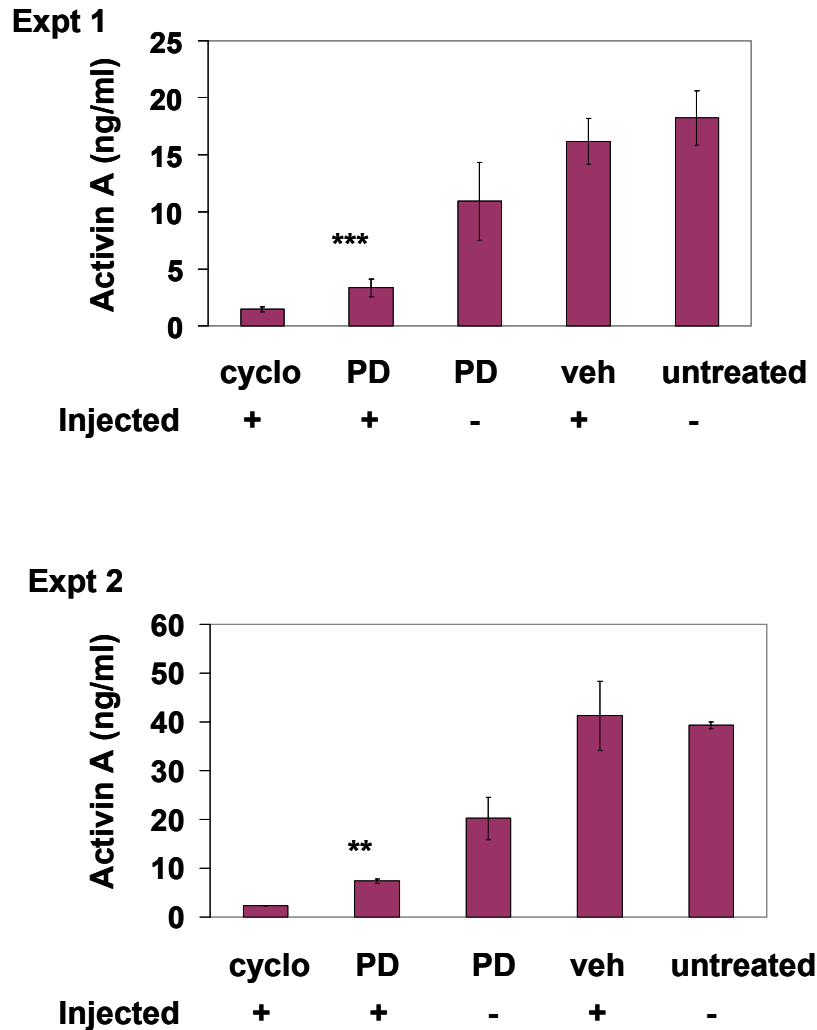


Figure 3.19. The effect on activin A production by cartilage of injection of pharmacological inhibitors into joints prior to explantation.

Intact porcine MCP joints were injected (+) with 3ml serum-free medium (veh) or medium containing 10µg/ml cycloheximide (cyclo), the FGF receptor inhibitor PD173074 250nM (PD) or not injected (-) and equilibrated at 37°C for 2h. ~0.5g cartilage per point was then explanted into 2ml medium/g tissue containing fresh inhibitor (cycloheximide, PD) or medium alone (veh, untreated) and cultured for 16h prior to collection of the medium. This was clarified by centrifugation and assayed for activin A by ELISA. % activin A production was calculated for each sample, compared with untreated cartilage. 2 different experiments are shown. Bars show mean +/- S.D. from 4 cultures in Expt 1 and 3 cultures in Expt 2.

Expt 1: ***=P<0.0001 vs. untreated and P=0.005 vs. PD (not injected), by unpaired t-test. **Expt 2:** **=P<0.0001 vs. untreated and P=0.018 vs. PD (not injected), by unpaired t-test

Injection of the joints with 0.1% DMSO in medium did not affect the production of activin A following explantation. (After this finding was replicated on 3 occasions, this control was not included in further experiments) (Fig 3.20A). PD173074 inhibited activin A production on explantation. In 4 similar experiments, the mean inhibition with PD173074 was 82% (Table 3.1). Some variation in efficacy was seen in initial experiments because an old batch of inhibitor had been used. The partial inhibition by the FGFR inhibitor suggested that FGF-independent signalling pathways might also be involved in the induction of activin A on explantation. The p38 MAPK inhibitor SB202190 did not significantly inhibit activin A secretion after explantation, whereas the MEK inhibitor U0126 (20 μ M) did cause inhibition (Fig 3.20A). U0126 was also effective at a lower concentration of 10 μ M (Fig 3.20B). This suggested that the ERK pathway was required for the production of activin A following injury. This was likely to be downstream of FGF-2. Although the experiment shown in Figure 3.20A suggests that the effects of U0126 are comparable to PD173074, the mean inhibition over 6 experiments was lower at 51% (Table 3.1).

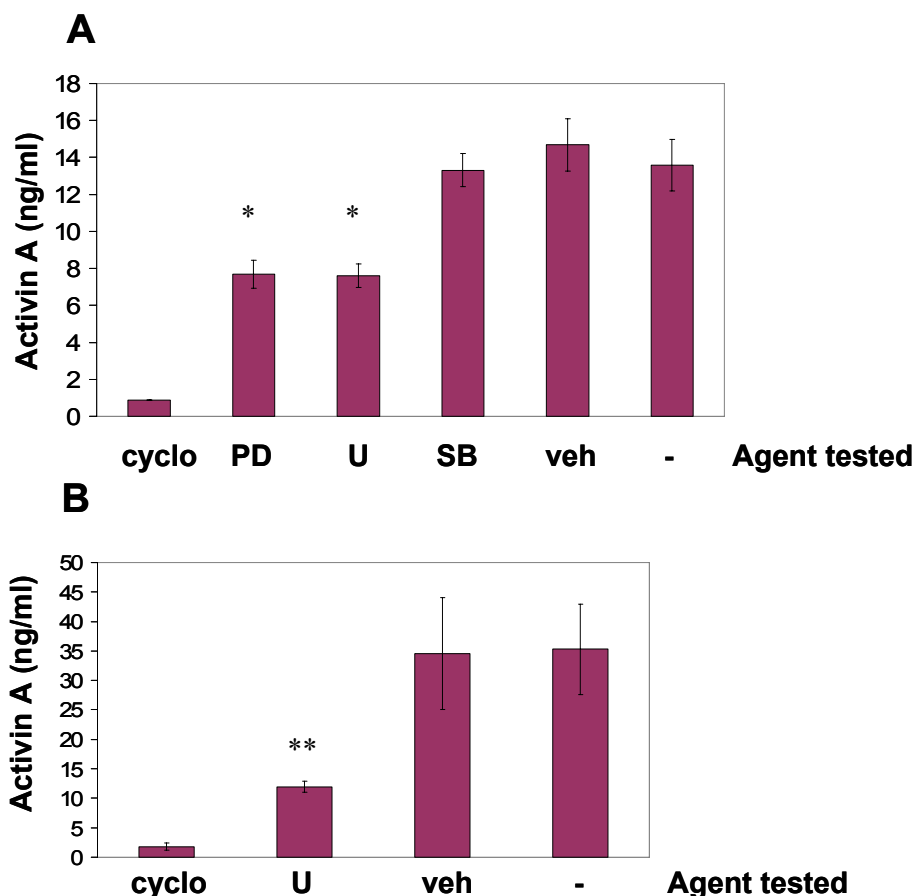


Figure 3.20. ERK inhibition and FGF receptor inhibition both reduce activin A production following explantation. (A) & (B) show 2 independent experiments.

A. Intact porcine MCP joints were injected with 3ml of serum-free medium containing cycloheximide 10µg/ml (cyclo), the FGF receptor inhibitor PD173074 250nM (PD), the MEK inhibitor U0126 20µM (U), the p38 inhibitor SB202190 20µM (SB), the vehicle (medium + 0.2% DMSO) (veh) or were not injected (-) and equilibrated at 37°C for 2h. ~0.5g cartilage per point was then explanted into 2ml medium/g tissue containing fresh inhibitors, vehicle, or medium alone (-) and cultured for 16h prior to collection of the medium. This was clarified by centrifugation and assayed for activin A by ELISA.

B. Intact porcine MCP joints were injected with 3ml serum-free medium plus cycloheximide 10µg/ml (cyclo), the MEK inhibitor U0126 10µM (U), the vehicle medium + 0.1% DMSO (veh) or not injected (-) and equilibrated at 37°C for 2h. Cartilage was then explanted into 2ml medium/g tissue +/- fresh inhibitors and cultured for 16h prior to collection of medium. This was clarified by centrifugation and assayed for activin A by ELISA.

Bars show mean +/- S.D. from triplicate cultures. *=P=0.03 & **=P=0.006, vs. untreated cultures (-), by unpaired t-test.

Table 3.1. Effects of signalling inhibitors on activin A production after explantation.

Inhibitor	Target pathway	Concentration	n	% inhibition, mean (range)	S.E.M
PD173074	FGFR	250nM	4	82 (79-84)	1.44
U0126	MEK(ERK)	10µM	6	51 (34-66)	5.71
MG132	NFκB (proteasome)	10µM	7	49 (28-70)	5.10
BMS345541	NFκB (IKK)	10µM	4	71 (63-78)	3.58
PP2	Src family kinases	10µM	3	60 (52-66)	4.22

For each experiment with a particular inhibitor, a % inhibition of activin A production compared with untreated samples was calculated. The mean % inhibition, range of % inhibition and standard error of the mean (S.E.M) for a number of experiments (n) is shown.

The inhibition of activin A by U0126 or PD173074 was never to baseline levels, despite these inhibitors effectively preventing ERK activation following explantation (Fig 3.18). This suggested that additional signalling pathways activated by injury also contributed to the regulation of expression of the cytokine. One candidate was NFκB, so I therefore tested the effects of inhibitors of this pathway.

3.12 NFκB is also required for activin A production after explantation of cartilage

The effects of the proteasome inhibitor MG132 and the IKK inhibitor BMS345541 on activin A production following cartilage injury were investigated. Porcine MCP joints were injected with inhibitors and equilibrated, prior to dissection and culture for 16h in medium with and without inhibitor. MG132 inhibited activin A production (Fig 3.21A). This effect could have been due to NFκB inhibition or non-specific effects: blocking the proteasome must affect many cellular processes. However, BMS345541 also inhibited activin A production to a similar degree (Fig 3.21A). The extent of inhibition was similar to that seen with PD173074 (Table 3.1).

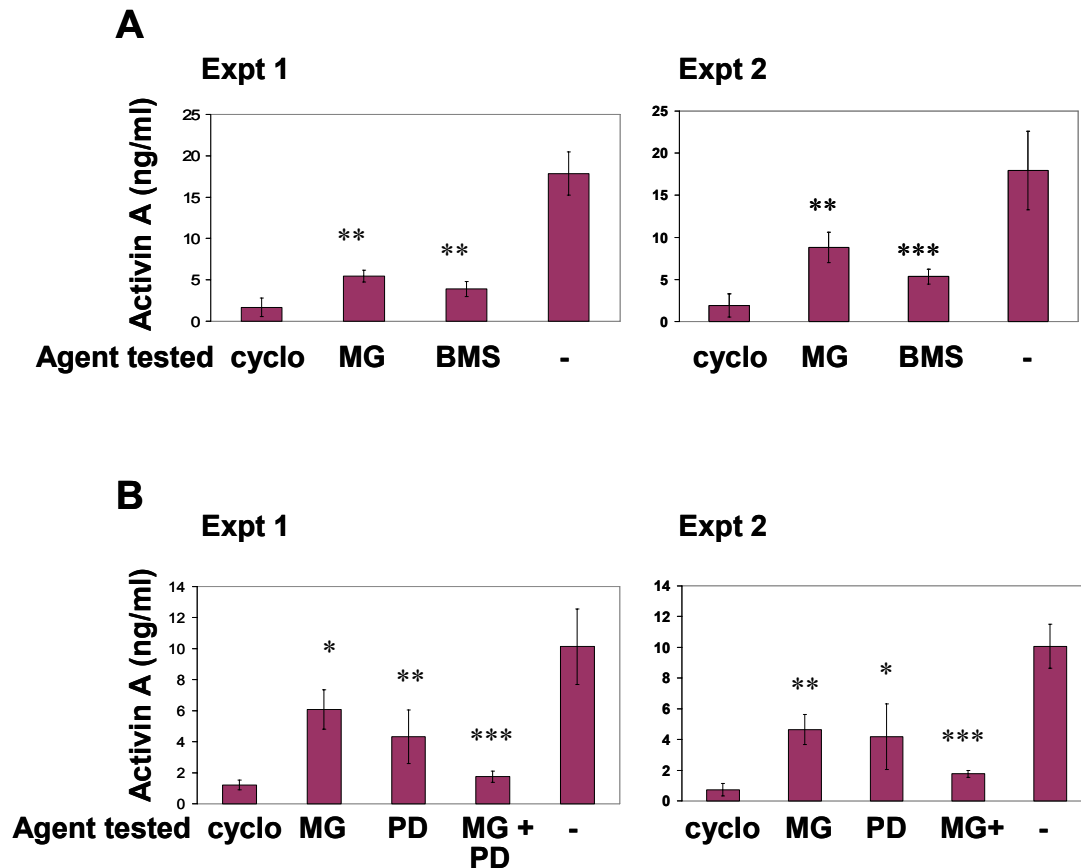


Figure 3.21. NF κ B pathway inhibitors reduce activin A production on explantation.

A. Intact porcine MCP joints were injected with 3ml serum-free medium plus 10 μ g/ml cycloheximide (cyclo), the proteasome inhibitor MG132 (MG) 10 μ M, the IKK inhibitor BMS345541 (BMS) 20 μ M or not injected (-) and equilibrated at 37°C for 2h.

B. Intact porcine MCP joints were injected with 3ml serum-free medium plus 10 μ g/ml cycloheximide (cyclo), the proteasome inhibitor MG132 (MG) 10 μ M, the FGF receptor inhibitor PD173074 (PD) 250nM, a combination of both inhibitors (MG+PD) or not injected (-) and equilibrated at 37°C for 2h.

In both **A.** and **B.** ~0.5g cartilage per point was then explanted into 2ml medium/g tissue containing fresh inhibitors or medium alone (-) and cultured for 16h prior to collection of the medium. Medium was clarified by centrifugation and assayed for activin A by ELISA. Bars show mean $\pm$ S.D. from quadruplicate cultures. For both **A.** and **B.** 2 different experiments are shown.

A. Expt 1 **= $P=0.002$; **Expt 2** **= $P=0.015$, ***= $P=0.003$ all vs. untreated cultures (-), by unpaired t -test

B. Expt 1 *= $P=0.03$; **= $P=0.008$; ***= $P=0.0005$; **Expt 2**, **= $P=0.0008$, *= $P=0.004$, ***= $P=0.0001$ all vs. untreated cultures (-), by unpaired t -test.

The effect of inflammatory signalling (NF κ B) and FGF-dependent signalling seemed likely to be additive. This possibility was tested by treating porcine joints with both PD173074 and MG132 compared with each inhibitor in isolation. The combination of FGF receptor inhibition and proteasome inhibition reduced the production of activin A on injury to baseline levels (Fig 3.21B). This suggested that NF κ B, in addition to FGF-dependent pathways, was required for maximal activin A production upon injury.

The experiment was repeated using PD173074 and the alternative NF κ B inhibitor BMS345541 both alone, and in combination. Combining the IKK inhibitor with the FGFR inhibitor did not have a significantly greater effect than either agent alone, although there was already marked suppression in these experiments with the individual inhibitors (Fig 3.22A). Since NF κ B is anti-apoptotic, it was possible that the reduction in activin A production caused by inhibitors of NF κ B over a period of 16h was due to cell death. The effect of these inhibitors on mRNA levels of inhibin β A (the subunit of activin A) following the dissection was therefore studied. Cartilage was either dissected immediately into TRI reagent, or into medium in the presence or absence of inhibitors for 4h prior to RNA extraction. Some inhibition of inhibin β A mRNA was seen at 4h after dissection into PD173074 or the IKK inhibitor BMS345541, compared with dissection into medium alone (Fig 3.22B). The combination of these two inhibitors suppressed activin A mRNA to low levels. It therefore appeared that the IKK inhibitor was probably not toxic to the chondrocytes, and that the FGF pathway and NF κ B pathways were both necessary for the production of activin A mRNA and protein following injury.

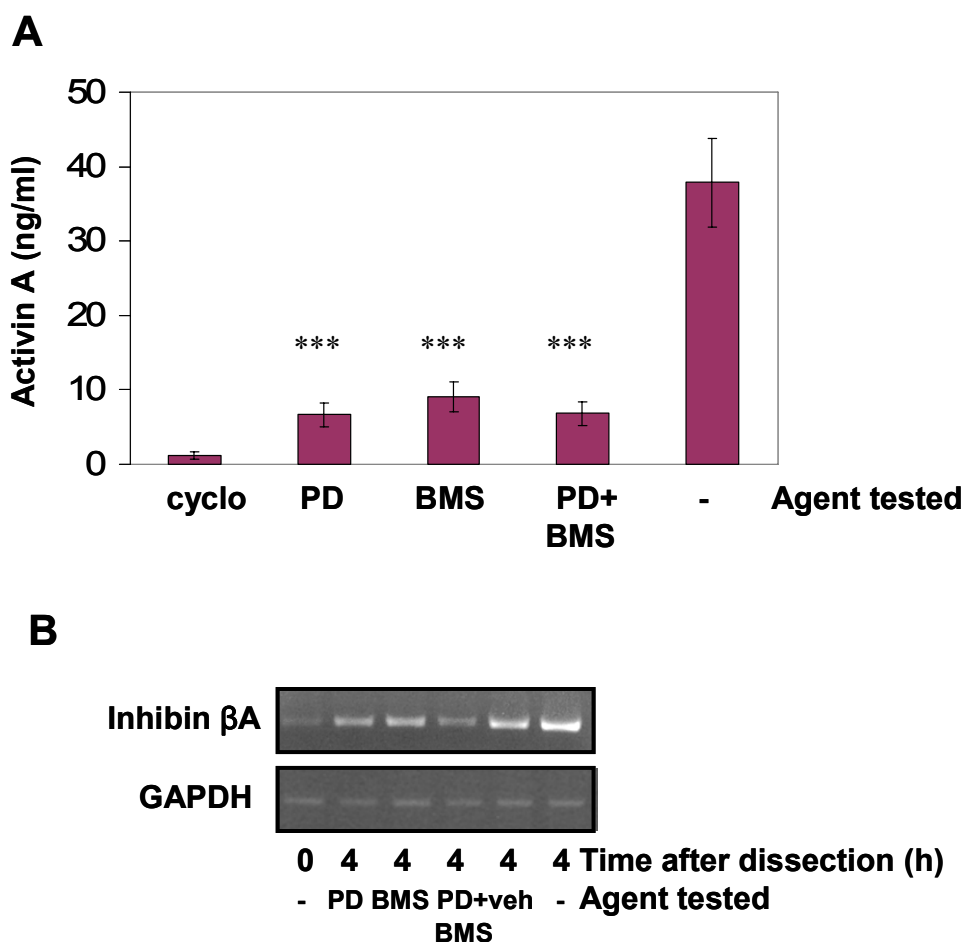


Figure 3.22. The effect of a combination of FGF receptor and NF κ B pathway inhibitors on activin A (A) protein and (B) mRNA following explantation.

Porcine MCP joints were initially injected with 3ml of serum-free medium containing the vehicle medium + 0.1% DMSO (veh), cycloheximide 10 μ g/ml (cyclo), 250nM FGF receptor inhibitor PD173074 (PD), 10 μ M IKK inhibitor BMS345541 (BMS) or a combination of both inhibitors (PD+BMS), or left untreated (-). Following equilibration at 37°C for 2h:

A. Cartilage was dissected into 1g/ml culture medium +/- fresh inhibitor and cultured for 16h. Activin A in the medium was measured by ELISA. Bars show mean +/- S.D. from quadruplicate cultures. ***=P=0.0001 vs. untreated cultures (-), by unpaired *t*-test.

B. Cartilage was either dissected directly into ice cold TRI Reagent, or dissected and cultured for 4h in serum-free medium +/- fresh inhibitor, prior to homogenisation of cartilage explants, RNA extraction and RT-PCR for inhibin β A and GAPDH.

A. and **B.** are each representative of 2 experiments.

3.13 Src family kinases are implicated in activin A production after explantation of cartilage

The FGF receptor inhibitor was highly efficient at reducing activin A levels following explantation by on average 80% (Fig 3.19, Fig 3.22A & Table 3.1). It was possible that ERK was not the only FGF-dependent pathway involved in the regulation of this cytokine. Stimulation of the FGFR activates several signalling pathways. In addition to the ERK MAPK pathway, protein kinase C (PKC), PI3 kinase and src family kinases are all likely to be activated by FGF (Ch 1, Fig 1.1). The effects of inhibitors of these pathways on activin A production were tested. The protein kinase C (PKC) inhibitors Gö6976 and Gö6983 inhibit the classical, novel and atypical PKC enzymes differentially. However, neither reduced activin A production following explantation (Fig 3.23A).

To investigate whether tyrosine kinases such as the src family are involved in the response, genistein, a broad inhibitor of tyrosine kinases was used, as well as PP1 and PP2 (closely related pyrazolopyrimidine compounds which have been widely used as inhibitors of src family kinases). They are specific (having significantly lower efficacy against the related kinase csk), but do not discriminate between src family members (Bain et al., 2003). Genistein and PP1 both caused inhibition of activin A secretion when tested in the explantation system (Fig 3.23B). PP2 caused on average a 60% inhibition of activin A production which was highly reproducible at 10 μ M (Fig 3.24 & Table 3.1). This was in contrast to the PI3 kinase inhibitor LY294002, which had no effect on activin A production. PP2 did not inhibit the FGF-induced activation of ERK in chondrocytes (Fig 3.25).

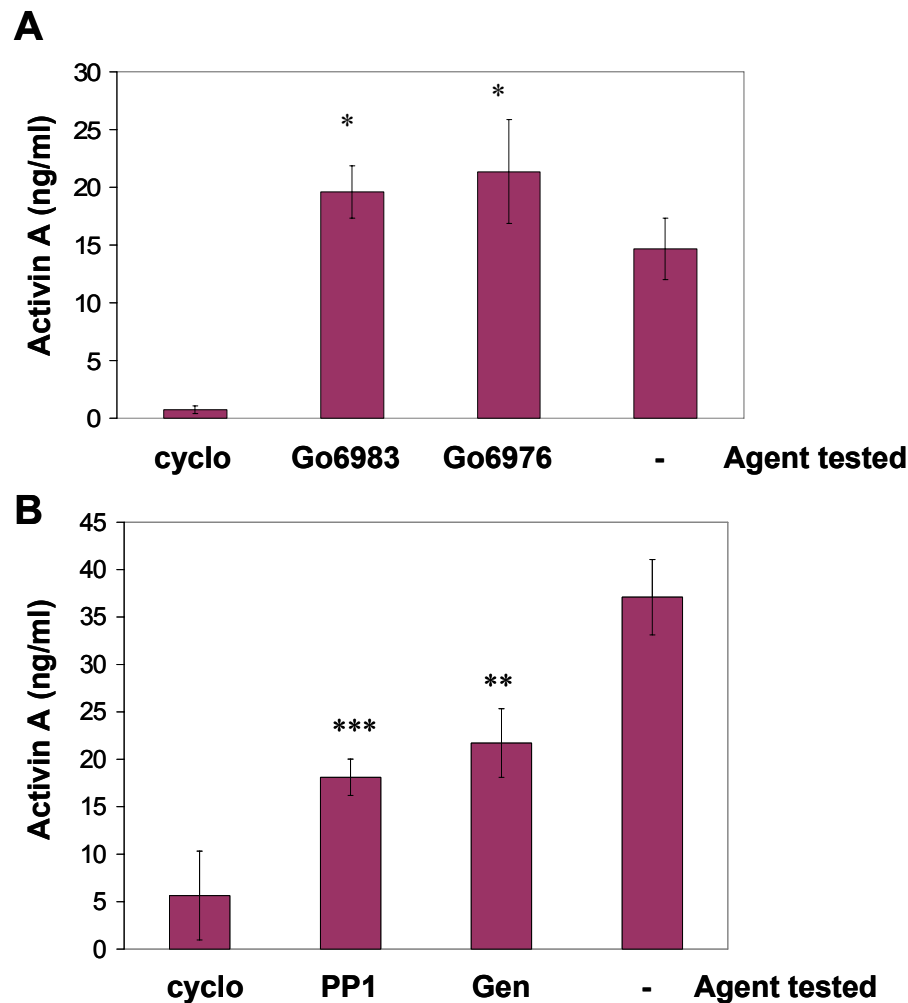


Figure 3.23. The effect of inhibiting PKC and tyrosine kinase pathways on activin A production.

A. Intact porcine MCP joints were injected with 3ml serum-free medium plus cycloheximide 10µg/ml (cyclo), the PKC inhibitors Gö6983 100nM (Go6983) or Gö6976 100nM (Go6976) or left untreated (-) and equilibrated at 37°C for 2h.

B. Intact porcine MCP joints were injected with 3ml serum-free medium plus cycloheximide 10µg/ml (cyclo), the src kinase inhibitor PP1 10µM (PP1), the tyrosine kinase inhibitor genistein 100µM (Gen) or left untreated (-) and equilibrated at 37°C for 2h.

For both **A.** & **B.**, ~0.5g cartilage per point was then explanted into 2ml medium/ g tissue containing fresh inhibitors or medium alone (-) and cultured for 16h prior to collection of the medium. This was clarified by centrifugation and assayed for activin A by ELISA. Bars show mean +/- S.D. from quadruplicate cultures. *= $P=0.04$; **= $P=0.001$; ***= $P=0.0001$, all vs. untreated cultures (-), by unpaired *t*-test. **A.** and **B.** are each representative of 2 experiments.

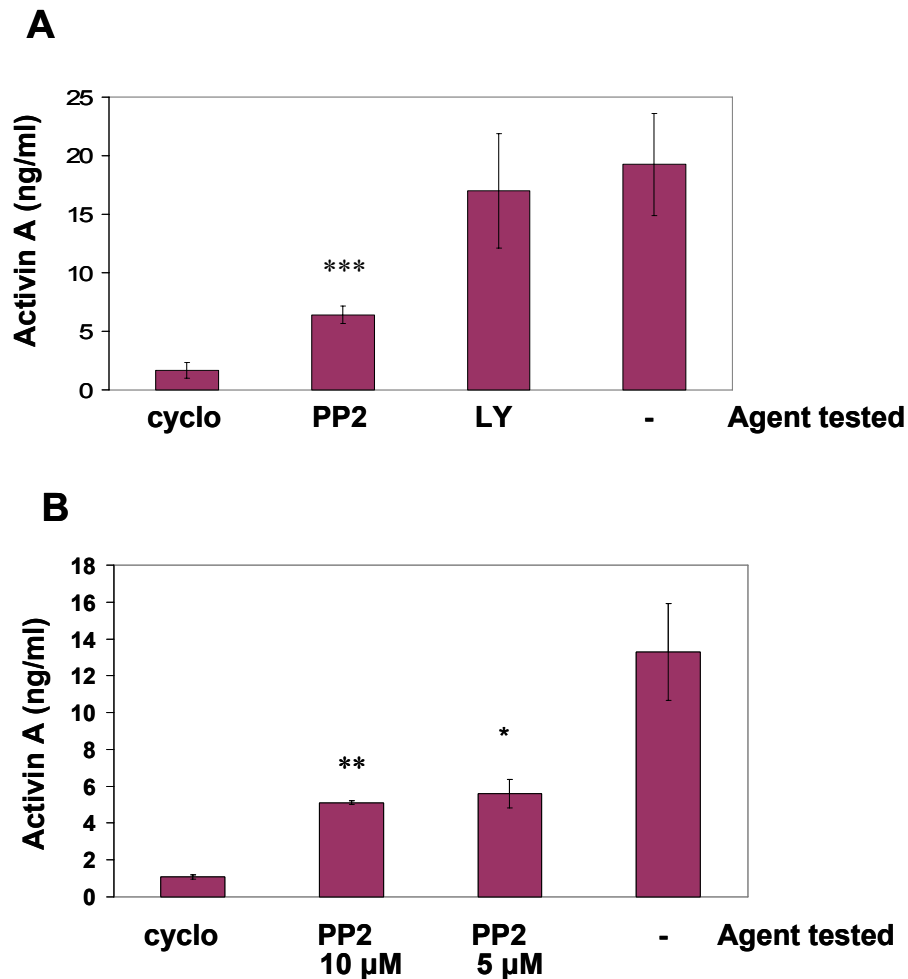


Figure 3.24. Src family kinase inhibition reduces activin A secretion following explantation whereas PI3 kinase inhibition has no effect.

A. Intact porcine MCP joints were injected with 3ml serum-free medium plus 10µg/ml cycloheximide (cyclo), the src kinase inhibitor PP2 10µM (PP2), the PI3 kinase inhibitor LY294002 10µM (LY) or left untreated (-) and equilibrated at 37°C for 2h.

B. Intact porcine MCP joints were injected with 3 ml serum-free medium plus 10µg/ml cycloheximide (cyclo), the src kinase inhibitor PP2 10µM or 5µM, or left untreated (-) and equilibrated at 37°C for 2h.

In both **A.** and **B.**, ~0.5g cartilage per point was then explanted into 2ml/g culture medium containing fresh inhibitors or medium alone (-) and cultured for 16h prior to collection of the medium. This was clarified, and assayed for activin A by ELISA. Bars show mean +/- S.D. from quadruplicate cultures.

A. ***=P=0.001 vs. untreated cultures (-), by unpaired *t*-test.

B. **=P=0.002 and *=P=0.003 vs. untreated cultures (-), by unpaired *t*-test.

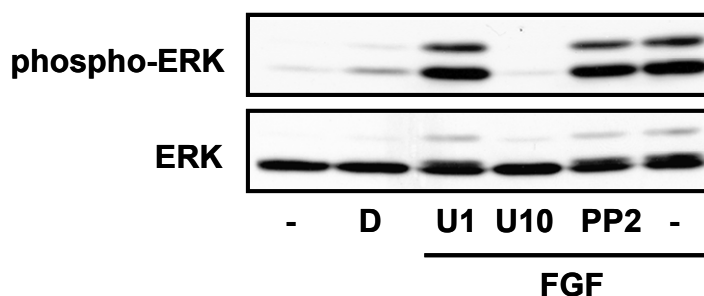


Figure 3.25. The src family kinase inhibitor PP2 does not inhibit FGF-induced activation of ERK in chondrocytes.

Porcine primary chondrocytes (2 million cells per point) were serum starved for 3h, then pre-incubated for 30min with medium alone (-) or medium containing 0.1% DMSO (D), the MEK inhibitor U0126 1 μ M (U1), U0126 10 μ M (U10) or the src family kinase inhibitor PP2 10 μ M (PP2) and then treated for 30min with serum-free medium alone +/- 0.1% DMSO (D), medium +/- fresh inhibitors plus FGF-2 20ng/ml (FGF). The medium was then aspirated and 250 μ l dissociative lysis buffer added per well. Chondrocyte lysates were assayed for phosphorylated ERK by western blotting. The membrane was stripped and re-probed for total ERK. This is representative of 2 experiments using different batches of chondrocytes.

These experiments suggested that non-receptor tyrosine kinases such as the src family kinases may also be important pathways in the response to articular cartilage injury. It was not clear whether these pathways were FGF-dependent or independent, or how they might relate to other inflammatory signalling pathway activation. Certain members of the src family are known to be activated by FGF-2 in other tissues, although little is known about their regulation in articular cartilage (Bromann et al., 2004; Kilkenny et al., 2003).

3.14 COX-2 protein expression following explantation is mediated by NFκB and FGF, and depends upon ERK and p38 MAPK

COX-2 protein had also been shown to be induced in articular cartilage following explantation (Fig 3.13). It was possible that its induction following the insult would be mediated by different pathways to activin A. To test whether FGF-dependent pathways or NFκB were necessary for COX-2 expression following explantation, the FGF receptor inhibitor PD173074 or the IKK inhibitor BMS345541 were injected into porcine MCP joints prior to dissection of cartilage into medium containing fresh inhibitor and culture for 16h (the time of maximal COX-2 expression, Fig 3.13). The FGF receptor inhibitor prevented COX-2 expression (Fig 3.26, Expt 1). In comparison, the IKK inhibitor BMS345541 reduced COX-2 expression after explantation by around 50%. The combination of the two inhibitors was not more potent than the PD173074 alone. A second similar experiment is shown. Both FGF-dependent pathways and NFκB appeared to be important in the regulation of COX-2 production after cartilage injury.

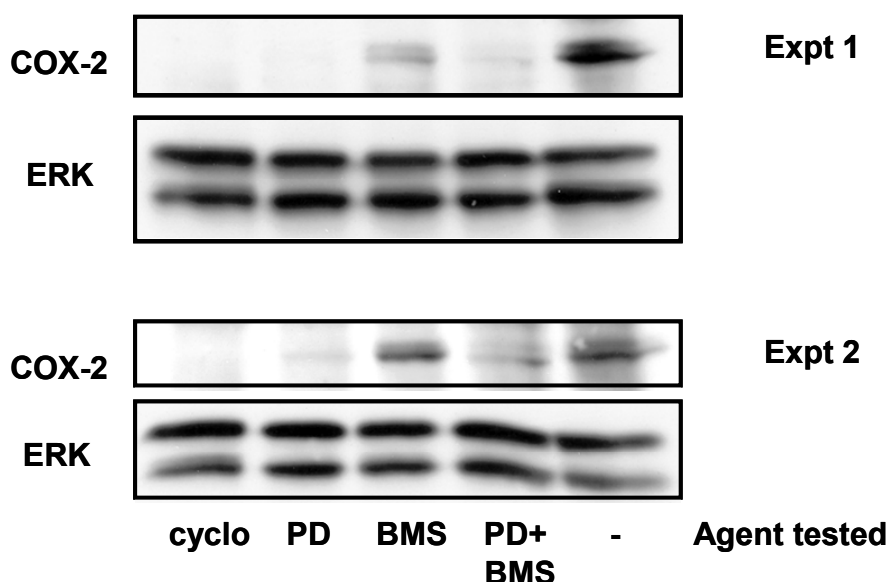


Figure 3.26. The effect of the FGF receptor inhibitor PD173074 and the IKK inhibitor BMS345541 on the production of COX-2 protein by cartilage following explantation.

Porcine MCP joints were initially injected with 3ml serum-free culture medium containing 10µg/ml cycloheximide (cyclo), 250nM FGF receptor inhibitor PD173074 (PD), 10µM IKK inhibitor BMS345541 (BMS), a combination of the two inhibitors, or left untreated (-). Following equilibration at 37°C for 2h, ~0.5g cartilage was dissected and cultured for 16h in serum-free medium +/- respective inhibitors prior to lysis. COX-2 in the cartilage lysates was assayed by western blotting. The membrane was stripped and re-probed for ERK to ensure equal loading. The results of two separate experiments are shown.

The two MAPK pathways regulated in chondrocytes by FGF-2 are ERK and p38 MAPK. We therefore investigated whether either of these pathways was required for COX-2 expression. Both U0126 (Fig 3.27A) and SB202190 (Fig 3.27B) markedly reduced COX-2 protein levels after explantation, but not quite to baseline (cycloheximide). NF κ B and FGF-2-induced pathways (ERK and p38 MAPK) were therefore likely to be responsible for induction of COX-2 expression after cartilage injury.

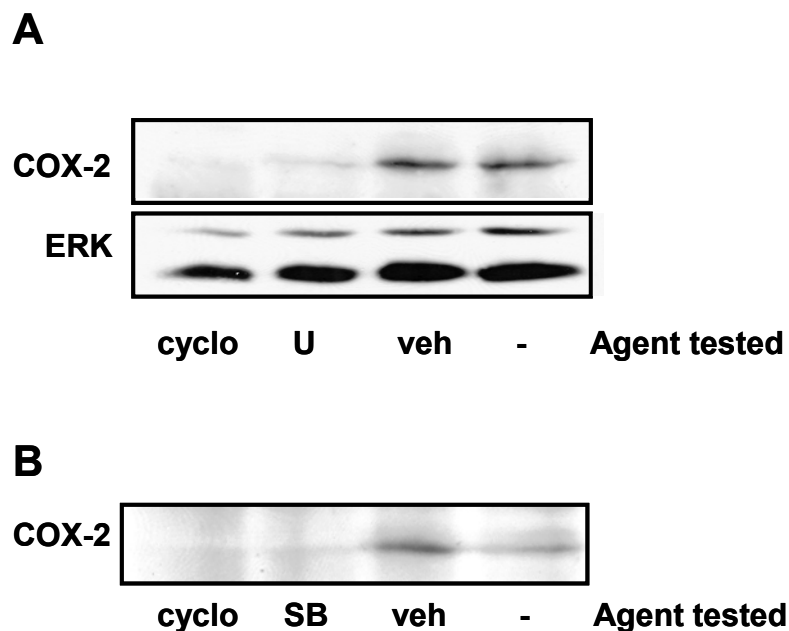


Figure 3.27. The effect of inhibitors of (A) MEK and (B) p38 MAPK on the production of COX-2 protein by cartilage following explantation.

Porcine MCP joints were left untreated (-) or injected with 3ml serum-free culture medium containing 10µg/ml cycloheximide (cyclo), the vehicle alone 0.1% DMSO (veh) or either (A) 10µM MEK inhibitor U0126 (U) or (B) 10µM p38 MAPK inhibitor SB202190 (SB). Following equilibration at 37°C for 2h, ~0.5g cartilage was dissected and cultured overnight in serum-free medium +/- inhibitors prior to lysis. COX-2 in the cartilage lysates was assayed by western blotting. The membrane was stripped and re-probed for ERK to ensure equal loading.

3.15 Discussion

These experiments show that in addition to MAPKs, other intracellular signalling pathways including PI3 kinase and IKK are activated by dissection of cartilage. The induction of IKK activity is strong and rapid (within 30sec). Re-cutting rested cartilage does not re-activate this pathway although the tissue responded to IL-1. Given this, and the lack of response to medium conditioned with freshly dissected extensively diced tissue, or to exogenous FGF, it was not possible to attribute the activation of IKK by injury to a soluble mediator released from the cartilage.

IKK is the most upstream kinase that I have investigated. It is not clear whether IKK α or β , or both, is the active kinase, because the assay detects both enzymes. Although IKK β is considered to be the main kinase in the canonical activation of NF κ B by inflammatory stimuli, IKK α may be equally important in the activation of the pathway by some stimuli such as IL-1 (Solt et al., 2007). This could be investigated by western blotting the phosphorylated forms of the kinases in cartilage lysates (Muddasani et al., 2007). The activation of IKK is not well understood. TAK-1 is the major kinase activated by inflammatory stimuli that phosphorylates IKK, but preliminary experiments suggest that TAK-1 activity is not increased in response to explantation (experiments carried out in collaboration with B. Brennan & J. Dean at the Kennedy Institute). Understanding the mechanism of this IKK activation would give insight into how cells detect tissue injury.

Fewer inflammatory response genes are induced following cartilage injury than by IL-1 (8 out of 12 tested). These include COX-2, activin A and IL-6, as well as collagenases such as MMP-1 and -13 and the aggrecanase ADAMTS-4. Induction of proteinases could be catabolic for the extracellular matrix, and therefore provide a link between cartilage injury and degeneration. My experiments did not give clear

evidence of induction of ADAMTS-5. This proteinase has been implicated as the major aggrecanase in murine models of injury-induced osteoarthritis, but it is not clear if it has the same importance in other species (Glasson et al., 2005; Stanton et al., 2005). ADAMTS-4 or other enzymes may have an equally important role in man (Song et al., 2007).

The increased expression of activin A in cartilage following injury is interesting because recent work in our laboratory has shown that it inhibits IL-1-induced aggrecan degradation in cartilage *in vitro* (Alexander et al., 2007), see also Appendix. This suggests that activin A may be an anti-catabolic autocrine cytokine produced by injured cartilage, and that injury induces anti-catabolic and catabolic (ADAMTS-4, IL-1) molecules. In a recent microarray study of a rat model of joint injury, inhibin β A mRNA was up-regulated over 8 fold (Appleton et al., 2007).

In some experiments, the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was noted to be regulated by explantation or stimulation with FGF. Recent reports highlight that regulation of such genes is recognised in certain circumstances, causing difficulty with normalisation of experiments (Fundel et al., 2008). For a more detailed assessment of absolute changes in mRNA levels, quantitative PCR would be necessary. However, normalisation can still be an issue. 18S, a ribosomal RNA which is present at high abundance in total RNA samples is often used for normalisation of q-PCR. This may have proved a better internal control than GAPDH in this setting.

FGF-dependent signalling is required for induction of two inflammatory response genes, activin A and COX-2 in response to cartilage injury. Both genes appear to be strongly regulated by FGF, as judged by the effect of the inhibitor of the receptor tyrosine kinase. However, in the case of activin A, FGF stimulation (exogenous

stimulation with FGF, or re-cutting of rested explants) is not sufficient to induce a significant increase in activin A, implying that other pathways must be necessary. ERK appears important for induction of both COX-2 and activin A, but is likely to be down-stream of FGF. p38 MAPK was not required for activin A production, but is important in the induction of COX-2 protein. This p38-dependence may be related to the fact that COX-2 mRNA contains AU-rich elements in its 3'-UTR and requires p38 activity for its stabilisation (Dean et al., 2003). Results with both MG132 and BMS345541 suggest that the NF κ B pathway may be needed for both activin A and COX-2 induction. Interestingly, the effects of FGFR and NF κ B inhibition seem to be additive. The earlier observation that the NF κ B pathway is not activated by re-cutting resting explants could explain why re-cutting had a significant but smaller effect on increasing activin A production. However, the specificity of these agents is obviously important when effects such as these are detected: MG132 may have off-target effects because of its action on the proteasome and the specificity of BMS345541 for IKK is not well-characterised.

A further pathway implicated in the injury response in the case of activin A is src. PP2 has been found by others to be quite specific for src family kinases (Bain et al., 2003). Srcs have been shown to be expressed in chondrocytes, but their role in cartilage is largely unexplored (Islam et al., 2001; Liu et al., 2004). Although FGF-2 activates SFKs in many cell lines, whether it does so in primary chondrocytes has not been investigated (Bromann et al., 2004). It is not clear from the effect of PP2 on activin A production whether src family kinases represent another FGF-dependent pathway, or are activated by some other means.

4 Investigation of tyrosine phosphorylation in cartilage following injury

4.1 Introduction

In chapter 3, I have shown that a physical injury to cartilage not only activates several intracellular signalling pathways, but also induces a number of inflammatory response genes. In the case of production of both activin A and COX-2 after injury, FGF-2 appears to be necessary, but to work in conjunction with other pathways. The effect of PP2 on activin A production strongly suggested that src family kinases were needed. It was possible that src kinases mediate in part the effect of FGF. Alternatively, a src family member may be involved in the injury response independently of FGF. Non receptor tyrosine kinases are commonly involved in surface receptor signalling other than that of receptor tyrosine kinases. For example signalling through T and B cell receptors, through interferon receptors and the receptors of the IL-6 group of cytokines all involve tyrosine kinases.

Because of our lack of understanding of the mechanism by which chondrocytes detect physical injury, and because of the significant effect of PP2 in inhibiting activin A, I decided to examine whether there is tyrosine kinase activation in cartilage in response to injury and to what extent it is FGF-independent.

4.2 Tyrosine phosphorylation rapidly follows explantation of cartilage

A common approach to detect changes in protein tyrosine kinase or phosphatase activity is to western blot tyrosine-phosphorylated proteins in cell extracts. A number of antibodies which recognize tyrosine-phosphorylated proteins have been successfully employed, for example see (Hinsby et al., 2003; Zhang et al., 2005). 4G10 is a mouse IgG kappa monoclonal antibody which has been widely used either alone, or in combination with other phosphotyrosine antibodies for immunoprecipitation and western blotting.

To investigate whether there was change in protein tyrosine phosphorylation in the cartilage in response to explantation, porcine trotters were equilibrated at 37°C prior to cartilage being dissected either directly into a tube on dry ice or into serum-free culture medium for increasing times, prior to lysis in RIPA buffer. Lysates were clarified and mixed with sample buffer prior to electrophoresis on an 8% acrylamide gel, and western blotting with 4G10 antibody. There was clear induction of a number of bands following explantation, in particular at 190 kD, 100 kD, 80 kD, 60 kD and 55 kD (Fig 4.1). The phosphorylations declined gradually after 20min. The pattern of induction of bands was reproducible and rapidly followed the injurious stimulus. In another experiment these bands were induced 30sec after injury (Fig 4.2). The membrane was stripped and re-probed with antibody to ERK to check loading. This showed that the 5min sample was under-loaded, accounting for the weaker phosphotyrosine staining at this time. Usually, tyrosine phosphorylation peaked between 10-30min and was still present at 60min (Fig 4.1). No temporal separation of these bands was possible by western blotting: they were all evident within 20-30sec of explantation.

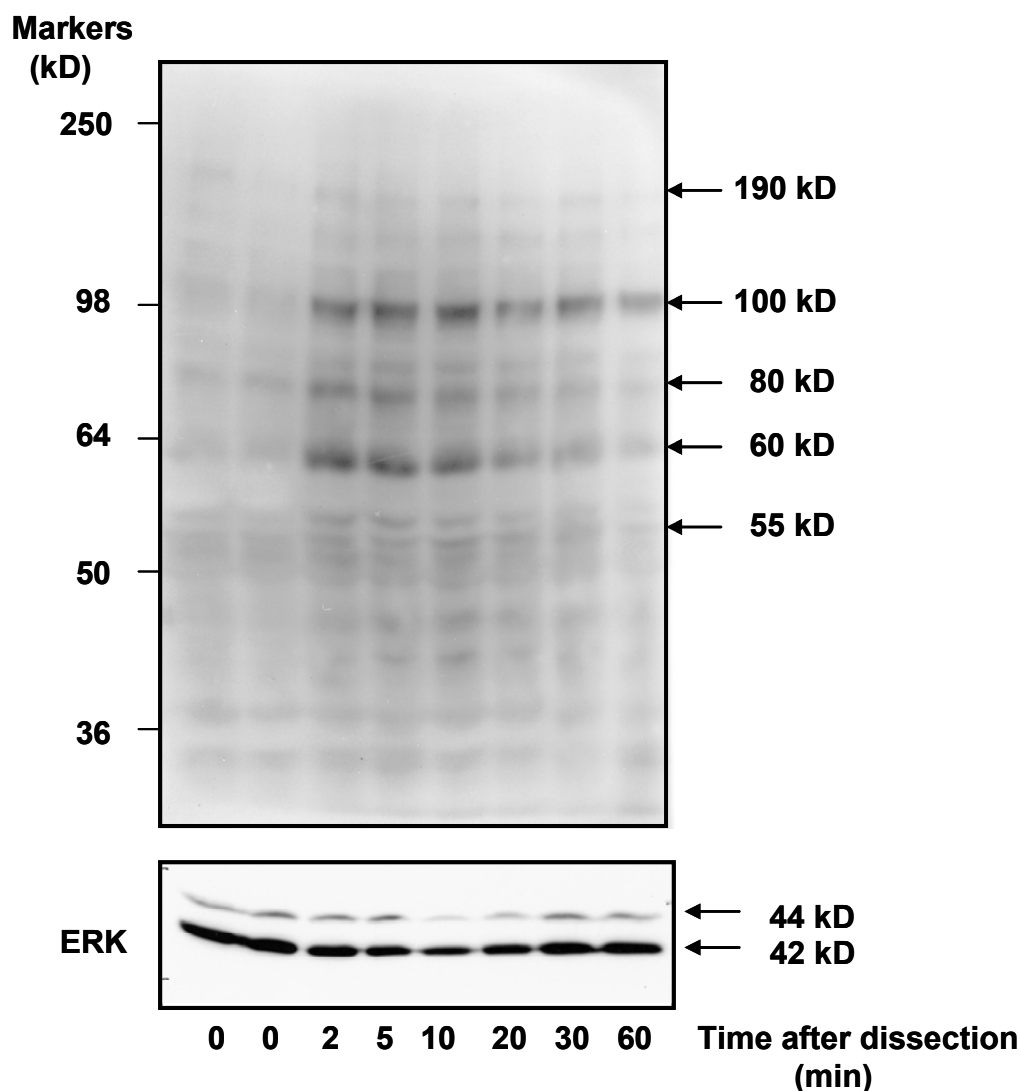


Figure 4.1. Tyrosine phosphorylation patterns of articular cartilage lysates following explantation of the tissue.

Porcine MCP joints were equilibrated at 37°C for 4h. ~0.5g cartilage (from a separate joint for each time point) was dissected into serum-free medium for the time specified. At the end of the incubation, cartilage was washed 3 times in ice cold PBS prior to snap freezing. For 0min samples, cartilage was dissected into a tube on dry ice. Cartilage lysates were boiled with SDS sample buffer, electrophoresed on an 8% gel and assayed for phosphotyrosine residues by western blotting with the 4G10 antibody (upper panel). The membrane was stripped and re-probed for ERK (lower panel). The arrows indicate the positions of the major induced bands. These results are representative of 4 experiments.

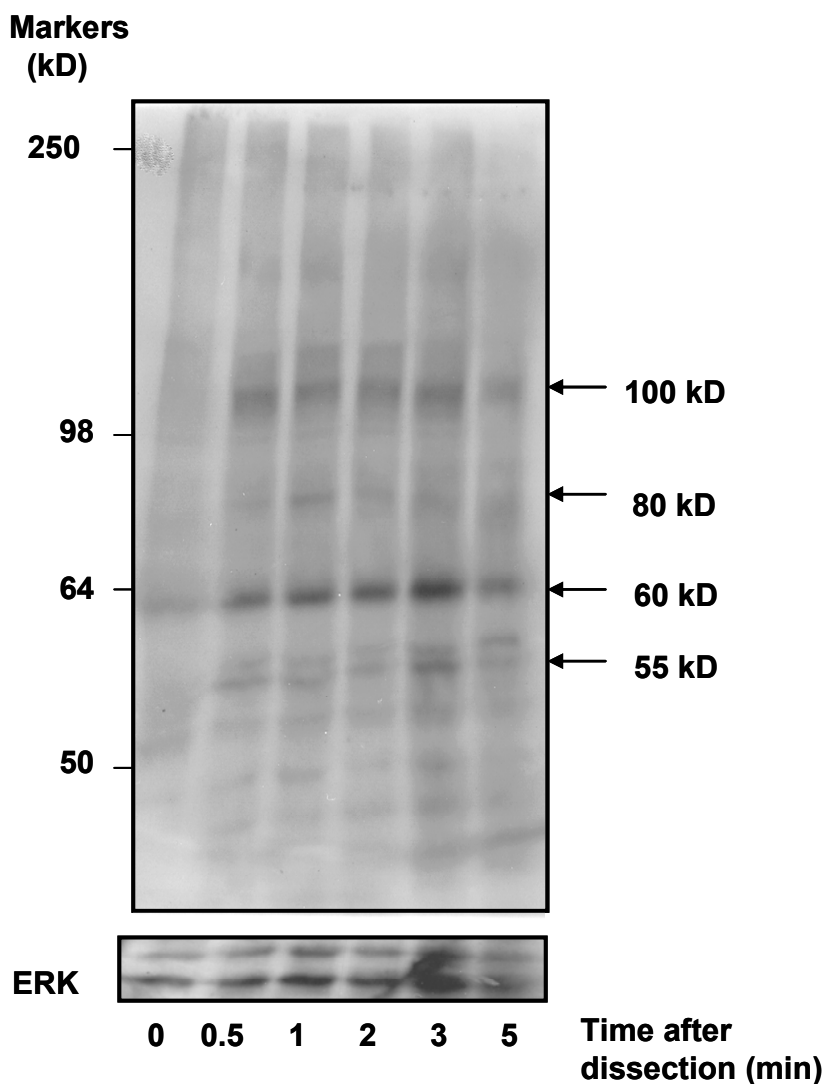


Figure 4.2. Tyrosine phosphorylation patterns of explanted articular cartilage. Porcine MCP joints were equilibrated at 37°C for 4h. ~0.5g cartilage from an individual joint was dissected into serum-free medium for the times specified. At the end of the incubation, cartilage was washed 3 times in ice cold PBS prior to snap freezing. For 0min sample, cartilage was dissected into a tube on dry ice. Cartilage lysates were assayed for phosphotyrosine residues by western blotting with the 4G10 antibody. The membrane was stripped and re-probed for ERK to ensure equal cellular protein loading.

4.3 Induction of tyrosine-phosphorylated proteins following cartilage injury is not affected by culture medium

All culture medium in these experiments was serum-free, as growth factors present in serum could stimulate tyrosine kinases. It was conceivable that the induction of these bands was related to contact with culture medium. To test this possibility, cartilage was dissected from joints equilibrated at 37°C into a dry tissue culture dish and incubated at 37°C without medium for increasing times prior to lysis and western blotting for phosphotyrosine residues. Induction of the same bands was evident, although in some cases their appearance was delayed until 2min after dissection and they were maximal at the later time of 60min (Fig 4.3). Because cartilage was not washed prior to lysis in this experiment, serum albumin is present (as well as other plasma proteins of synovial fluid) and distorts the pattern in the 55-60 kD region.

Conceivably, a factor in synovial fluid could have been responsible for inducing tyrosine phosphorylation by coming in contact with chondrocytes via the cuts in the cartilage. However this possibility was not supported by the observation that the activation was reduced neither by increasing washing of cartilage prior to culture nor by increasing the volume of medium used for culture (data not shown). It was also possible that the removal of cartilage from the articular surface, rather than the sharp injury *per se* caused activation of tyrosine kinases. To investigate this, porcine trotters were opened and equilibrated at 37°C prior to scoring the articular surface as previously described (see Materials & Methods for details, section 2.2.2). Joints were replaced in the incubator for increasing times indicated. The surface was then washed rapidly (10 sec) with ice cold PBS prior to rapid dissection and lysis of the cartilage. Again, induction of the same bands was evident, particularly a complex of bands between 100 and 120 kD and the 60 kD band (Fig 4.4).

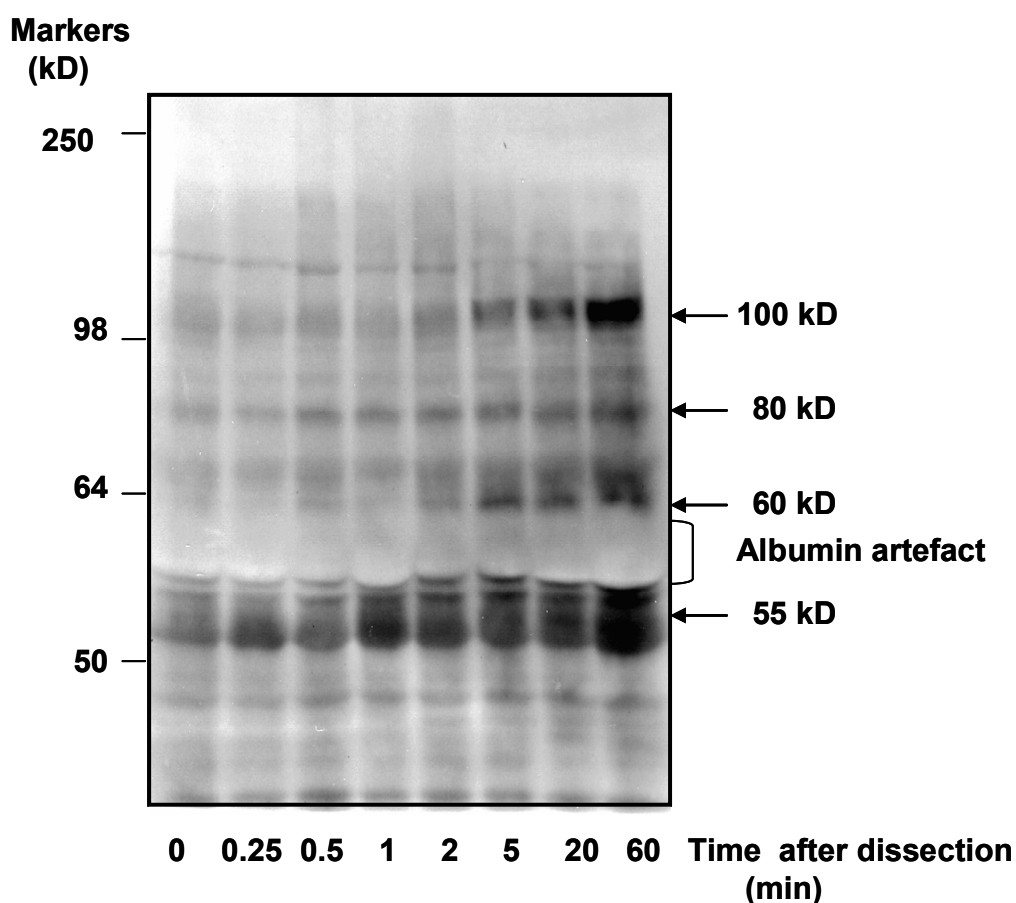


Figure 4.3. Tyrosine phosphorylation following explantation of articular cartilage is not dependent on contact with culture medium.

Porcine MCP joints were equilibrated at 37°C for 4h. 3 cartilage pieces from a single joint surface were dissected into a dry tissue culture dish and incubated at 37°C for increasing times specified, and points performed in duplicate. For 0min samples, cartilage was dissected directly into a tube on dry ice. Samples were pooled for lysis. Cartilage lysates were assayed for phosphotyrosine residues by western blotting. This is representative of 2 experiments.

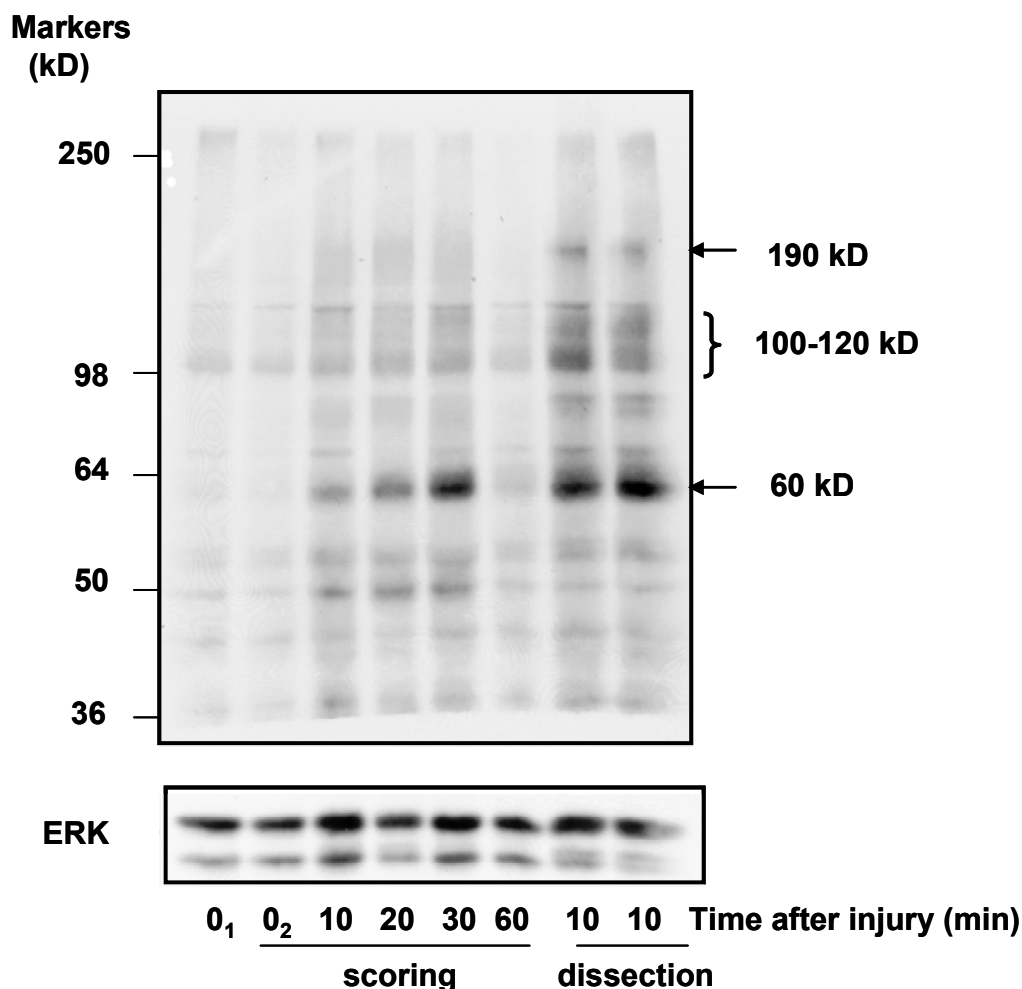


Figure 4.4. Protein tyrosine phosphorylation is induced in cartilage by scoring of the articular surface.

Trotters were opened and equilibrated at 37°C. ~0.2g cartilage was dissected from the articular surface directly on to dry ice (0₁) or scored immediately prior to dissection (0₂), or the articular surface extensively scored (1 x 1mm) and the joint closed and incubated at 37°C for the times indicated prior to dissection into a tube on dry ice (scoring). As controls, an equal amount of cartilage was dissected from the surface into serum-free culture medium and cultured for 10min prior to snap freezing (dissection). Samples were lysed and western blotting with 4G10 antibody carried out. The membrane was re-probed for ERK to ensure equal loading. This is representative of 2 experiments.

The induction of tyrosine phosphorylations by scoring was somewhat weaker than that seen following dissection and culture for 10min (shown as a control in the experiment, Fig 4.4). This could have been because the surface area of tissue injury was proportionately less. In addition, an inducible band at around 190 kD is evident in these samples. This was seen faintly in Fig 4.1 and is increasingly apparent in later experiments, where samples have not been stored prior to lysis and electrophoresis, and where transfer time has been extended to enhance the transfer of larger proteins on to the membrane.

4.4 Induction of tyrosine phosphorylation following cartilage injury is not dependent on FGF-2

Since FGF-2 is released upon cartilage injury, it was entirely possible that the inducible protein tyrosine phosphorylations were FGF-dependent. This was investigated.

4.4.1 The FGF receptor inhibitor does not prevent the tyrosine phosphorylation following explantation

To test whether FGF signalling was necessary for the initial explantation-induced tyrosine phosphorylation, intact joints were equilibrated at 37°C, having been injected with 250nM FGF receptor tyrosine kinase inhibitor PD173074, or vehicle alone, or not injected. Cartilage was then dissected either immediately into a tube on dry ice or into medium containing PD173074, or medium alone for 5min prior to snap freezing, lysis and western blotting with 4G10 antibody. There was strong induction of the bands of interest following explantation, and this was unaffected by the presence of the FGF receptor inhibitor (Fig 4.5A). The membrane was re-probed for ERK protein to ensure equal loading. To ensure that the FGF receptor inhibitor was having an effect on the cartilage in this experiment, the samples were re-run, and western blotting for phosphorylated ERK was carried out. This showed partial inhibition of

ERK activation in response to explantation in the presence of the inhibitor. This was consistent with FGF-2 activation of FGFR being responsible for some of the ERK activation caused by explantation. The results indicate that the tyrosine phosphorylation is likely to be independent of FGF-2.

Because the src family kinase inhibitor PP2 inhibited activin A production after explantation, I investigated whether the tyrosine phosphorylation induced by cartilage injury would be suppressed by this compound. In a similar experiment to Fig 4.5A, joints were equilibrated at 37°C, having been injected with 10µM PP2, or the vehicle 0.1% DMSO alone, or not injected. Cartilage was then dissected either immediately into lysis buffer (0) or into medium containing PP2, or into medium containing 0.1% DMSO for 20min prior to lysis and western blotting with 4G10 antibody. In contrast to the FGF receptor inhibitor, PP2 suppressed the induction of essentially all tyrosine phosphorylation following explantation (Fig 4.5B). For this experiment the tissue was lysed 20min after dissection but the effect of the inhibitor was seen at earlier times in other experiments. This suggested (a) that src, or a src-like kinase, was necessary for the tyrosine phosphorylation induced by explantation, and (b) that this kinase activity was not dependent on the action of the FGF-2 released.

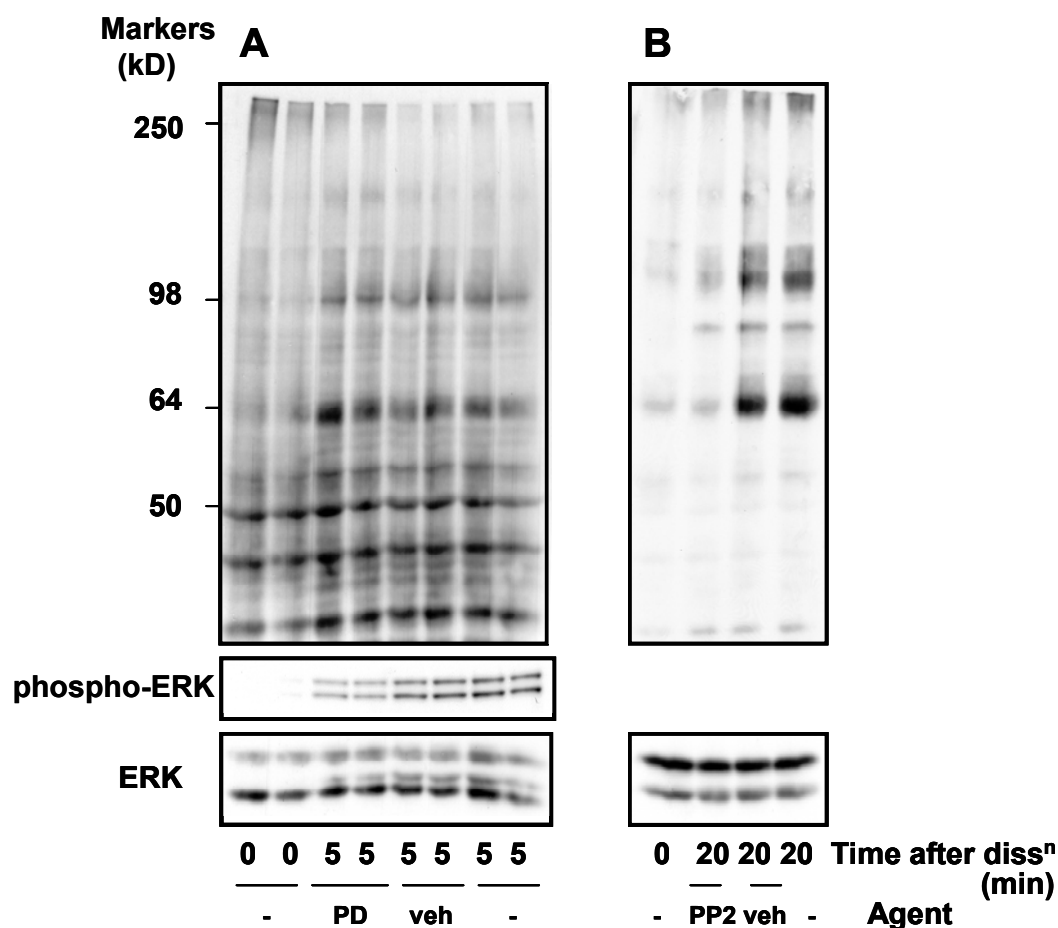


Figure 4.5. The FGF receptor inhibitor PD173074 does not prevent tyrosine phosphorylation following cartilage explantation (A), whereas the src family kinase inhibitor PP2 does (B).

A. Porcine MCPJs were injected with 250nM FGF receptor inhibitor PD173074 (PD) or the vehicle culture medium alone (veh) or not injected (-) and equilibrated at 37°C. ~0.5g cartilage was then dissected either immediately into a tube on dry ice (0) or into serum-free medium alone or medium containing PD173074 and cultured for 5min prior to washing in ice cold PBS and snap freezing.

B. Porcine MCP joints were injected with medium containing 10μM src family kinase inhibitor PP2 (PP2) or the vehicle 0.1% DMSO (veh) or not injected (-) and equilibrated at 37°C. ~0.5g cartilage was then dissected either directly into lysis buffer (0), or into medium alone, medium containing 0.1% DMSO or PP2 and cultured for 20min prior to lysis.

For both **A.** and **B.**, lysates were assayed for phosphotyrosine content by western blotting with 4G10 antibody. The membrane was stripped and re-probed with antibodies to ERK to ensure equal cellular protein loading. For **A.** samples were re-run and western blotting for phospho-ERK was carried out.

A. is representative of 2 experiments and **B.** is representative of 3 experiments.

4.4.2 FGF-2 stimulation of cartilage explants or chondrocytes does not increase tyrosine phosphorylation

The effect of stimulation with exogenous FGF-2 on tyrosine phosphorylation in cartilage was also examined. Cartilage explants were rested in serum-free medium for 24h prior to stimulation with 50ng/ml FGF-2 for increasing times prior to lysis. Some explants were treated with 10mM pervanadate for 10min as a positive control to induce strong tyrosine phosphorylation. As predicted, this phosphatase inhibitor strongly up-regulated tyrosine phosphorylation (Fig 4.6A, most right hand lane). This is despite this lane being underloaded on blotting with ERK as a loading control. In comparison, there was no change in the levels of tyrosine-phosphorylated proteins in response to FGF-2, other than the induction of two bands around 40 kD which were thought likely to be tyrosine-phosphorylated ERK. Weak constitutive tyrosine phosphorylation was seen in all samples. The membrane was re-probed for phosphorylated ERK to confirm activation of ERK by FGF-2. As a further test, cartilage explants were re-cut. This stimulus also led to ERK activation but did not increase levels of tyrosine-phosphorylated proteins above baseline either (data not shown).

The effect of FGF-2 on tyrosine phosphorylation in primary porcine chondrocytes was also examined. Chondrocytes were serum starved prior to stimulation with FGF-2 for increasing times prior to lysis. Lysates were run on a 4-12% bis tris minigel, and so the pattern and the relative positions of the markers are different to Fig 4.6A. Constitutive tyrosine phosphorylation in the resting serum starved chondrocytes was evident (Fig 4.6B). Many bands were present, but no change in their intensity was seen in response to FGF-2. In contrast, strong induction of phosphorylated ERK in response to this stimulus was noted when the membrane was stripped and re-probed.

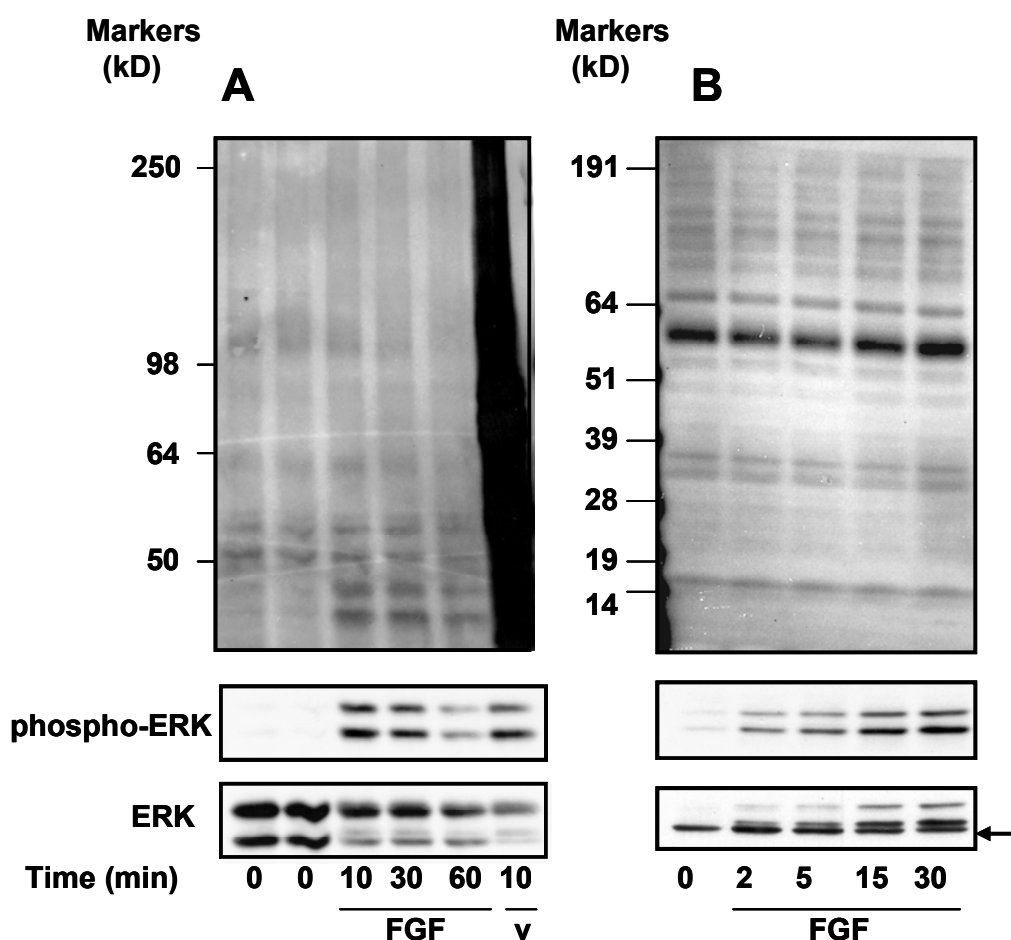


Figure 4.6. FGF-2 does not induce tyrosine phosphorylations in (A) cartilage explants or (B) primary porcine chondrocytes.

A. Cartilage was dissected and rested for 24h in serum-free medium. Then, equal amounts of cartilage were either snap frozen (0) or stimulated with medium containing 250ng/ml FGF-2 (FGF) for increasing times indicated, or medium containing 10mM sodium pervanadate for 10min (v) prior to snap freezing and lysis.

B. Primary porcine chondrocytes were prepared and rested in serum-containing medium for 48h. 2 million cells per point were then serum starved for 3h prior to immediate lysis (0) or stimulation with 20ng/ml FGF-2 for the times indicated prior to lysis.

For both **A.** and **B.**, lysates were clarified and western blotting with 4G10 antibody was carried out. Membranes were re-probed with phospho-ERK antibodies and then stripped and re-probed with total ERK, to ensure equal cellular protein loading. **A.** is representative of 4 experiments and **B.** is representative of 2 experiments.

The SAK2 ERK antibodies used in Fig 4.6B only recognised the lower 42 kD isoform of ERK and showed equal loading of cellular protein (indicated with arrow). However, the secondary antibodies also recognized un-stripped phospho-ERK from the previous blot. In Fig 4.6A, the membrane was stripped and re-probed with Santa Cruz ERK antibodies (See Materials & Methods, Table 2.1). Here the antibodies bound less well where phospho-ERK antibody had previously bound to the membrane, giving the impression of under-loading of lanes 3-6.

4.5 Immunoprecipitation of tyrosine-phosphorylated proteins

The rapidity and extent of the tyrosine phosphorylation caused by injury, together with its apparent independence of FGF-2 was intriguing. The fact that the src inhibitor PP2 prevented it and also inhibited activin A expression suggested it may be an important factor in the response. I therefore decided to identify the tyrosine-phosphorylated proteins by mass spectrometry. I immunoprecipitated them from lysates with the 4G10 antibody covalently linked via protein A to agarose beads (called '4G10 beads'). Immunoprecipitation prior to western blotting would purify and concentrate tyrosine-phosphorylated proteins in the samples.

4.5.1 Preliminary experiments

Immunoprecipitation with 4G10 beads was first tried with porcine primary chondrocyte lysates. 10 million chondrocytes per point were either left unstimulated, or treated with sodium pervanadate for 20min, prior to lysis in RIPA buffer and immunoprecipitation overnight with 4G10 beads. Beads were then washed in RIPA buffer, boiled with SDS sample buffer and western blotting of the supernatants for phosphotyrosines was carried out with 4G10 antibody. (Gels used to analyse the immunoprecipitates were 4-12% bis tris mini gels, and therefore the protein patterns are somewhat different to the 8% acrylamide gels shown previously). A number of

bands were immunoprecipitated from chondrocyte lysates (Fig 4.7). The phosphatase inhibitor sodium pervanadate strongly up-regulated immunoprecipitable tyrosine-phosphorylated proteins, and therefore served as a good positive control for this procedure.

Next, lysates from cartilage which had either been dissected immediately from the articular surface (0), or into serum-free medium and cultured for 2min, were incubated overnight with either 4G10 antibody or 'non-immune' mouse immunoglobulin pre-incubated with protein A agarose beads. A number of bands were immunoprecipitated from cartilage lysates only by the 4G10 antibody, some of which (notably bands at 60 kD and 100-120 kD) appeared to be regulated by injury (Fig 4.8). It was possible that greater regulation would have been seen at longer times after explantation, and this was taken in to account in later experiments, where a time of 10min was selected (see Figs 4.1& 4.4). Non specific bands at ~27 and ~52 kDa present in all samples were likely to represent immunoglobulin light and heavy chains respectively.

4.5.2 Optimisation of methods for 4G10 immunoprecipitation from cartilage lysates

Preparation of lysates: The length of time for lysis of cartilage in order to obtain maximum extraction of tyrosine-phosphorylated proteins was assessed. Equal amounts of cultured cartilage were lysed in RIPA buffer for increasing times. Relatively little phosphotyrosine was detected after 15min of extraction. Markedly more was seen after 45min, and 2h extraction showed improved extraction of the 190kD band (Fig 4.9). I decided to use 2h routinely for extraction.

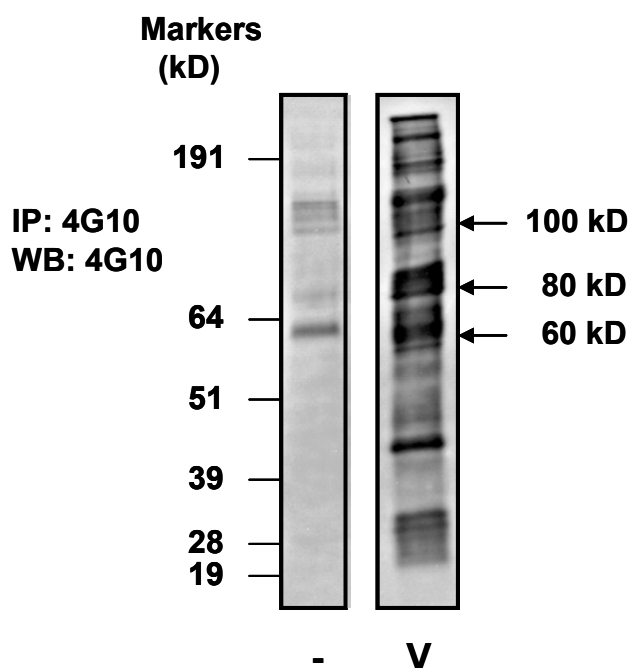


Figure 4.7. Tyrosine-phosphorylated proteins can be immunoprecipitated from primary chondrocyte lysates by 4G10 antibody bound to agarose beads.

Serum starved primary porcine chondrocytes (10 million per point) were either left unstimulated (-), or stimulated for 20min with 10mM sodium pervanadate (V), prior to lysis with RIPA buffer. Lysates were incubated overnight with protein A agarose beads cross-linked to 4G10 (4G10 beads). Beads were washed 5 times in RIPA buffer, then boiled for 10min with SDS sample buffer and the supernatant blotted for protein phosphotyrosines using conjugated 4G10-HRP antibody.

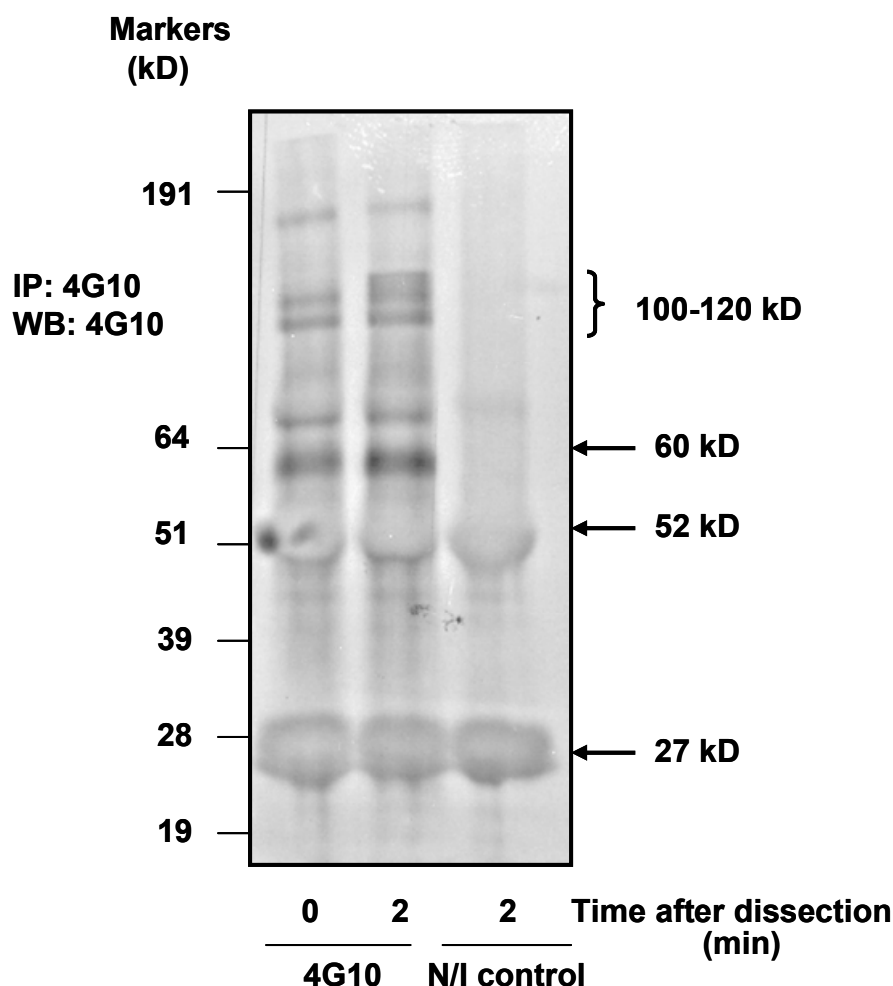


Figure 4.8. Tyrosine-phosphorylated proteins can be immunoprecipitated from cartilage lysates.

Intact joints were equilibrated at 37°C. 2g cartilage per point was dissected either immediately into a tube on dry ice (0) or into culture medium and incubated for 2min, prior to washing 3 times with cold PBS and snap freezing. Cartilage was lysed in RIPA and lysates incubated overnight at 4°C with either 4G10 beads (4G10) or protein A agarose pre-incubated with mouse immunoglobulin (N/I control). Beads were washed 5 times in RIPA buffer, then boiled for 10min with SDS sample buffer and the supernatant examined for protein tyrosine phosphorylations by western blotting with the 4G10-HRP antibody.

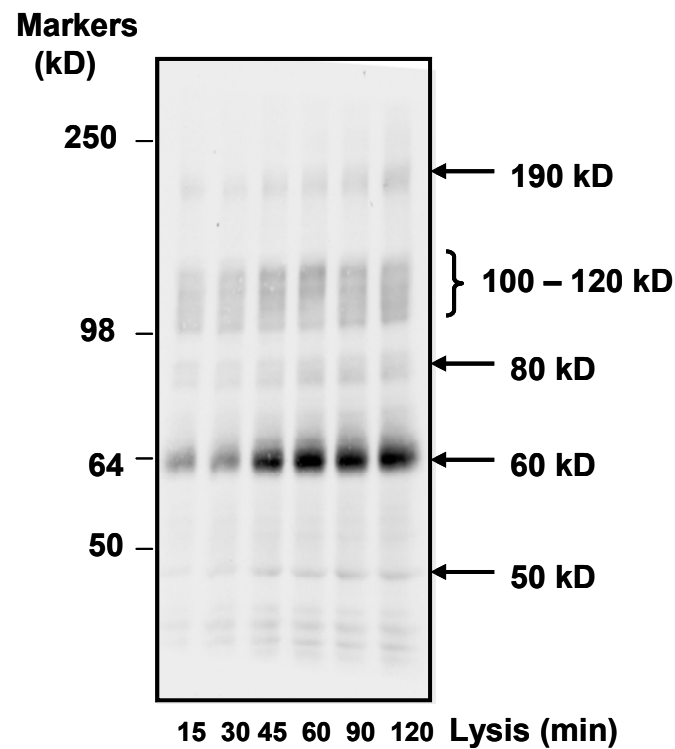


Figure 4.9. Investigating optimal time of cartilage lysis prior to phosphotyrosine immunoprecipitation.

~0.5g cartilage was dissected per point into culture medium for 10min, prior to lysis in RIPA buffer, shaking at 4°C for the increasing times indicated. Lysis buffer was then removed, clarified and western blotting of the lysates with 4G10-HRP antibody to detect their protein phosphotyrosine content was carried out.

Selective elution of phosphoproteins from 4G10 beads: There was some evidence of non-specific precipitation of proteins from tissue lysates (Fig 4.8). In order to elute phosphorylated proteins selectively from the beads 0.2M phenyl phosphate was used (Peirce et al., 2005). To recover efficiently all of the phenyl phosphate solution from the beads after elution, the slurry was passed through a spin column (0.22 μ M cellulose acetate).

To test how selective this elution step was, in an initial experiment 2 aliquots of 600 μ l of cultured cartilage lysate were each incubated with 10 μ l 4G10 beads overnight, and the beads eluted with either RIPA alone, or RIPA containing 0.2M phenyl phosphate for 2h. Phosphorylated proteins were only evident in the eluate containing phenyl phosphate on 4G10 western blotting (Fig 4.10A).

It was important to investigate the efficiency of the immunoprecipitation and elution step at removing tyrosine-phosphorylated proteins. Lysates prior to immunoprecipitation (pre-IP), following immunoprecipitation (post IP) and the eluates from immunoprecipitates (IP) were all assessed for phosphotyrosine content by 4G10 western blotting. The immunoprecipitation and phenyl phosphate elution markedly concentrated the tyrosine phosphorylation within the samples, and marked regulation of phosphorylation following injury was still evident (Fig 4.10B 'IP'). Some tyrosine phosphorylation was still present in lysates after immunoprecipitation. This could have been because the 4G10 immunoprecipitation was inefficient at depleting all tyrosine-phosphorylated proteins from the lysates. Alternatively, the avidity of 4G10 antibody binding on western blotting meant that this procedure was not able to assess any depletion quantitatively.

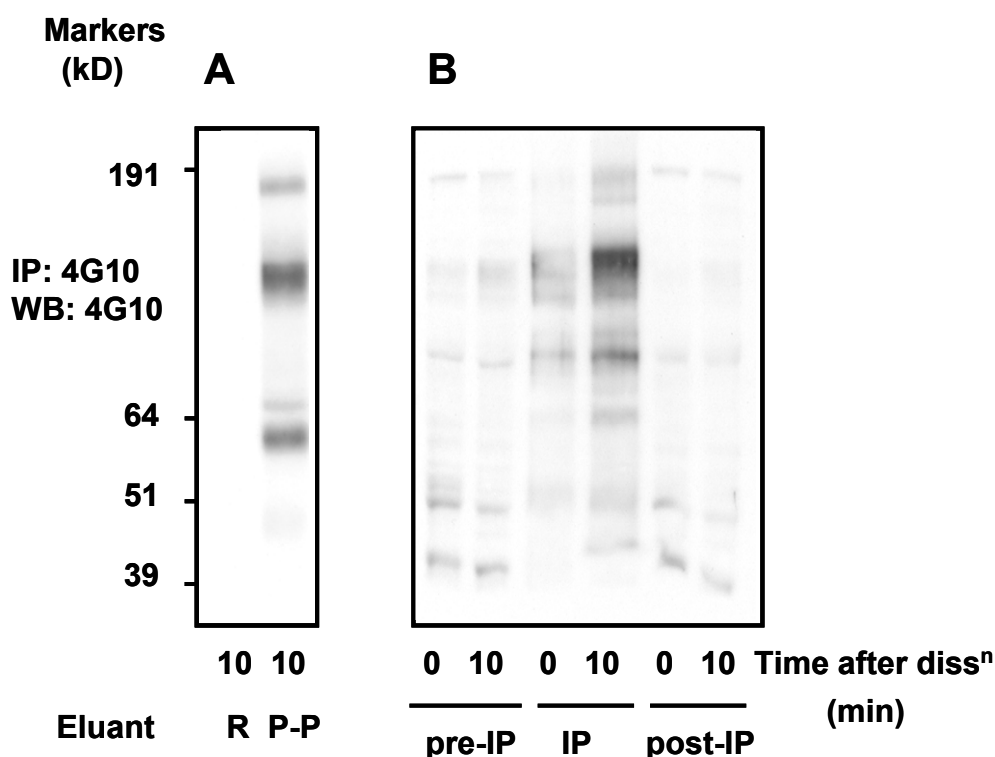


Figure 4.10. Selective elution of phosphorylated proteins from 4G10 beads.

A. 2x~0.5g cartilage was dissected into serum-free medium for 10min, prior to washing 3 times in ice cold PBS and lysis in RIPA buffer. Lysates were pooled, vortexed, pre-cleared with protein A agarose, and then incubated overnight with 4G10 beads. Beads were washed 5 times in RIPA, and then eluted for 2h in 20 μ l of either RIPA alone (R) or RIPA containing 0.2M phenyl phosphate (P-P). Eluates were boiled with SDS sample buffer and western blotting with 4G10-HRP antibody carried out.

B. ~0.5g cartilage from each of 4 MCP joints was either dissected immediately into a tube on dry ice (0) or into serum-free medium for 10min prior to washing 3 times and lysis (10). Respective lysates were pooled, and aliquots either boiled immediately with SDS sample buffer (pre-IP) or incubated overnight with 4G10 beads and eluted with phenyl phosphate (IP). Further aliquots of lysate which had undergone 4G10 immunoprecipitation were mixed with sample buffer (post-IP). Western blotting with 4G10-HRP antibody was carried out.

Reduction of serum protein contamination: In Figure 4.8, strong bands at ~27 and ~52 kD were likely to represent the light and heavy chains of immunoglobulin respectively, as they were also precipitated by the non-immune IgG. Contaminating serum proteins such as this were likely to hinder identification of proteins of interest, which lay between 50 and 190 kDa. By ‘running off’ the samples from the minigel to the 39 kD marker, the light chain was removed and spreading of the remaining bands was achieved. In addition, cartilage was rapidly washed 4 times to remove as much synovial fluid as possible prior to lysis. For ‘0’ time points, the articular surface was rapidly washed with 250ml ice cold buffered saline prior to dissection.

Protein A has a high affinity for immunoglobulin. ‘Pre-clearing’ the lysates with protein A agarose beads was therefore likely to deplete immunoglobulin and reduce non-specific binding during subsequent immunoprecipitation with 4G10 beads (which contained protein A). It was important to check that incubation with protein A agarose beads did not reduce the phosphorylated protein content of lysates. Increasing volumes of protein A agarose beads or Blue Sepharose beads (which are known to bind serum albumin and other serum proteins) were incubated with equal aliquots of pooled cartilage lysate prior to 4G10 immunoprecipitation and western blotting. The highest volume of the protein A beads (500µl of slurry in PBS) and 100µl Blue Sepharose beads caused depletion of tyrosine phosphorylation in the lysates (Fig 4.11). Up to 100µl protein A agarose beads was subsequently used for pre-clearing 600µl of lysate.

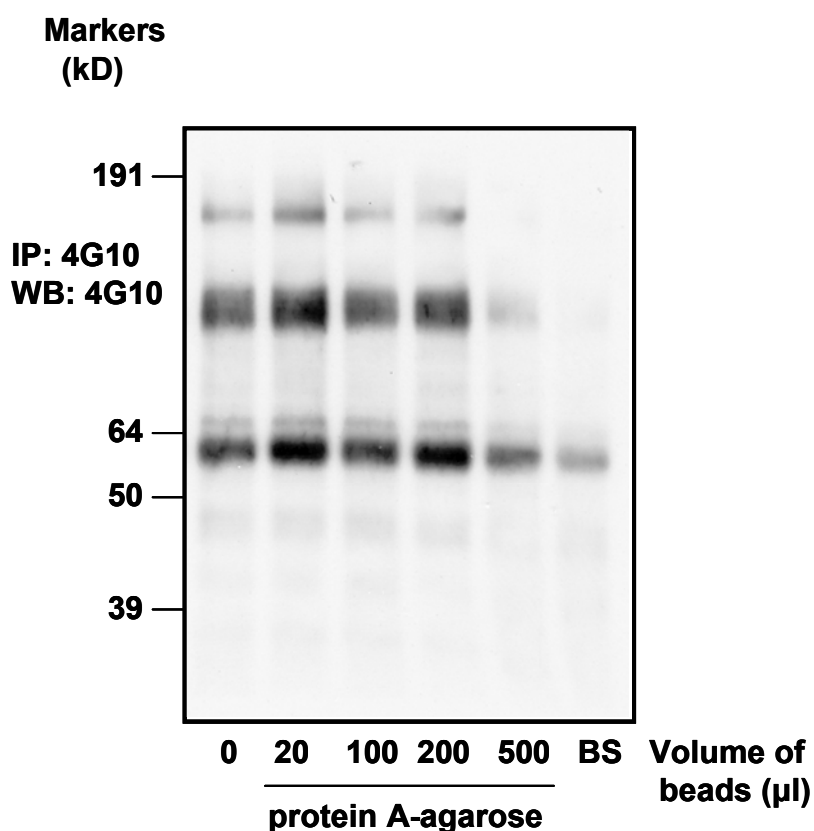


Figure 4.11. Does pre-clearing with protein A agarose or blue sepharose beads affect phosphotyrosine content of lysates?

~0.5g cartilage per point was dissected and cultured for 10min in serum-free culture medium, washed 3 times in PBS, then lysed in RIPA buffer. 600μl of clarified, pooled lysate was rotated for 1h at 4°C with increasing volumes of a 50% slurry of protein A agarose beads (protein A-agarose), or 100μl blue sepharose beads (BS). Supernatants were then incubated overnight with 10μl 4G10 beads. Beads were washed 5 times in RIPA, and then eluted with phenyl phosphate. Eluates were boiled with SDS sample buffer. Western blotting with 4G10-HRP antibody was carried out.

Stability & concentration of tyrosine-phosphorylated proteins: Some reports suggest an immunoprecipitation time of 1-2h for 4G10 antibody is optimal, although this clearly depends on the buffer system and the concentration of antigen. Cartilage lysates were incubated with 4G10 beads for either 2h or 18h, prior to 4G10 immunoprecipitation and western blotting. There were stronger bands and greater regulation after 18h of immunoprecipitation compared with 2h (Fig 4.12). Future immunoprecipitations were therefore carried out overnight (~18h). This optimised protocol, using 4G10 immunoprecipitation and phenyl phosphate elution is referred to henceforth as “standard 4G10 immunoprecipitation” (see Materials & Methods, Fig 2.3 for summary).

To be able to carry out further purification of tyrosine-phosphorylated proteins, it was important to ascertain whether they were stable in the eluted material. Following 4G10 immunoprecipitation from cartilage lysates, aliquots of pooled phenyl phosphate eluate were either mixed immediately with sample buffer (Lane 1, Fig 4.13) or kept at various temperatures for 24h prior to the addition of sample buffer and 4G10 western blotting. Compared with freshly prepared samples (Lane 1), those kept at room temperature lost their phosphotyrosines (Lane 2), and there was also some loss at 4°C (Lane 3, Fig 4.13).

Given the low relative concentration of tyrosine-phosphorylated proteins, a concentration procedure was likely to be necessary to make positive identifications by mass spectrometry. Any such procedure needed to conserve as far as possible the phosphotyrosine content of the samples. Possibilities were limited because of the presence of RIPA (and therefore SDS) in the samples (TCA precipitation was not possible). Poor stability made strategies such as freeze drying the samples inadvisable (Fig 4.13).

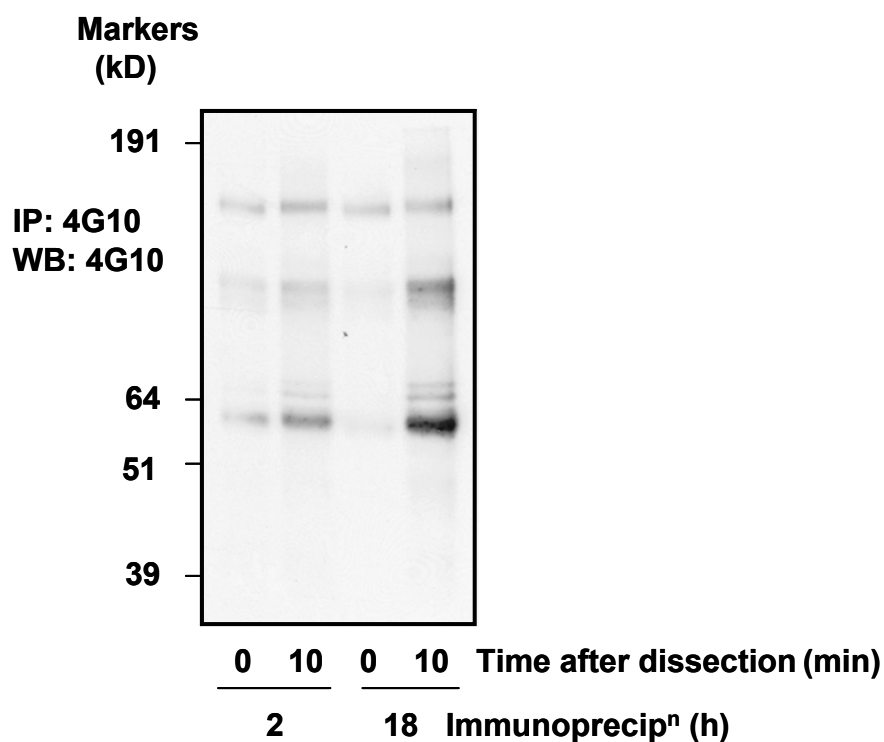


Figure 4.12. Investigating the optimal length of phosphotyrosine immunoprecipitation from cartilage lysates.

~0.5g cartilage was dissected either directly into ice cold lysis buffer (0) or into culture medium for 10min (10) prior to lysis. Lysates were incubated with 4G10 beads for either 2 or 18h prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate and boiling the eluate with SDS sample buffer. Samples were electrophoresed and assayed for phosphotyrosine content by western blotting with 4G10-HRP antibody. This is representative of 2 different experiments.

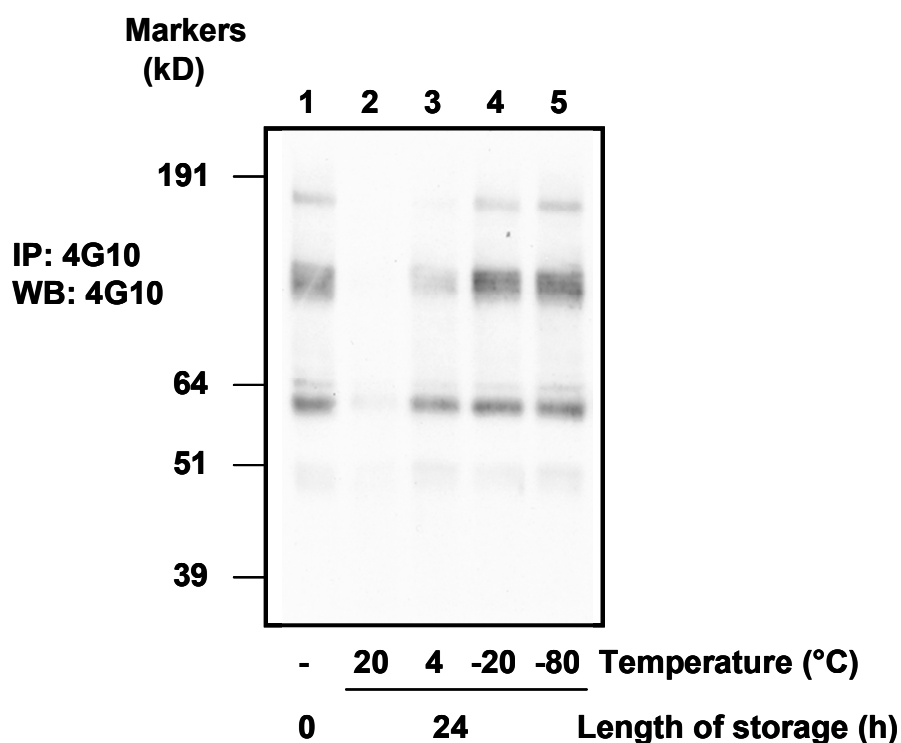


Figure 4.13. Stability of protein tyrosine phosphorylation in eluates from cartilage immunoprecipitates on storage.

5 x ~0.5g cartilage was dissected into culture medium for 10min prior to lysis. Lysates were incubated with 4G10 beads overnight, prior to washing the beads 5 times in RIPA and eluting in phenyl phosphate. Eluates were pooled, mixed and then aliquoted into 5 samples. The first sample was boiled with SDS sample buffer immediately (0). The remaining samples were stored for 24h at the various temperatures indicated, prior to boiling with SDS sample buffer. Samples were electrophoresed and assayed for phosphotyrosine content by western blotting with 4G10-HRP antibody.

Acetone precipitation carried out on eluates led to significant loss of phosphorylation on 4G10 western blotting compared with unconcentrated material (Fig 4.14, Lane 2). Using an electrophoresis clean-up kit (GE), there was preservation of tyrosine phosphorylation (Fig 4.14, Lane 3). This kit incorporated an acetone precipitation step, but also had several buffers to aid re-solubilisation of the pellet in low volumes. It took an additional 3-4 hours, with samples kept either on ice or at -20°C. This method was selected for the concentration of material in subsequent scale-up experiments.

4.5.3 Rapidity of injury-induced tyrosine phosphorylation following immunoprecipitation

It was important to see if the apparent rapid up-regulation of tyrosine phosphorylation after explantation seen on western blotting of cartilage lysates was still evident following optimised 4G10 immunoprecipitation. Trotters were equilibrated prior to dissection of cartilage either on to dry ice, or into culture medium for the times indicated prior to lysis. Standard 4G10 immunoprecipitation and 4G10 western blotting was carried out. Tyrosine phosphorylation of proteins was rapidly induced within 15-30 sec of injury (Fig 4.15A). The band at 60 kD, the upper bands of the complex at 100-120 kD and the 190 kD band were all seen at the earliest time point. These bands probably all corresponded to bands of similar sizes noted in earlier 4G10 western blotting experiments using the large gel system (Figs 4.1 & 4.4).

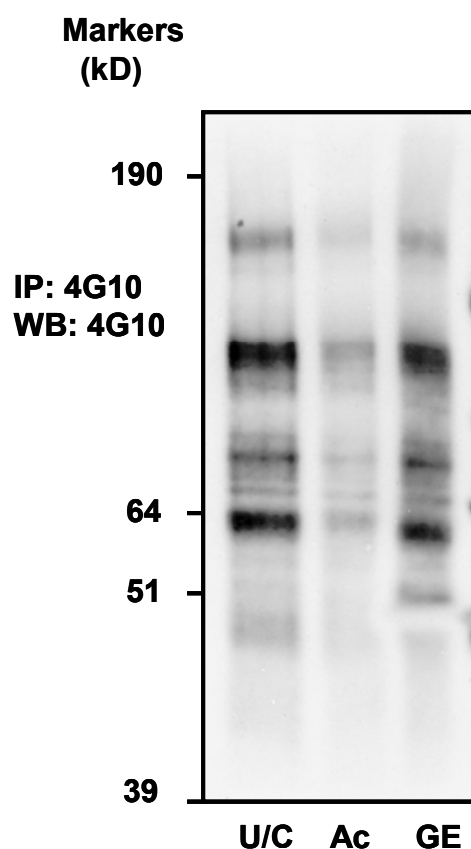


Figure 4.14. Concentration of eluates from 4G10 immunoprecipitates.

6g cartilage was dissected into culture medium for 10min prior to lysis (0.5g per sample). Lysates were incubated with 4G10 beads overnight prior to washing the beads 5 times in RIPA, and eluting in phenyl phosphate. Unconcentrated eluate was pooled (U/C). 40% of this material was concentrated by acetone precipitation (Ac). 40% of the material was concentrated using an electrophoresis clean up kit (GE). Equal amounts (cf. starting material) of unconcentrated and concentrated material (Ac, GE) were electrophoresed and phospho-western blotting with 4G10-HRP antibody carried out.

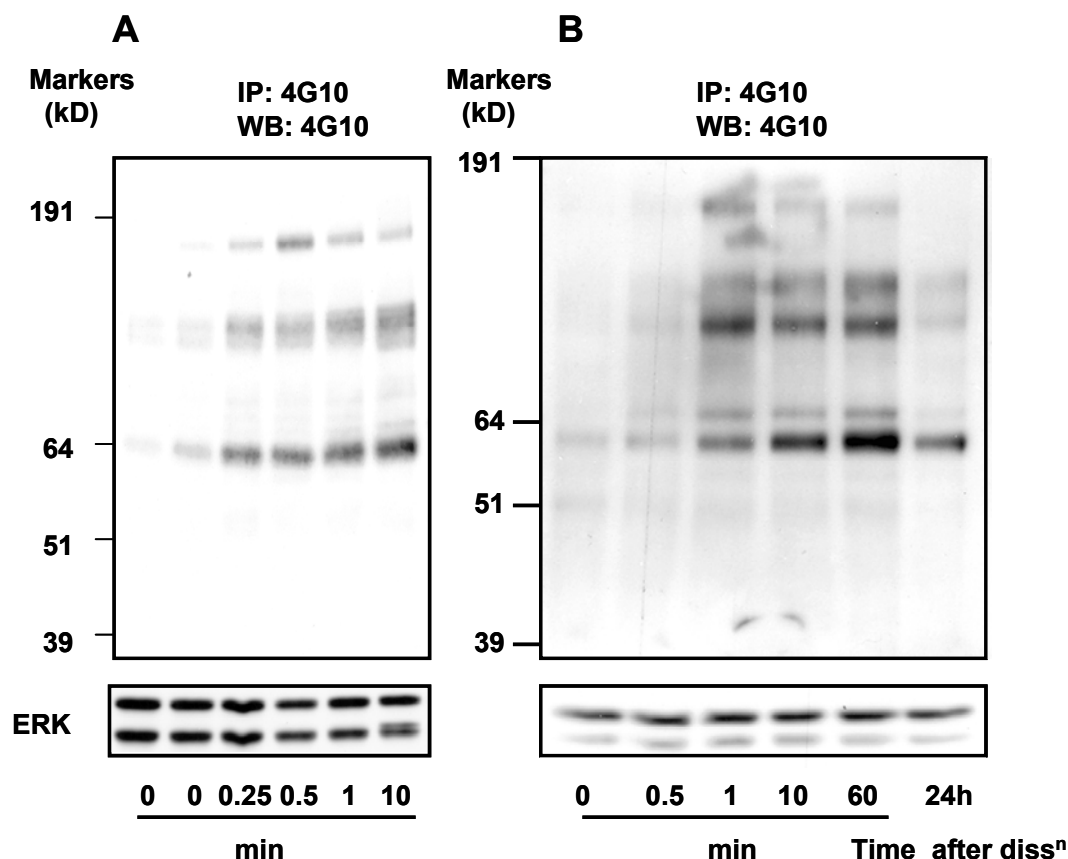


Figure 4.15. Induction of protein tyrosine phosphorylation in 4G10 immunoprecipitates of lysates is seen within seconds of cartilage injury. (A) and (B) are two separate but similar experiments.

For both A. & B., trotters were opened and equilibrated at 37°C. 3 cartilage pieces from a single joint surface were dissected either directly into a tube on dry ice (0) or into serum-free medium for increasing times. Points were performed in duplicate and pooled prior to lysis. Lysates were incubated with 4G10 beads overnight prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate solution and boiling the eluate with SDS sample buffer. Western blotting with 4G10-HRP antibody was carried out. Lysates were kept and electrophoresed separately and western blotting for ERK carried out to ensure equal amounts of cellular protein was used for immunoprecipitation.

In a separate experiment, cartilage was cultured for longer times prior to lysis and 4G10 immunoprecipitation. The phosphotyrosine pattern remained strong at 60min (Fig 4.15B). At 24h it was diminished, although had not returned to control levels, consistent with findings in previous experiments on rested explants (Fig 4.6A). In all experiments performed, the bands of interest were reproducibly present and strong at 10min, so this time point was chosen for further experiments.

4.6 Identification of tyrosine-phosphorylated proteins from pervanadate-stimulated cartilage

To identify proteins from gels, they would need to be visible on silver staining and be present in sufficient quantities for detection by mass spectrometry. The phosphatase inhibitor sodium pervanadate had caused marked up-regulation of immunoprecipitable tyrosine-phosphorylated proteins in chondrocytes (Fig 4.7). I investigated whether explantation into sodium pervanadate, or explantation alone, induced tyrosine phosphorylation of proteins which could be detected by silver staining following standard 4G10 immunoprecipitation.

Cartilage was either dissected immediately from the articular surface into RIPA buffer, or into medium with or without 10mM sodium pervanadate for 10min. Aliquots of lysates were incubated with either a non-immune mouse immunoglobulin control on protein A agarose beads (N/I control) or 4G10 beads. Eluates were mixed with SDS sample buffer and electrophoresed for either phospho-western blotting with 4G10 antibody or silver staining. On 4G10 western blotting, there was clear regulation of bands following explantation into medium alone. However, the cartilage which had been dissected into pervanadate showed strongly increased phosphorylation (~100 fold greater than cartilage dissected into medium alone) (Fig 4.16, left panel). No immunostaining was present in the non-immune precipitates on

phospho-western blotting. However, the non-immune beads did precipitate a number of proteins visible on silver stain, some of which aligned with silver stainable bands seen following 4G10 immunoprecipitation (Fig 4.16, right panel). The weak silver-staining bands in 4G10 immunoprecipitates from cartilage explanted into medium alone mostly appeared to be non-specific (i.e. some were reduced in the 10min injury lane compared with *ex-vivo* (0), as the former could be washed more extensively). In contrast, the 4G10 immunoprecipitates of cartilage explanted into pervanadate contained many silver-staining bands which were not present in the non-immune precipitates (Fig 4.16, right panel).

The large quantity of tyrosine-phosphorylated proteins evident on silver staining of immunoprecipitates from pervanadate-treated cartilage made pervanadate an attractive stimulus for preliminary attempts to identify cartilage phosphoproteins. ~0.75g cartilage was dissected directly from the articular surface into a tube on dry ice. Cartilage was also dissected into either serum-free medium for 10min, or into medium containing 10mM pervanadate for 20min prior to lysis and standard 4G10 immunoprecipitation. 4G10 western blotting or silver staining of the phenyl phosphate eluates was carried out. Explantation into pervanadate markedly induced tyrosine phosphorylation of many proteins as judged by western blotting. These bands were many fold stronger than the bands seen following explantation alone (Fig 4.17, left panel, 2 exposures are shown).

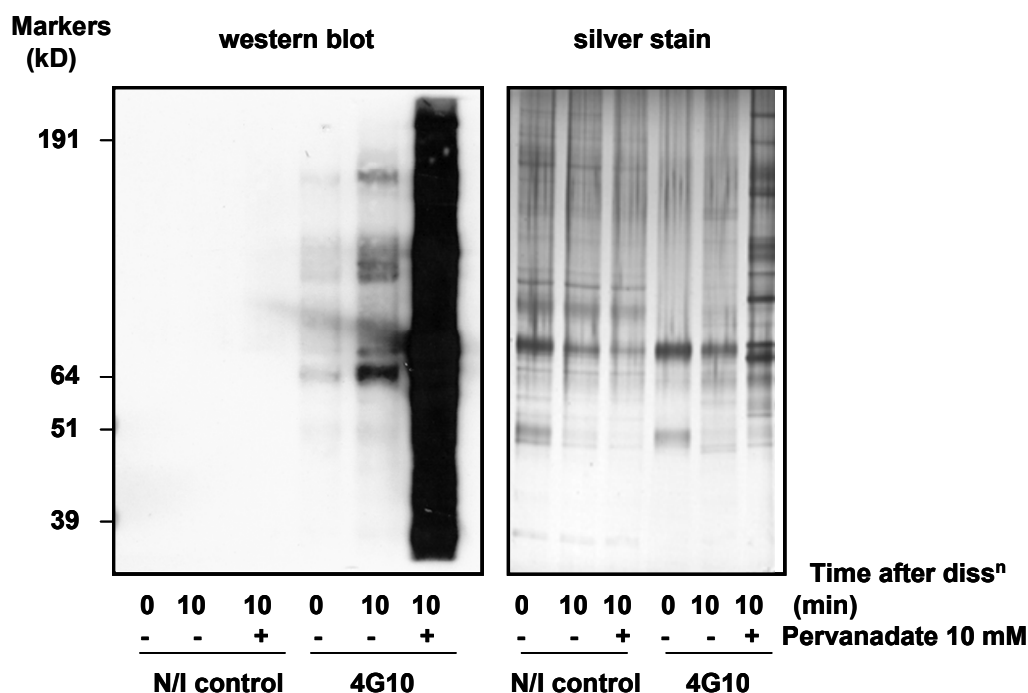


Figure 4.16. Explantation of cartilage into pervanadate solution induces strong protein tyrosine phosphorylation and specific bands on silver staining.

~0.5g cartilage was dissected either directly into ice cold lysis buffer (0) or into culture medium with (+) or without (-) 10mM sodium pervanadate for 10min prior to lysis. Lysates were incubated overnight with 4G10 beads (4G10) or protein A agarose pre-incubated with mouse immunoglobulin (N/I control) prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate and boiling the eluate with SDS sample buffer. Samples were electrophoresed and either assayed for phosphotyrosine content by western blotting with 4G10-HRP antibody (western blot) or silver stained (silver stain).

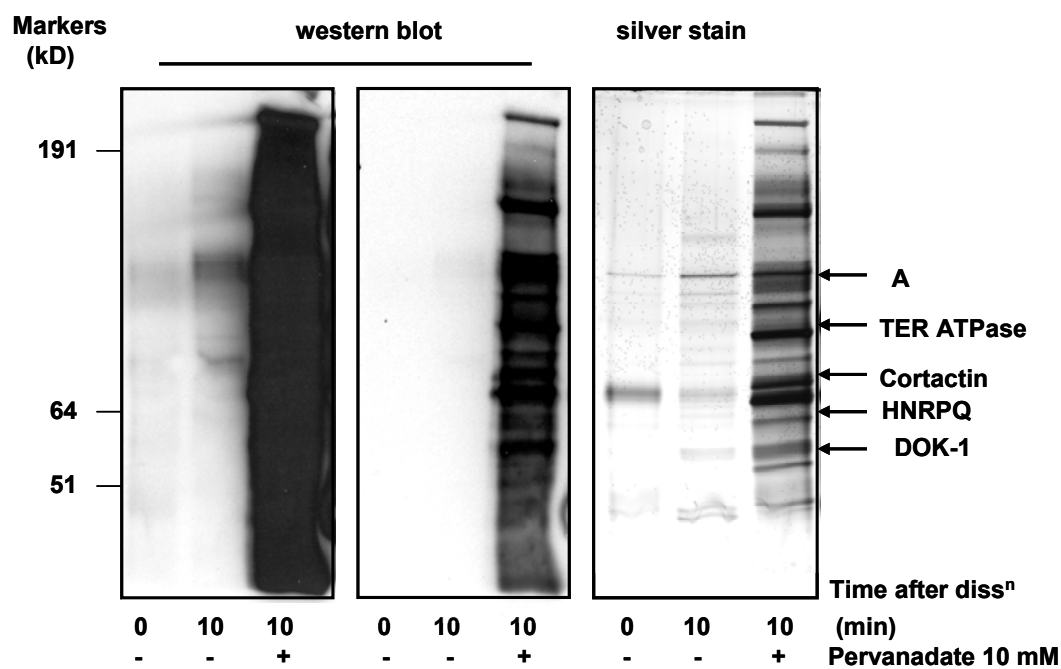


Figure 4.17. Identification of tyrosine-phosphorylated proteins induced by pervanadate.

~0.75g cartilage per lane was dissected either immediately into a tube on dry ice (0) or into serum-free medium with (+) or without (-) 10mM sodium pervanadate and cultured for 10min, prior to lysis. Lysates were incubated with 4G10 beads overnight prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate and boiling the eluate with SDS sample buffer. Samples were electrophoresed and either assayed for phosphotyrosine content by western blotting with 4G10-HRP antibody (western blot, 2 different exposures shown) or silver stained (silver stain). Bands from the silver stained gel were cut out, digested with trypsin overnight, and analysed by mass spectrometry.

Abbreviations: Transcriptional endoplasmic reticulum (TER) ATPase; heterogeneous nuclear ribosomal protein (HNRP)Q; downstream of tyrosine kinase (DOK)-1; unidentified band (A)

Multiple strong bands were seen on silver staining of samples from the pervanadate-stimulated cartilage (Fig 4.17, right panel). All bands were excised, trypsin digested and analysed by mass spectrometry. All of the proteins identified were known to be tyrosine-phosphorylated, many with multiple sites for tyrosine phosphorylation (Table 4.1). These identifications were all confirmed in subsequent experiments, with many of the proteins such as cortactin identified from multiple bands on further scale-up because of their abundance.

Table 4.1. Identification of phosphoproteins from pervanadate-stimulated cartilage by mass spectrometry.

Protein Name/ Accession number	Size (kDa)	Function	Unique peptides	Coverage %	Ion score
Transitional endoplasmic reticulum ATPase (TERA_HUMAN)	89	Golgi stacking	3	4.6	181
Cortactin (SRC8_HUMAN)	80/85	Src substrate; scaffolding protein	1	2.4	90
Heterogeneous nuclear riboprotein Q (HNRPQ_HUMAN)	69	mRNA processing	1	2.1	66
Docking protein 1 (DOK1_HUMAN)	62	Adaptor protein	1	4.8	33

MS identifications from gel shown in Fig 4.17 are listed. Ion score is $10 \cdot \log(P)$ where P is the probability that the observed match is a random event. Individual ion scores > 58 indicate identity or extensive homology ($P < 0.05$).

The identification of phosphoproteins validated the approach but none of them aligned with the 60 or 100 kD bands induced by explantation alone on the 4G10 blot. Some bands e.g. A in the pervanadate lane did align with bands which appeared to be regulated by injury alone, but could not be identified. Disappointingly, none of the

bands seen on silver stain of the immunoprecipitates made from explanted cartilage were identified by mass spectrometry (Fig 4.17, right panel). There was likely to be far less phosphotyrosine present in these samples, and silver-stainable bands were probably below the limit of detection for mass spectrometry (~100-500 fM). (On testing, silver staining had a sensitivity of about 60 fM, data not shown).

4.7 Identification of injury-induced tyrosine-phosphorylated proteins by mass spectrometry

To obtain sufficient tyrosine-phosphorylated proteins to allow their identification, it was necessary to scale-up the procedure. Loading larger volumes of sample on large non-gradient gels did not increase the intensity of the bands. It was not possible to load more sample on a minigel (50µl well capacity) without concentrating the sample.

Fig 4.18A shows a 16 fold scale-up of *ex vivo* versus explanted cartilage compared with Fig 4.17. Following 4G10 immunoprecipitation, phenyl phosphate eluates were pooled and concentrated using the GE kit. 1/6 of the concentrated sample was electrophoresed for western blotting with 4G10 antibody. This confirmed preservation of regulation of tyrosine phosphorylation following this procedure (Fig 4.18A, left panel). 5/6 of the sample was electrophoresed on the same gel, which was stabbed before being divided so that the resulting nicks on the western blot and the silver stained gel could be re-aligned. Silver staining of the slab was carried out. A large number of protein bands were seen in both lanes (Fig 4.18A, right panel). The 100-120 kD complex on 4G10 blotting directly aligned with a quadruplet of bands on silver stain which appeared to be regulated by injury. All of the bands were manually excised and digested with trypsin. A number of proteins were identified by electrospray tandem mass spectrometry (Fig 4.18B). The mass spectrometry and identification was performed by Dr R.Wait of the Kennedy Institute.

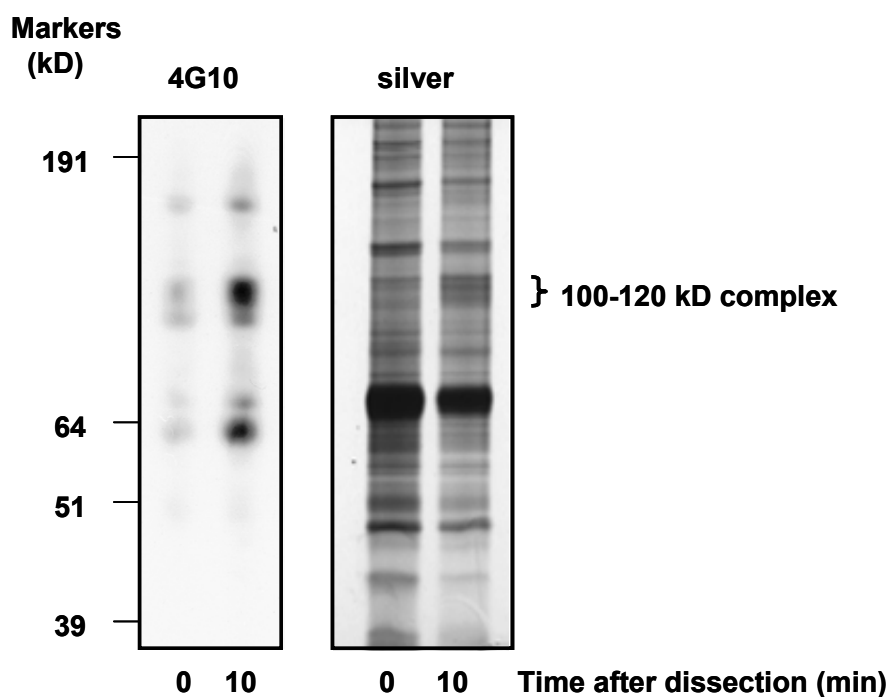
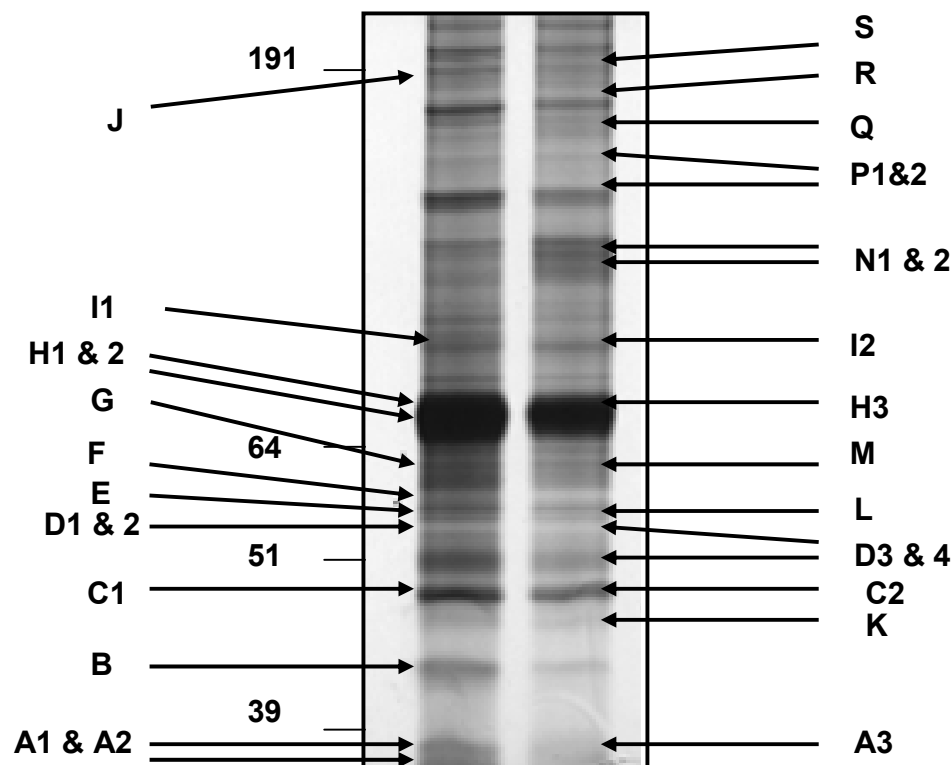


Figure 4.18A. Further scale-up and identification of tyrosine-phosphorylated proteins in injured cartilage.

~12g (0.5g x 24 samples) of porcine cartilage was dissected from MCP joints either directly into tubes on dry ice (0) or into serum-free medium and cultured for 10min (10) prior to lysis. Samples were lysed separately, pre-cleared once with 50 μ l protein A agarose and standard 4G10 immunoprecipitation carried out. Phenyl phosphate eluates were pooled and concentrated with the clean up kit (GE) prior to boiling samples with SDS sample buffer. 1/6 of the concentrated material for each sample was electrophoresed and assayed for phosphotyrosine content by western blotting with 4G10 antibody (left panel). 5/6 of the concentrated material was electrophoresed on the same gel, the gel divided, and silver staining carried out (right panel). The figure shows alignment of the two pieces of gel. Bands from the silver stained gel were cut out, digested with trypsin overnight, and analysed by mass spectrometry.



A	Glyceraldehyde 3 phosphate dehydrogenase
B	Actin
C	alpha-enolase
D	EGF-like repeat and discoidin 1-like domain containing protein 3
E	Glucose 6 phosphate isomerase
F	Protein disulphide isomerase kinase
G	Albumin
H	Porcine immunoglobulin (IgM)
I	Cartilage oligomeric matrix protein
J	Collagen type II
K	Collagen binding protein 2
L	Pyruvate kinase
M	Paxillin
N	Focal adhesion kinase
P	Keratin
Q	Epidermal growth factor receptor
R	Porcine immunoglobulin
S	Polyubiquitin

Figure 4.18B. Identification of tyrosine-phosphorylated proteins in injured cartilage by mass spectrometry.

The top two bands of the quadruplet which aligned with the 100-120 kD complex on 4G10 blotting were identified as the tyrosine kinase focal adhesion kinase (FAK) (Bands N1 and N2, Fig 4.18B). 3 peptides were identified, the second of which had the highest probability of being from FAK alone (Fig 4.19A&B). The alignment was with human, dog or chicken FAK, as the full porcine FAK amino acid sequence is not known. It does appear to be highly conserved in mammals, however.

A band on silver stain which aligned with the 60 kD regulated band on phospho-western was identified as paxillin, a substrate of FAK (Band M, Fig 4.18B). Only a single peptide was detected, but this was 30 amino acids long, increasing the probability of it being from paxillin alone (Fig 4.20A&B). (The alignment was with human paxillin, for the same reasons as above). Both FAK and paxillin are known to be tyrosine-phosphorylated, and both were found only in the 10min 'injured' cartilage lane, although were likely to be at the lower limit of detection for the mass spectrometer. None of the peptides detected contained a known tyrosine site (phosphorylation of the peptides is not confirmed by this technique in any case).

In addition, a peptide was found in the digest of the gel slice from the 190 kD region of the injury lane which was possibly from the epidermal growth factor receptor (EGFR) (Band Q, Fig 4.18B). However this finding was not confirmed in repeat experiments. Subsequent western blotting of unconcentrated lysates of injured cartilage or 4G10 immunoprecipitates with antibodies to total EGFR, phospho-tyr 1068 and phospho-tyr 845 EGFR (tyr 845 is a src-dependent tyrosine phosphorylation of EGFR) did not confirm EGFR phosphorylation on injury.

A Human PTK-2 (Focal adhesion kinase) amino acid sequence

1 maaayldpnl nhtpsnstkt hlgagversp gamervlkvf hyfenssepa twasiirhgd
 61 atdvrgiiqk ivdshkvkhv acygrfrshl rseevhwihl dmgsnsvrek yelahppeeew
 121 kyelrirykp **kgflnqfted kptlnffyyqq vks**dymseia drvdqialk lgcleirrsy
 181 wemrgnalek ksnyevlekd vglkrffprs lldsvkaktl rkliqqtfrrq fanlnreesi
 241 lkffeilspv yrfdkecfkc algsswiisv elaigpeegi syltdkgcnp thladfnqvq
 301 tiqysnsedk drkgmlqli agapeplvt apsltiaenm adlidgycrl vngatqsfii
 361 rpqkegeral psipklanse kqgvrtahv vsetddyaie ideedtytmp strdyeiqre
 421 rielgrcige gqfgdvhqgv ymspenpala vaiktcknct sdsvrekflq ealtmrqfdh
 481 phivkligvi tenpvwiime lctgelrsf lqvrkysldl aslilyayql stalaylesk
 541 rfvrhdiaar nvlssndcv klgdflsry medstyykas kgklpikwma pesinfrfft
 601 sasdvwmfgy cmweilmhgv kpfqgvknnd vigrienger lpmppncppt lyslmtkcwa
 661 ydpsrrprft elkaqlstil eekvqqeer mmesrrqvt vsdsggsde appkpsrpgy
 721 psprsegfy pspqhmvtqn hyqdsaalld rgigqvlpth lmeerlirqq qemeedqrwl
 781 ekeerflkpd vlrsgrsidr edgslqgptg nqhiyqpvgk pdpaappkpp prpgapghlg
 841 slaslsspgd synegvklqp qeispptan ldrsndrye nvtglvkavi emsskiqpap
 901 peeyvpmvke vglal**rtlla tvdetlpvlp asthre**iema qklinsdlge linkmklaqq
 961 yvmtslqqey kkqmltaaha lavdak**nlld vidqarl**ml gptrph

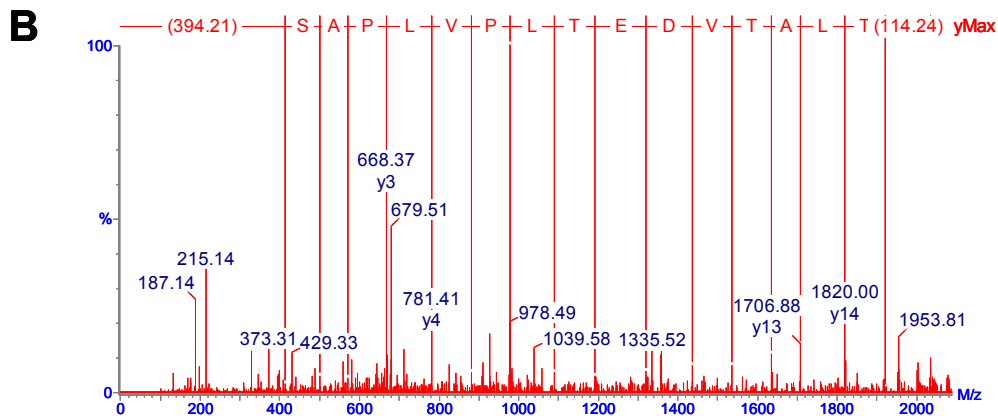


Figure 4.19. Identification of focal adhesion kinase as a tyrosine-phosphorylated protein induced by cartilage injury.

A. The full human FAK amino acid sequence is shown (Q81YN9_HUMAN). The peptide sequences identified by tandem mass spectrometry are highlighted in red. A search against the NCBI nr database retrieved FAK with a score of 78, 60 and 40 for each of the peptides respectively. (Ion score is $10 \times \log(P)$ where P is the probability that the observed match is a random event. Individual ion scores > 58 indicate identity or extensive homology ($P < 0.05$). The sequence coverage was 5.6% of the total sequence.

B. Tandem mass spectrum of the second FAK peptide RTLLATVDETLPLVLPASTHRE is shown.

A Human paxillin amino acid sequence

1 mddldallad lestshnik rpvlseetp ysytgnhty qeivappvp pppssealing
61 tildpldqwq psgsrhqq pqssspyvgs saktssvsnp qdsvgspcsr vgeeehvysf
121 pnkqksaeps ptvmstslgs nlseldrill elnavqhnp gfpadeanss pplpgalspl
181 ygvpetnspl gkgagpltke kpkrnggrgl edvrpsvesl ldelessvps pvpaitvnqg
241 emsspqrvts tqqrtrisas satreldeim aslsdfkgs w pleevllvs isssvqegek
301 yphpcaarhr tpslrspdqp ppcpqfmaag ktgssppgg ppkpgsqlds mlgslsqdln
361 klgvatvakg vcgackkpia qgvvtamgt whpehfvcth cqeegsmf ferdgqpyce
421 kdyhnlfspr cyycngpild kvvtaldrw hpehffcaqc gaffgpegfh ekdgkaycrk
481 dydfmfapkc ggcarailen yisaltlwh pecfvcrecf tpfvngsffe hdgqpycevh
541 yherrgsics gcqkpitgrc itamakfhfp ehfvcafclq qlnkgtfkeq ndkpycqncf
601 lkffc

B

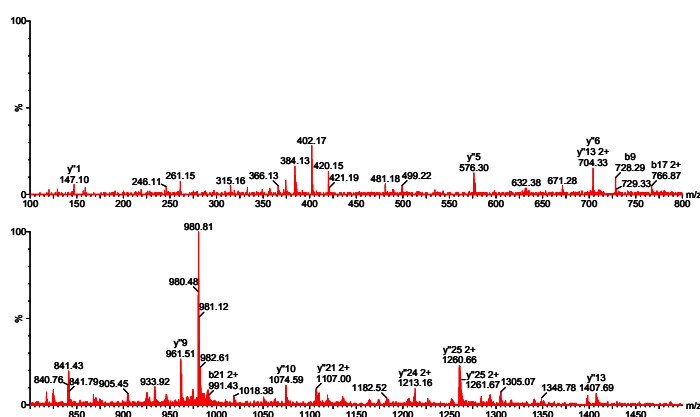


Figure 4.20. Identification of paxillin as a tyrosine-phosphorylated protein induced by cartilage injury.

A. The full human paxillin amino acid sequence is shown (P49023 PAXI_HUMAN). The peptide sequence identified by tandem mass spectrometry is highlighted in red.

B. Tandem mass spectrum of the peptide TGSSSPGGPPKPGSQLDSMLGSLQSDLNK from paxillin. The measured m/z of the triply protonated peptide was 980.52, corresponding to a deconvoluted Mr of 2938.5283 (calculated value = 2938.4342). A search against the NCBI nr database (20090228 release; 7,930,315 sequences) retrieved paxillin with a score of 74. (Ion score is $10 \times \log(P)$ where P is the probability that the observed match is a random event. Individual ion scores > 58 indicate identity or extensive homology $P < 0.05$).

Some proteins were identified from the gel in the uninjured lane only, or in both uninjured and injured lanes (Fig 4.18B). A number of serum, house-keeping and cartilage matrix proteins (not known to be tyrosine-phosphorylated and therefore likely to be contaminants) were identified. Some phosphoproteins including α -enolase and EGF-like repeat and discoidin 1-like domain containing protein 3 (EDIL-3) were present in both lanes. These phosphoproteins were not investigated further on the basis that their tyrosine phosphorylation status was not proven to be regulated by injury.

The identification of FAK and paxillin from the gel was of particular interest as they aligned with two of the four regions of interest defined by western blotting of phosphotyrosines. In a repeat of the experiment, FAK was again identified by mass spectrometry, but paxillin was not found and nor were any other further regulated phosphoproteins (data not shown). Validation of the injury-induced regulation of FAK and paxillin is carried out by immunoprecipitation and western blotting experiments described in the next chapter.

4.8 Discussion

Tyrosine kinase activation following cartilage injury is early and reproducible. Multiple tyrosine phosphorylations in cartilage occur within 30 seconds of explantation. The identification by mass spectrometry of FAK and paxillin as two proteins which are phosphorylated in response to injury is interesting. FAK is required for cell spreading (the formation of focal adhesions), cell proliferation and motility. Knockout of FAK in mice results in an embryonic lethal phenotype due to defects in mesodermal tissues and defects in angiogenesis (Shen et al., 2005). The mechanism by which FAK becomes activated is not well understood. In response to various stimuli including growth factors, integrin binding or cell stress, FAK auto-phosphorylates on tyr 397 (Lietha et al., 2007). This brings about a conformational change in the protein, which allows binding of the auto-phosphorylated site and a nearby PxxP motif to src, via src's SH2 and SH3 domains (Lietha et al., 2007). Subsequent phosphorylation of further tyrosine sites in the activation loop of FAK by src (tyr 576 and tyr 577) leads to full activation of FAK (Calalb et al., 1995). It is this FAK/src complex which then phosphorylates substrates including paxillin (at two main sites) (Schaller and Parsons, 1995) and p130cas (crk associated substrate – another adaptor protein seen at focal adhesions). The FAK/src complex can also associate with PI3 kinase and phospholipase C (Schaller and Schaefer, 2001; Turner, 2000). Phosphorylation of paxillin creates binding sites for SH2 domains of other signalling proteins at focal adhesions, including src, another adaptor protein Crk and c-src kinase (Csk – a negative regulator of srcs) (Schaller and Parsons, 1995).

Whether such tyrosine kinase activity could be part of a mechano-transducing mechanism is unknown. There are no reports of FAK phosphorylation in response to direct tissue injury. One group showed that stretch of mid-palatal suture cartilages in

rats induced FAK expression, formation of stress fibres and ERK activation (Takahashi et al., 2003). Another group noted tyrosine phosphorylation after mechanical stimulation of cultured chondrocytes, and by investigation of potential candidates demonstrated that FAK was phosphorylated (Lee et al., 2000). This phosphorylation was suggested to be integrin-dependent.

The peptide identification by mass spectrometry identifies proteins, and not phospho-proteins, or phospho-sites. The technique does not confirm that a phospho-protein was identified, although the identifications of FAK and paxillin were only made from the injured cartilage immunoprecipitates. Mass spectrometry can identify phosphorylated amino acids but higher concentrations of the proteins than used here are needed for successful identification. In order to confirm the identifications made by mass spectrometry, antibodies to the proteins and their phosphorylation sites are used in the next chapter.

2 out of the 4 major protein bands on 4G10 immunoprecipitation were identified by mass spectrometry. It is notable that both FAK and paxillin are substrates of src: FAK activation is dependent upon phosphorylation by src, and src also appears to be capable of phosphorylating paxillin independently of FAK *in vitro* (Schaller and Schaefer, 2001). In addition, a fragment of the EGF receptor was found in the third band at 190 kD in one experiment. Like FAK and paxillin, this is another src substrate (Wu et al., 2002) but the identity of this band could not be verified immunologically. The fourth band at 80 kD was in a similar region of the gel to the src substrate cortactin which had been identified in pervanadate-treated cartilage lysates. Cortactin is a doublet of 80/85 kD. It acts as a scaffolding protein, promoting actin polymerization and is also involved in receptor mediated endocytosis and vesicular trafficking (Tehrani et al., 2007; Zhu et al., 2007). Further experiments to investigate whether

cortactin was phosphorylated upon cartilage injury are also described in the next chapter.

It was surprising that so few bands were seen in injured cartilage 4G10 immunoprecipitates. Given the complex pattern on 4G10 blotting, one might have anticipated more. The low number of proteins might have been because of incomplete phosphotyrosine immunoprecipitation. The proteins immunoprecipitated and identified probably represented the most abundant and most highly phosphorylated (FAK and paxillin both have multiple tyrosine phosphorylation sites). The immunoprecipitation process was likely to preferentially enrich multiply phosphorylated proteins in this way, and these were not necessarily the most important. Various other strategies might have been incorporated to increase the yield of phosphoproteins. It has been reported that 4G10 may not immunoprecipitate certain phospho-tyrosine motifs, for example where arginine is an adjacent residue (Bergstrom Lind et al., 2008). Other groups favour alternative antibodies such as PY100, or mixtures of phosphotyrosine antibodies which increase the number of identifications, and such a combination could have been tested (Bergstrom Lind et al., 2008; Hinsby et al., 2003). Another strategy to purify phosphoproteins is immobilised metal ion affinity chromatography (IMAC). Here, a column of gallium or iron preferentially binds phosphorylated amino acids (Hinsby et al., 2004). However, this would preferentially enrich the more abundant serine and threonine phosphorylations in preference to tyrosine phosphorylations. It also requires prior digestion to a peptide mixture. It was not felt to be suitable for analysis of complex solutions from tissue lysates.

It was possible that other less abundant proteins of a similar size were induced and over-lay the identified proteins using this 1D gel system, and have not been identified.

The possibility of 2D electrophoresis was considered but would have required further scale-up.

The observation that the induction of tyrosine phosphorylation following injury was rapid and strong on western blotting would suggest that chondrocyte activation in the tissue may not be restricted to the cut edge: cells distant to the injury may also be activated. To test this possibility, phosphorylated proteins would need to be visualised in the cartilage by immunolocalisation. Phosphorylation of ATF-2 and translocation of the p65 subunit of NF κ B 30 minutes after explantation from the articular surface has been previously demonstrated throughout the mid and deep zones of cartilage (see A. Didangelos, Ph.D. thesis, 2008 and Figure 1.5). However, it is not clear whether the immediate response is localised to the cut edge, or whether distant cellular activation is seen immediately after the injury. Immunolocalisation of a tyrosine-phosphorylated protein after injury to another connective tissue is shown later in Chapter 6.

5 The role of tyrosine kinases in the response of cartilage to injury

5.1 Introduction

Dissection of cartilage induced within seconds phosphorylation of tyrosine residues on a number of proteins, as visualised by phosphotyrosine immunoprecipitation and western blotting. These phosphorylations were prevented by the src inhibitor, PP2. By phospho-selective purification from cartilage lysates, two known phosphoproteins, focal adhesion kinase (FAK) and its substrate, paxillin were identified from silver stained gels. It was important to verify that these proteins accounted for two of the regulated bands of interest seen on 4G10 antibody staining. Their phosphorylation in response to injury was therefore investigated by the use of antibodies to the proteins and to specific phosphorylation sites. It was also important to investigate whether FAK or other tyrosine kinases such as src family kinases might be upstream of MAPK or IKK activation seen following injury.

5.2 Verification of mass spectrometry identifications by western blotting

The presence of the proteins in the 4G10 immunoprecipitates was investigated by western blotting the precipitates with antibodies. Western blotting for FAK showed a band at around 120 kD whose intensity was increased following dissection (FAK is a 125 kD protein) (Fig 5.1A), and for paxillin a similarly regulated band at 60 kD (paxillin is a 68 kD protein) (Fig 5.1B). The membranes were re-probed with 4G10 antibody (right hand panels). The FAK band aligned with the top 2 bands of the 100-120 kD cluster on 4G10 western blotting. It did not account for the lower part of the 4G10-stained complex. The paxillin band on western blot aligned exactly with the 4G10-stained 60kD band.

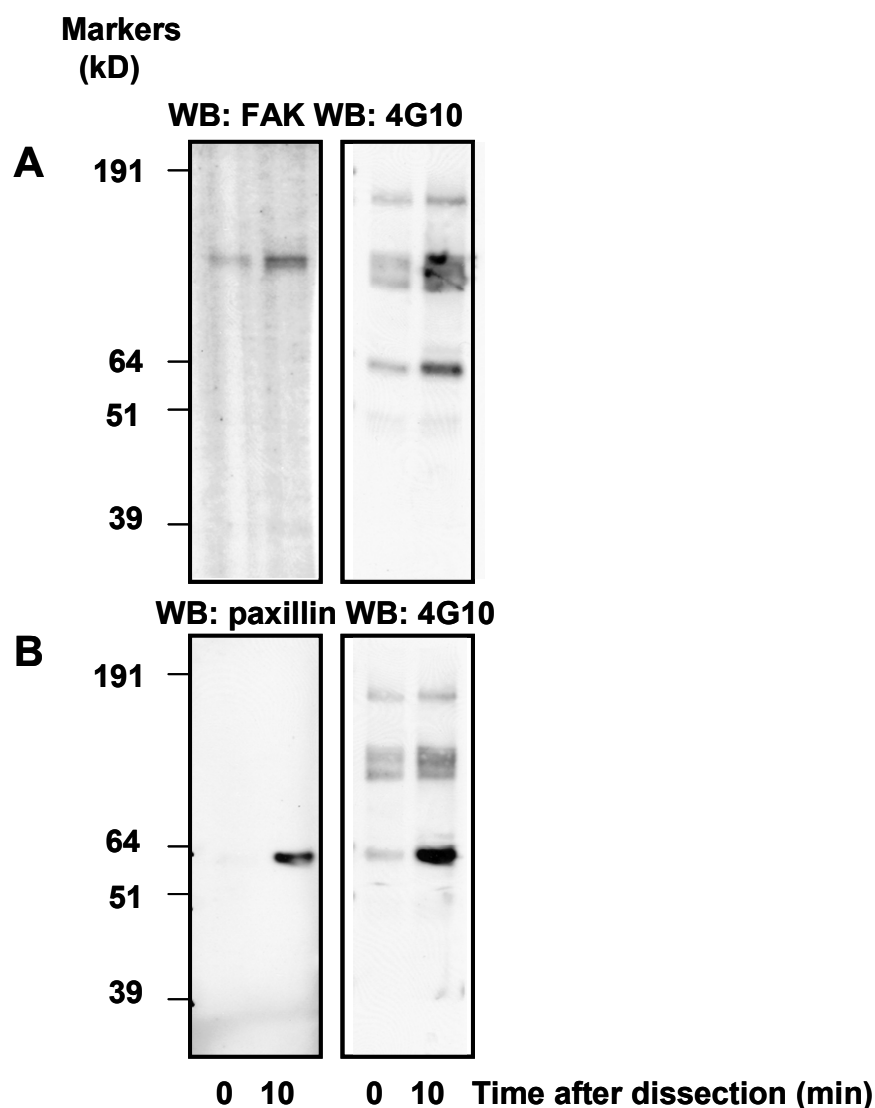


Figure 5.1. Western blotting of 4G10 immunoprecipitates of injured cartilage for (A) FAK and (B) paxillin.

For both **A.** & **B.**, ~0.5g cartilage was dissected either directly into ice cold lysis buffer (0) or into culture medium for 10min (10) prior to lysis. Lysates were incubated with 4G10 beads overnight prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate and boiling the eluates with SDS sample buffer. Western blotting of the 4G10 immunoprecipitates with **A.** antibodies to FAK protein (left upper panel), or **B.** antibody to paxillin protein (left lower panel) was carried out. The membranes were re-probed with 4G10 antibody (right panels). **A.** and **B.** are both representative of 2 experiments.

The phosphorylation of tyrosine residues at 60 kD and 100-120 kD was seen within 20-30sec of injury by phospho-western blotting with 4G10 antibody. To ascertain whether the phosphorylation of FAK and paxillin was seen as rapidly after injury, freshly dissected cartilage was processed and precipitated with 4G10 as previously. Eluates of the precipitates were western blotted for FAK protein. The membrane was successively re-probed with antibody to total paxillin and then 4G10 antibody. Induction of a doublet was seen 20sec after dissection on western blotting for FAK. Induction was stronger at 10min (top panel, Fig 5.2). In contrast, western blotting with paxillin antibody showed increase of tyrosine-phosphorylated protein at 10min but not by 20sec (middle panel, Fig 5.2). These bands aligned with the respective bands stained by 4G10.

5.2.1 Cortactin is also phosphorylated following cartilage injury

Another src substrate, cortactin, had been identified from 4G10 immunoprecipitates of cartilage treated with sodium pervanadate (Ch 4, Fig 4.17). To test whether tyrosine phosphorylation of cortactin occurred after injury, cartilage was either snap frozen or dissected into medium for 10min prior to lysis and standard 4G10 immunoprecipitation. Eluates were western blotted with antibody to cortactin. Induction of cortactin tyrosine phosphorylation was seen in cartilage after explantation (Fig 5.3). This doublet aligned with the 80 kD band visible on 4G10 western blotting (see Chapter 4, Fig 4.17).

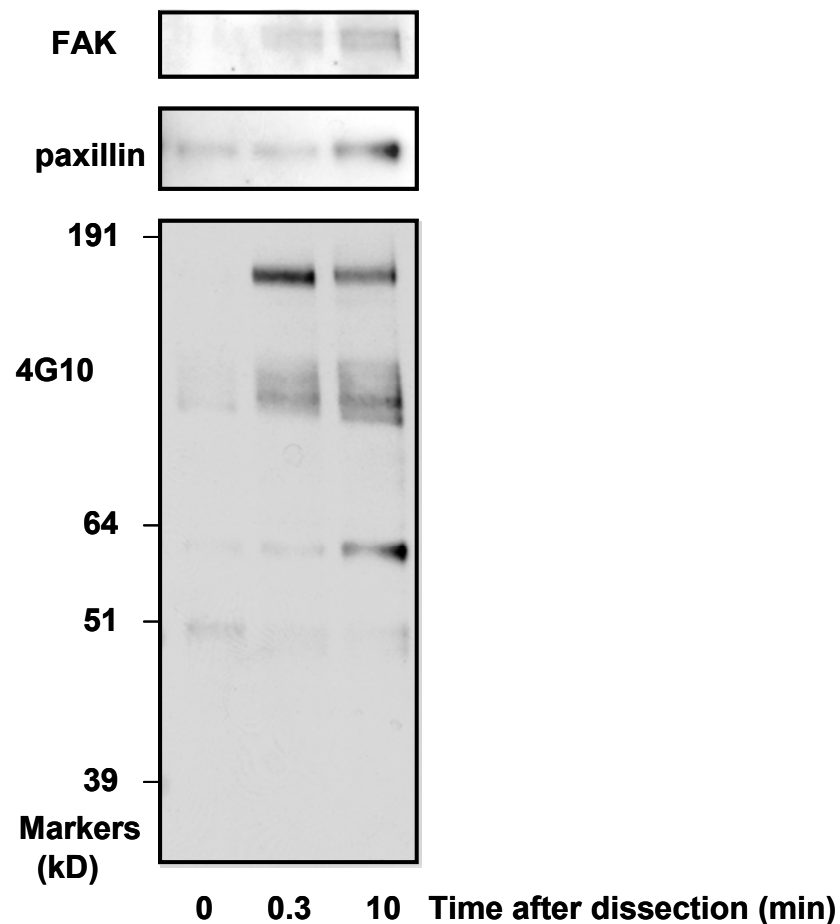


Figure 5.2. FAK is phosphorylated within seconds of explantation of articular cartilage.

Trotters were equilibrated at room temperature, and then a total of 8 pieces of cartilage (from 4 different trotters) was dissected either immediately into ice cold lysis buffer (0) or into medium and cultured for 20sec (0.3) or 10min prior to lysis. 4G10 immunoprecipitation and western blotting of the eluted material was carried out with antibodies to FAK. The membrane was then re-probed with paxillin antibody and also with 4G10 antibody (lower panel). This is representative of 2 experiments.

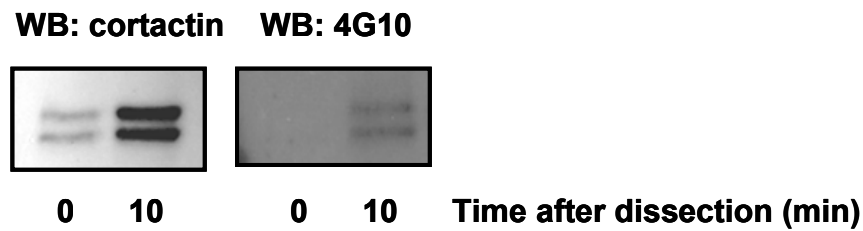


Figure 5.3. Phosphorylation of cortactin is induced by cartilage explantation.

~0.5g cartilage was dissected either directly into a tube on dry ice (0), or into medium and cultured for 10min (10) prior to snap freezing. Following lysis and 4G10 immunoprecipitation, western blotting of eluted material with antibody to cortactin was carried out (left panel). The membrane was re-probed with 4G10 antibody (right panel).

5.3 Investigation of FAK and paxillin phosphorylation in the response of cartilage to injury by the use of phospho-site specific antibodies

To confirm that phosphorylated forms of FAK could be detected in cartilage lysates as well as 4G10 immunoprecipitates, and to investigate the nature of the phosphorylation, antibodies to phosphorylation sites were employed. FAK has 6 known tyrosine phosphorylation sites. As discussed in Chapter 4, activation of FAK is thought to be initiated by auto-phosphorylation at tyr 397. This causes a conformational change in the molecule which unmask two further tyrosine phosphorylation sites (tyrs 576 and 577) which are targets for src (generally c-src in other cell systems). Their phosphorylation is required for full activation of FAK.

Western blotting of the cartilage lysates with phospho-tyr 397 (Biosource) and phospho-tyr 576 FAK (Upstate) antibodies was carried out. Phospho-tyr 397 antibodies stained a band at ~120 kD, which was not regulated by injury (Fig 5.4, upper panel). In comparison, increase of phospho-tyr 576 FAK staining was seen within 10min of dissection (Fig 5.4, middle panel). Phosphorylation was still evident at 60min, but had returned to the basal level (0min) by 24h. The membrane was re-probed with antibodies to ERK to check for equal loading of cellular protein. That phospho-397 antibodies did not detect a regulated band suggested either that this site was not being phosphorylated or that the antibodies might also detect un-phosphorylated FAK, and may not be specific for the phospho-form. Neither phosphorylated nor un-phosphorylated forms of the peptide used to raise the antibodies were available from the manufacturer, so their specificity could not be tested. A subsequent experiment where FAK immunoprecipitates from cartilage lysates were then probed with phospho-tyr 397 antibodies also showed a band which was not regulated by injury (data not shown).

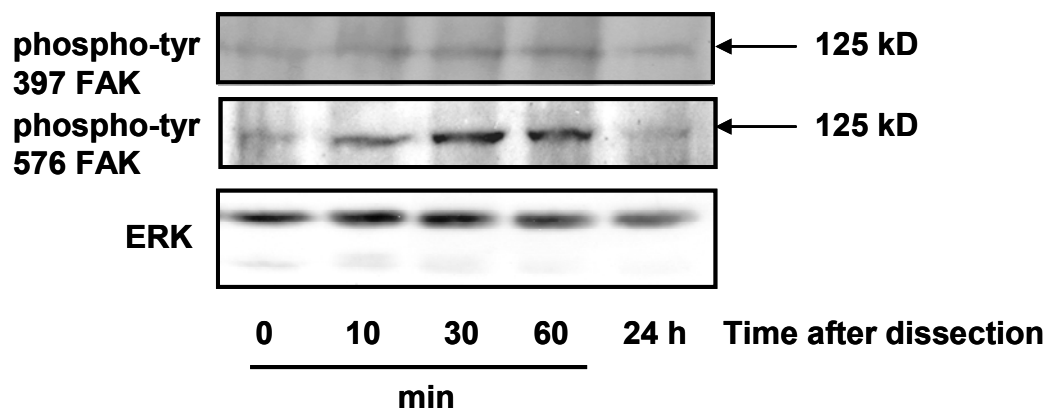


Figure 5.4. FAK is phosphorylated upon tyr 576 following explantation of cartilage.

~0.5g cartilage was dissected either directly into lysis buffer (0) or into serum-free medium and cultured for increasing times prior to lysis. Western blotting of the cartilage lysates with phospho-tyr 397 FAK antibodies or phospho-tyr 576 FAK antibodies was carried out. The membrane was re-probed with antibodies to ERK to ensure equal cellular protein loading.

In view of this uncertainty regarding the specificity of phospho-tyr 397 antibodies, they were not used in further experiments. They had been used successfully by other groups, for example in detecting a regulated band in lysates of human platelets treated with low density lipoprotein or thrombin (Relou et al., 2003).

5.3.1 Phospho-FAK in cartilage 4G10 immunoprecipitates

Antibodies to FAK protein had been tested on 4G10 immunoprecipitates (Fig 5.1A). Eluates of 4G10 immunoprecipitates were also blotted for phospho-tyr 576 FAK. The membrane was re-probed with 4G10 antibody. The phospho-tyr 576 FAK antibodies detected two strong bands induced by dissection which aligned with the top two bands of the 100-120 kD complex on 4G10 blotting (Fig 5.5). These experiments further supported the identification of FAK as a kinase which was phosphorylated upon cartilage injury.

5.3.2 Phosphorylation of paxillin is seen in response to explantation of cartilage

Paxillin is also a multiply phosphorylated protein, with 4 known tyrosine phosphorylation sites. Src and activated FAK form a complex which phosphorylates targets including paxillin (Schaller and Parsons, 1995). Tyr 118 (and tyr 31) are phosphorylation sites on paxillin thought to be dependent on FAK or src (Schaller and Schaefer, 2001). Antibodies to phospho-tyr 118 paxillin (Cell Signaling) were therefore employed in further experiments.

Cartilage was dissected either directly into lysis buffer (0) or into serum-free medium and cultured for either 5 or 10min, or into pervanadate for 10min prior to lysis. Western blotting of the cartilage lysates with phospho-tyr 118 paxillin antibodies (henceforth referred to as phospho-paxillin antibodies) was carried out. Induction of phosphorylation of paxillin was seen within 5min of explantation (Fig 5.6).

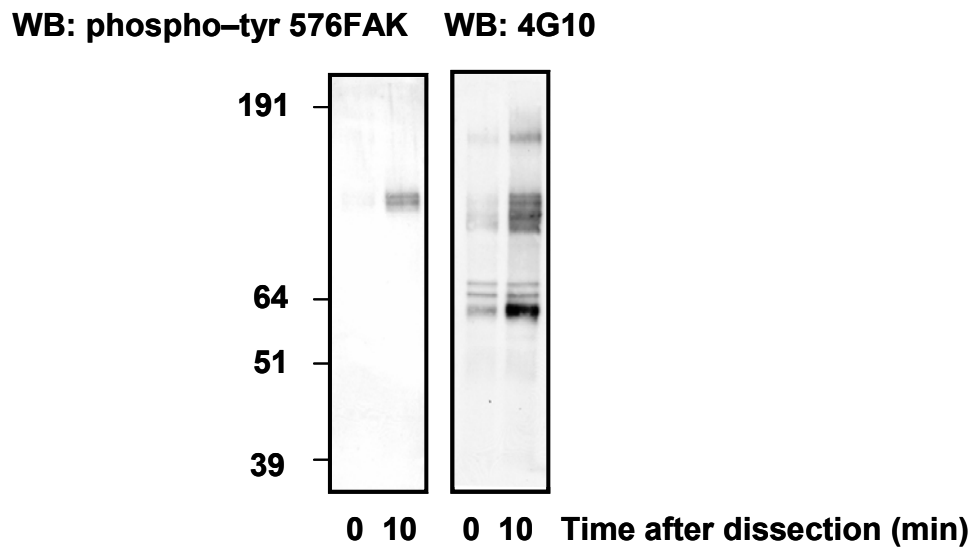


Figure 5.5. The 100-120 kD complex on 4G10 immunoprecipitation contains phosphorylated FAK.

~0.5g cartilage per point was dissected either directly into lysis buffer (0) or into serum-free medium for 10min prior to lysis and 4G10 immunoprecipitation. Beads were washed and eluted with phenyl phosphate, and western blotting carried out on the eluates with phospho-tyr 576 FAK antibodies. The membrane was re-probed with 4G10 antibody. This is representative of 3 experiments.

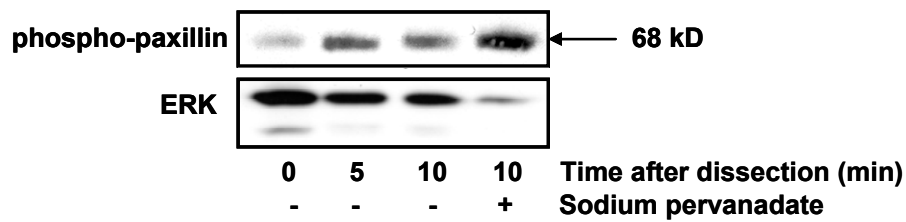


Figure 5.6. Paxillin is phosphorylated following explantation of cartilage.

~0.5g cartilage was dissected either directly into lysis buffer (0) or into serum-free medium and cultured for increasing times prior to lysis, or into medium containing 10mM sodium pervanadate for 10min prior to lysis. Western blotting of the cartilage lysates with phospho-tyr 118 paxillin antibodies was carried out. The membrane was re-probed with antibodies to ERK to ensure equal cellular protein loading.

Pervanadate, as expected, strongly increased tyrosine phosphorylation of paxillin, and was included as a positive control in future experiments (Fig 5.6). The ERK western blot suggests that the pervanadate sample is underloaded in this experiment.

The phosphorylated form of paxillin was also shown to be present in the 4G10 immunoprecipitates of the injured tissue (Fig 5.7A, left panel). The membrane was re-probed with 4G10. The phospho-paxillin band exactly aligned with the inducible 60 kD band on 4G10 blotting (Fig 5.7A, right panel).

The specificity of the band was checked by testing with the immunising phosphopeptide (obtained from Cell Signaling). Cartilage was dissected directly into lysis buffer (0) or into serum-free medium for 10min (10) prior to lysis and standard 4G10 immunoprecipitation. Eluted material for each experimental condition was pooled and 4 sets of equal aliquots electrophoresed, transferred on to nitrocellulose and then the membrane split into 4. The first membrane was incubated overnight with 1:1000 dilution of the phospho-paxillin antibodies. The second, third and fourth pieces of membrane were incubated overnight with 1:1000 antibodies which had been pre-incubated for 1h with various concentrations of the control peptide. The next day, all four membranes were developed with secondary antibodies and chemiluminescence together. In the absence of peptide, a regulated band at 60kD was seen on phospho-western blotting. At all concentrations of the peptide, the 60 kD stained band was not seen (Fig 5.7B). This confirmed the specificity of the antibody. No unphosphorylated peptide was available, so I was unable to prove that the antibodies were only recognising phosphorylated forms of paxillin. This seemed likely, given that little staining with the antibodies of uninjured cartilage lysates was seen (Fig 5.6).

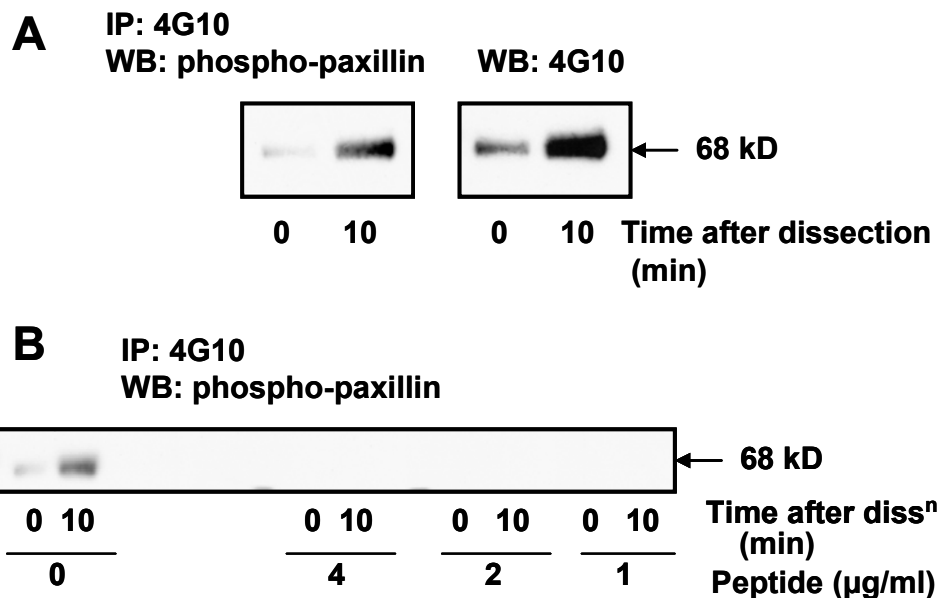


Figure 5.7. The recognition of the 60 kD protein on 4G10 immunoprecipitation by phospho-paxillin antibodies (A), is prevented by pre-incubation with the immunising phospho-paxillin peptide (B).

A. ~0.5g cartilage per point was either dissected directly into lysis buffer (0) or into serum-free medium for 10min (10) prior to lysis and 4G10 immunoprecipitation. Western blotting of eluates with phospho-paxillin antibodies was carried out.

B. 4 x 0.5g cartilage was either dissected directly into lysis buffer (0) or into serum-free medium for 10min (10) prior to lysis and 4G10 immunoprecipitation. Beads were eluted with phenyl phosphate. Eluted material was pooled, electrophoresed, transferred on to nitrocellulose and the membrane split into 4. The membranes were incubated overnight with 1:1000 dilution of the phospho-paxillin antibodies alone, or 1:1000 antibodies which had been pre-incubated for 1h with increasing concentrations indicated of the control phospho-paxillin peptide (peptide). All four membranes were then developed together, with secondary antibody and chemiluminescence.

5.3.3 A time course of tyrosine phosphorylation of FAK and paxillin in response to cartilage injury

The phospho-FAK and phospho-paxillin antibodies, in conjunction with 4G10 immunoprecipitation were used in further experiments to examine the induction of tyrosine phosphorylation after cartilage injury. Cartilage was lysed at increasing times after dissection and culture in serum-free medium. Eluted material from 4G10 immunoprecipitation was western blotted with antibodies to phospho-tyr 576 FAK and phospho-paxillin. The lysates were run separately and western blotting for ERK carried out, to ensure that equal amounts of cellular protein had been included in the immunoprecipitation. Weak induction of FAK phosphorylation was seen at 30sec after explantation in this experiment, confirming earlier findings with anti-FAK antibodies (Figs 5.8 & 5.2). Phosphorylation of paxillin was just visible at 30sec after explantation, and clearly present at 1min (Figs 5.8 & 5.2). The phosphorylation of FAK and paxillin was maximal between 10 and 60min. After 24h, FAK and paxillin phosphorylation had declined, but not to baseline levels (Fig 5.8). This was consistent with previous experiments which suggested increased tyrosine phosphorylation at 24h after explantation on 4G10 western blotting (see Ch 4, Fig 4.15).

5.3.4 FGF is not required for FAK activation following cartilage injury

Growth factors such as FGF are reported to activate FAK and src (discussed in Ch 1, section 1.6), so the activation of FAK and subsequent phosphorylation of paxillin could have been due to the release of FGF-2 following cartilage injury. To test this hypothesis, similar experiments were performed to those in Chapter 4, section 4.4, but western blotting with phospho-FAK and phospho-paxillin antibodies was carried out in place of 4G10 antibody.

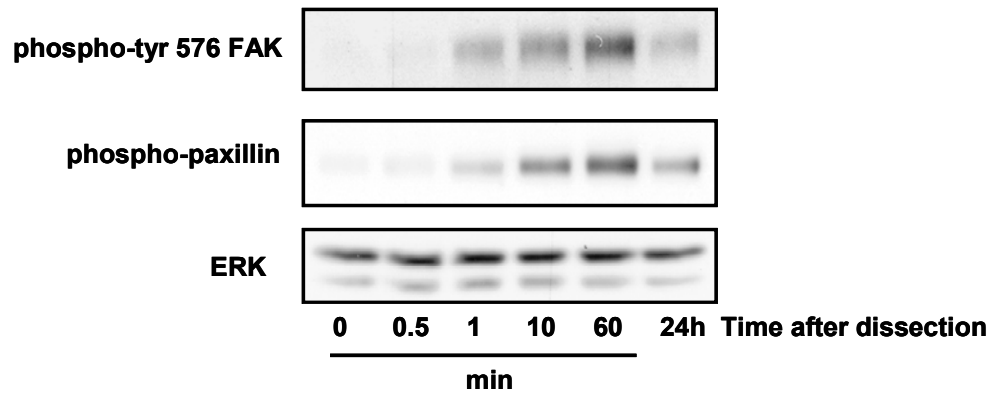


Figure 5.8. FAK and paxillin phosphorylation are rapid and sustained following explantation of cartilage.

Trotters were equilibrated at 37°C, and then a total of 8 pieces of cartilage (from 4 different trotters) was dissected either immediately into ice cold lysis buffer (0) or into medium and cultured for increasing times prior to lysis. 4G10 immunoprecipitation and western blotting of the eluted material was carried out with antibodies to phospho-tyr 576 FAK. The membrane was then re-probed with phospho-paxillin antibodies. Lysates were electrophoresed separately for western blotting with ERK antibodies, as a control for equal cellular protein loading. This is representative of 3 experiments..

No increase in phosphorylation of FAK or paxillin was seen in response to re-cutting of rested cartilage explants or treatment of rested explants with exogenous FGF-2 (data not shown).

The effect of the FGF receptor inhibitor PD173074 on the initial induction of FAK and paxillin phosphorylation following explantation was tested. Trotters were injected with culture medium with, or without, the FGF receptor inhibitor PD173074 and equilibrated at 37°C. Cartilage was then dissected into medium with or without the inhibitor and lysates were western blotted for phospho-tyr 576 FAK and phospho-paxillin. The phosphorylation of FAK induced by explantation was not inhibited by PD173074 (Fig 5.9, Expt 1). In contrast, the phosphorylation of paxillin was reduced by at least 50% in the presence of the inhibitor. As expected, the strong induction of ERK following explantation was also inhibited by around 50% by the FGF receptor inhibitor. The membrane was re-probed for total ERK. The results of a second experiment are shown in support of these findings (Fig 5.9, Expt 2). This supported the previous observation made by 4G10 western blotting of cartilage lysates, that the phosphorylation of the 120 kD protein following explantation (subsequently identified as FAK) was not primarily dependent on FGF signalling (Ch 4, Fig 4.5A). However, paxillin phosphorylation did appear to be partially FGF-dependent, whereas the induction of the 60 kD band on 4G10 blotting was not. Possible explanations include that there were also FGF-independent tyrosine phosphorylations of other paxillin tyrosine residues visualised by 4G10 blotting, or that another 60 kD phosphoprotein was also present.

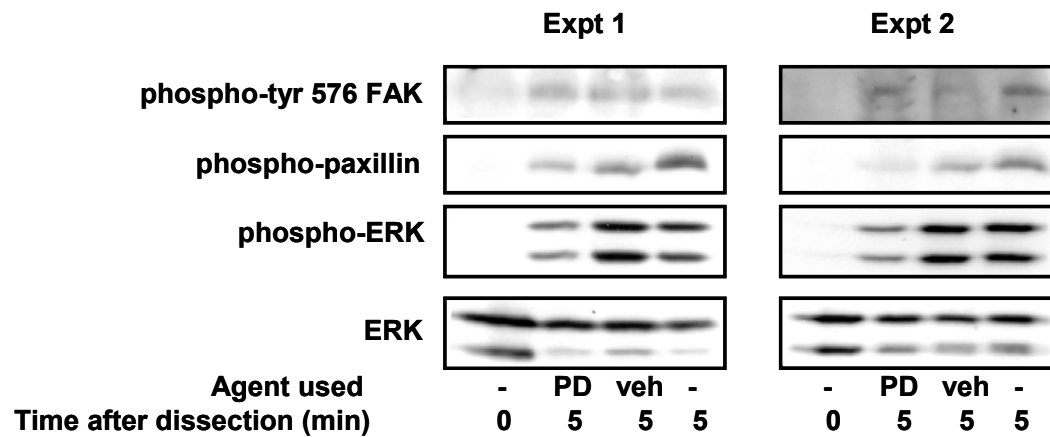


Figure 5.9. The effect of PD173074 on phosphorylation of FAK and paxillin after explantation of articular cartilage.

Porcine MCPJs were equilibrated at 37°C and then injected with medium containing 250nM PD173074 (PD) or medium alone (vehicle), or not injected (-). After 1h, ~0.2g cartilage was dissected from each of 2 trotters either directly into lysis buffer (0), or into serum-free medium alone (-, veh), or medium containing 250nM PD173074 (PD) and cultured for 5min prior to lysis. Cartilage lysates were assayed for phospho-tyr 576 FAK by western blotting. The membranes were stripped and re-probed for phospho-paxillin, phospho-ERK and ERK, the latter to check for equal cellular protein loading. Two different experiments are shown.

5.3.5 Attempts at inhibiting chondrocyte FAK activation

To investigate further the role of focal adhesion kinase in the activation of protein kinases in the response of cartilage to injury, a specific inhibitor was required. The only commercially available inhibitors of FAK, the tyrphostins, are non-specific (for example, they block the EGF pathway) and were not used (Farooki et al., 1998). A group from Pfizer recently reported a compound PF573228 as a FAK inhibitor (Slack-Davis et al., 2007). It was effective at 0.3-3 μ M at blocking the auto-phosphorylation of tyr 397 on FAK when tested on REF52 cells (an immortalised rat fibroblast cell line). 1 μ M PF573228 was tested on a panel of recombinant protein kinases. It was relatively selective, showing little significant inhibition of other kinases except the related kinase Pyk-2 (by 50%), cyclin-dependent kinase-1 and -7 (by around 80%) and glycogen synthase kinase 3 β (by 60%) (Slack-Davis et al., 2007). It was unclear whether this inhibitor would prevent phosphorylation of FAK by src. It had also not been tested on tissues. Pfizer kindly gave a sample of PF573228 for these experiments.

The inhibitor was first tested on primary chondrocyte monolayers. The ability of the inhibitor to block the constitutive phosphorylation of FAK and paxillin in adherent monolayer culture was examined. (This was the experiment described by J. Slack-Davis et al but in a different cell type). Resting primary porcine chondrocytes were incubated with either the vehicle DMSO or increasing concentrations of the FAK inhibitor PF573228 prior to lysis and standard 4G10 immunoprecipitation. Eluates were western blotted with antibodies to phospho-tyr 397 FAK, phospho-tyr 576 FAK and phospho-paxillin. Phosphorylation of FAK and paxillin was seen in resting chondrocytes and this was not affected by the presence of DMSO (Fig 5.10, Expt 1). The FAK inhibitor at concentrations of 0.3-3 μ M appeared to partially inhibit the

phosphorylation of paxillin. There was a suggestion of reduction of FAK 576 phosphorylation, but it was unconvincing. Paradoxically the inhibition appeared to be lost at the maximum concentration of PF573228 examined (10 μ M). Another similar experiment is shown (Fig 5.10, Expt 2).

To clarify whether the inhibitor would inhibit FAK phosphorylation in tissues, further experiments were performed on cartilage. Induction of FAK and paxillin phosphorylation was seen in response to explantation. Some inhibition of this response was evident when cartilage was dissected into 3 μ M PF573228, but this effect was lost in the presence of 10 μ M inhibitor (Fig 5.11). Explanations for this could be that the vehicle DMSO was activating FAK at higher concentrations (it did not appear to augment signalling when used as a vehicle control, however) or that the inhibitor was not having predictable effects. This could be because of variable tissue penetration of cartilage, although this paradoxical effect was only seen at higher concentrations of inhibitor. In view of the disappointing and variable results obtained with the inhibitor, it was not employed in further experiments.

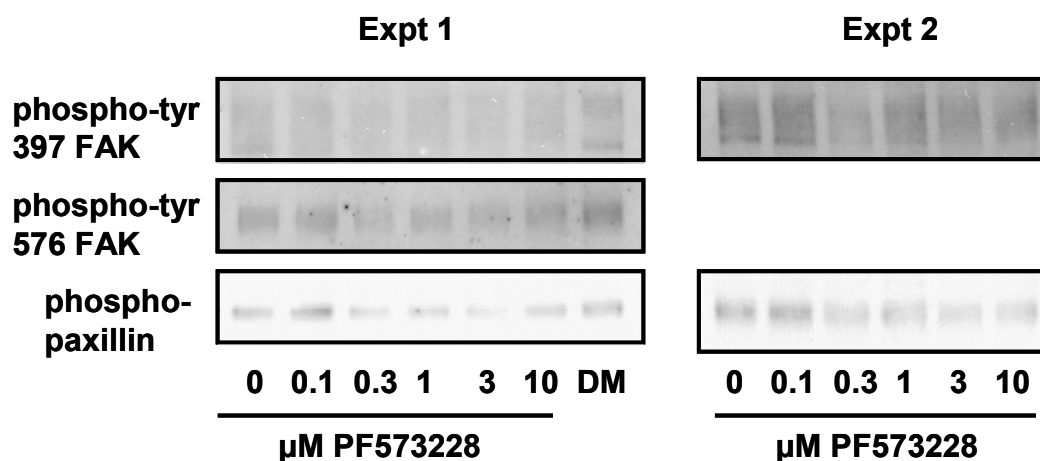


Figure 5.10. The effect of the FAK inhibitor PF573228 on phosphorylation of FAK and paxillin in adherent primary chondrocytes.

Primary porcine chondrocytes (4 million per point) were isolated and rested for 4 days, prior to serum starving for 3h. The vehicle (0.1% DMSO), or increasing concentrations of the FAK inhibitor PF573228 in serum-free medium were then added to the cells for 1h, prior to lysis and standard 4G10 immunoprecipitation. Eluted material was electrophoresed for western blotting with phospho-tyr 397 FAK antibodies and phospho-paxillin antibodies. Two separate experiments are shown. In experiment 1, samples were also assayed with phospho-tyr 576 FAK antibodies.

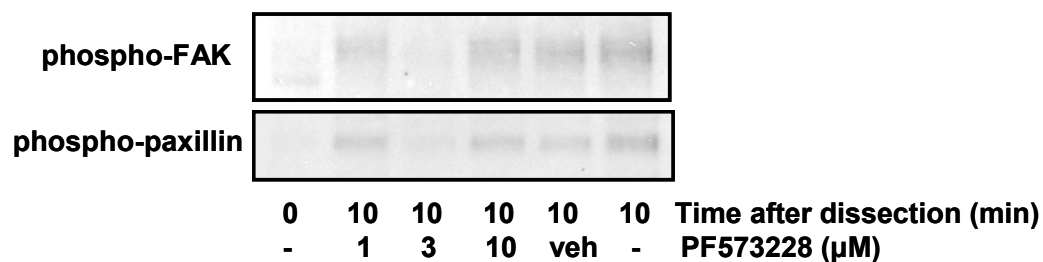


Figure 5.11. The effect of the FAK inhibitor PF573228 on phosphorylation of FAK and paxillin following explantation of cartilage.

Porcine MCPJs were opened, and joints equilibrated at 37°C for 4h. ~0.2g cartilage was then dissected either directly into a tube on dry ice, or into medium alone, medium containing the vehicle 0.1% DMSO (veh), or medium containing increasing concentrations of the FAK inhibitor PF573228 and cultured for 10min prior to snap freezing. Following lysis, 4G10 immunoprecipitation was carried out and western blotting with phospho-FAK 576 antibodies and subsequently phospho-paxillin antibodies was carried out. This is representative of 2 experiments.

5.4 The role of src family kinases in the response of cartilage to injury

A number of observations implicated src family kinases in the injury response in cartilage. FAK, paxillin and cortactin become phosphorylated upon injury, and all are known substrates of src. In the experiments shown earlier in this chapter, I had only been able to show a src-dependent phosphorylation of FAK, and had not established whether FAK was independently activated (Figs 5.4 & 5.5). The specific src kinase inhibitor PP2 had been previously noted to prevent all the injury-induced tyrosine phosphorylations of interest on 4G10 blotting (60, 80, 120 and 190 kD) (Ch 4, Fig 4.5B). Furthermore, I had shown that PP2 reduced the production of activin A after explantation (Ch 3, Fig 3.24). The possibility that src family kinases were activated after tissue injury was therefore investigated further.

5.4.1 Src expression and phosphorylation in cartilage

First, I investigated which src family members were expressed in porcine chondrocytes. Cells were serum starved, lysed and western blotting with antibodies to the non-haematopoietic src members (src, yes, and fyn) and hck was carried out. All 4 src members were detected (Fig 5.12).

To examine whether there was phosphorylation of src members in response to injury, pan-phospho src antibodies were employed, which recognise phosphorylation of tyrosine residues within certain motifs common to many src members. The 3 src tyrosine phosphorylation sites examined (-416,-215 and -418) were reported as being associated with src activation (Roskoski, 2005; Vadlamudi et al., 2003).

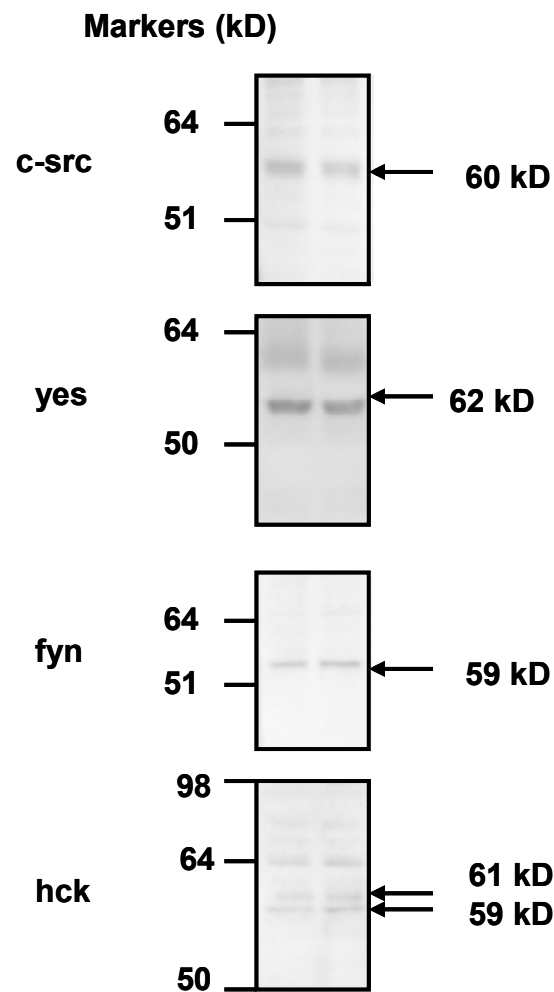


Figure 5.12. Expression of src family members in porcine primary chondrocytes.

Porcine chondrocytes were isolated and rested for 3 days, prior to being serum starved for 3h. They were lysed and western blotting was carried out with antibodies to the ubiquitous src members c-src (Santa Cruz), yes and fyn, and also to hck. See Chapter 2 for details of the antibodies used.

Testing the phospho-tyr 416 antibodies (which have probably been used most widely) by western blotting cartilage lysates, several non-specific bands were detected (data not shown). These included probable heavy chain immunoglobulin and phospho-ERK, but no band of the expected molecular size of 60 kD was seen. Phospho-tyr 215 src antibodies recognised a 60 kDa band in cartilage lysates, which was increased by pervanadate and weakly 10min after explantation (Fig 5.13A). However, the antibody also recognised a number of other proteins in cartilage lysates. Western blotting of the lysates with a total c-src antibody was carried out to ensure equal loading. A stronger band was seen in lysates stained with phospho-tyr 418 antibodies, particularly when the tissue was exposed to pervanadate, but this was not regulated by injury (Fig 5.13B).

Immunoprecipitation was carried out to concentrate tyrosine-phosphorylated srcs. Western blotting of 4G10 immunoprecipitates with either phospho-src 418 or total src (Upstate) antibodies was carried out. Conversely, c-src immunoprecipitates from cartilage were also assayed for phosphotyrosine content by 4G10 western blotting. In all experiments, a band which was increased by sodium pervanadate, but not by injury was seen (data not shown). It was likely that c-src would form a complex with activated FAK. However, using non-dissociative immunoprecipitation conditions, no evidence for co-immunoprecipitation of c-src with either FAK or paxillin on injury could be demonstrated (data not shown).

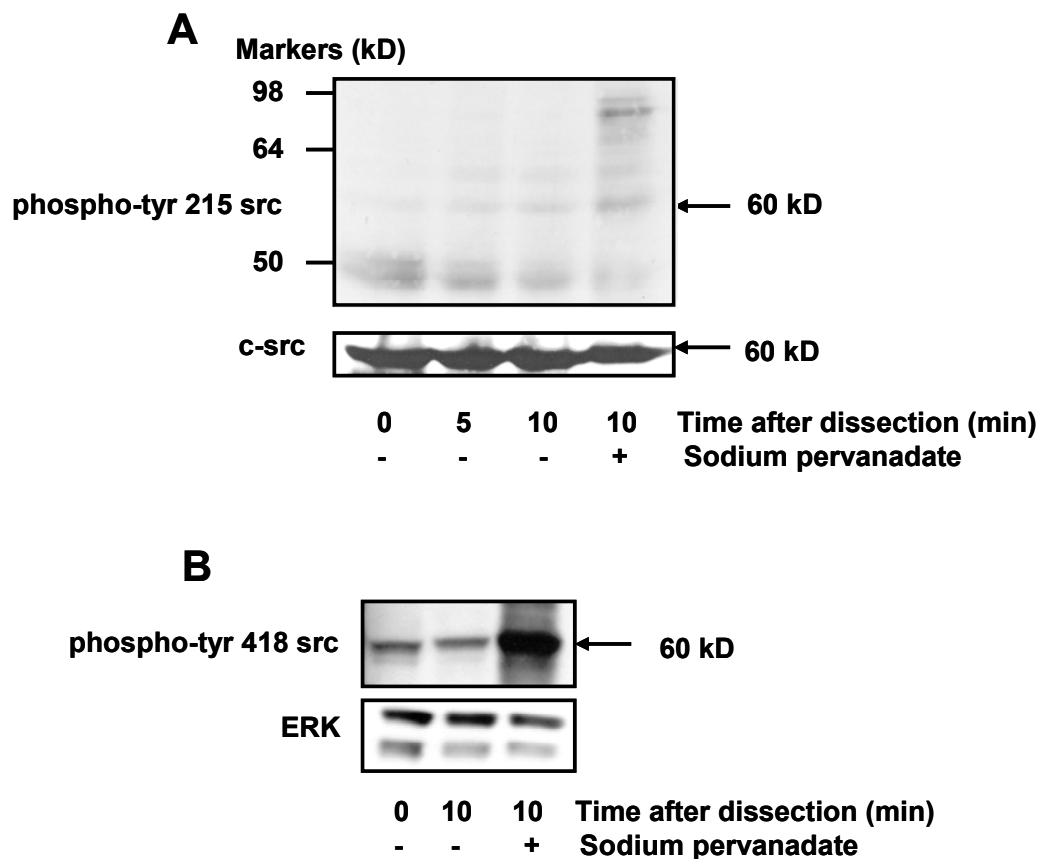


Figure 5.13. Testing phospho-src antibodies on cartilage lysates following dissection of the tissue and treatment with pervanadate.

For both **A.** & **B.**, in separate experiments, ~0.5g cartilage was dissected either directly into lysis buffer (0), or into medium and cultured for increasing times prior to lysis, or into medium containing 10mM sodium pervanadate for 10min prior to lysis.

A. Lysates were assayed for phospho-tyr 215 src by western blotting. The samples were also electrophoresed separately for western blotting with total c-src antibody (Upstate), as a loading control.

B. Lysates were assayed for phospho-tyr 418 src by western blotting, and the membrane re-probed with antibodies to ERK to check for equal cellular protein loading.

5.4.2 Immunoprecipitation and activity of c-src in cartilage lysates

Given that I had been unable to show convincing evidence of src phosphorylation upon tissue injury with either phospho-antibodies or immunoprecipitation-western blotting, I sought evidence of src activation by kinase assay. This measured the ability of c-src to auto-phosphorylate, and also to phosphorylate a known src substrate enolase (enolase had been detected in cartilage 4G10 immunoprecipitates by mass spectrometry, see chapter 4, Fig 4.18B). The conditions for the immunoprecipitation with a monoclonal antibody to c-src (Upstate) were optimised, and showed a band of appropriate size on western blotting of the immunoprecipitates with src antibody, but also detected heavy chain immunoglobulin, just below the band of interest (see later, Fig 5.15). Cartilage was either dissected directly on to dry ice, or into medium for 10min or 30min prior to snap freezing, or into medium containing 10mM sodium pervanadate for 10min as a positive control. A pre-clearing step with protein G agarose beads was included to deplete immunoglobulin in the samples, prior to immunoprecipitation for 1h with the c-src antibody (Upstate). The ability of the immunoprecipitate to phosphorylate enolase was assessed by [γ - ^{32} P]ATP kinase assay. Two bands were evident on autoradiography of the gel: an upper band at 60 kD, consistent with c-src auto-phosphorylation, and a lower band at 47 kD, likely to represent phosphorylated enolase (Fig 5.14). As controls for the assay, cartilage lysate was either incubated with an irrelevant mouse IgG monoclonal antibody (Lane 5) which gave a different (presumably non-specific) band to the two described, or the enolase was omitted and only the 60 kD band was seen (Lane 7). No bands were seen when lysate was excluded from the assay (Lane 6). Compared with uninjured cartilage, the lysates after injury at both 10 and 30min had reduced src activity in the assay, both in terms of auto-phosphorylation and phosphorylation of the substrate

enolase (Lanes 3&4, Fig 5.14). Dissecting and culturing cartilage in sodium pervanadate for 10min returned src activity to baseline levels (Lane 8).

This unexpected finding was made in three separate experiments. It suggested that constitutive c-src activity was reduced following injury. It was possible that either the amount or activity of c-src in lysates was reduced. Another possibility was that the src was less accessible to the antibody. To test these possibilities, cartilage was dissected into serum-free medium for increasing times prior to lysis. Lysates were pre-cleared and then western blotting of an aliquot of the lysate immediately carried out (top panel, Fig 5.15). This showed that equal amounts of c-src were present in the lysates prior to immunoprecipitation. The remaining lysate was incubated with monoclonal antibodies to c-src and protein G agarose for 1h. The immunoprecipitates were boiled with sample buffer, prior to electrophoresis and western blotting for c-src (second panel, Fig 5.15). The supernatant of the immunoprecipitates was assessed for residual c-src content by western blotting (third panel, Fig 5.15). Compared with *ex vivo* '0' samples, explantation for 5min or more affected the ability of the monoclonal antibody to immunoprecipitate c-src from the lysates (second panel, Fig 5.15). This phenomenon could be explained by conformational change in c-src, modification, or binding of another protein which masked the epitope, which included the SH2 site. The experiment supported the possibility that the src was less accessible to the antibody, rather than that its activity or concentration was reduced after injury.

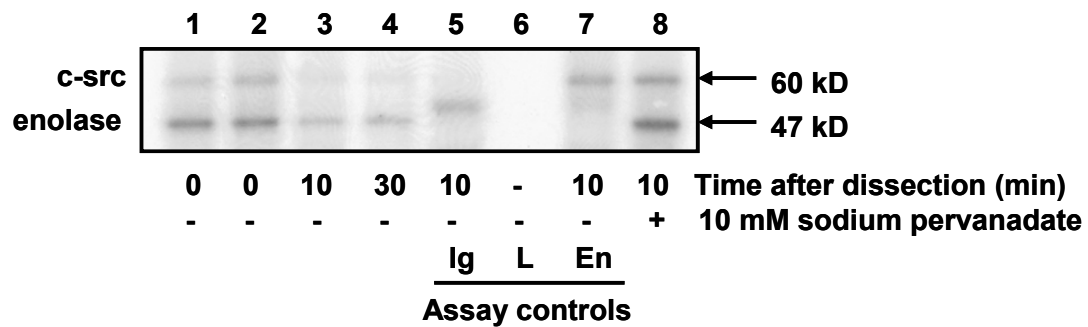


Figure 5.14. c-src activity by kinase assay appears reduced following explantation of cartilage.

~0.5g cartilage was either dissected immediately into a tube on dry ice (0), or into medium for 10 or 30min prior to snap freezing, or into 10mM sodium pervanadate for 10min as a positive control. Cartilage was lysed and pre-cleared with protein G agarose, prior to incubation with 4 μ g monoclonal antibody to c-src and protein G agarose beads for 1h. Beads were washed, and the ability of the immunoprecipitate to phosphorylate the substrate enolase (lower band) was assessed by [γ - ^{32} P]ATP kinase assay. As internal controls for the assay, further aliquots of pooled lysate from 10min-cultured cartilage were either incubated with an irrelevant mouse IgG monoclonal antibody and protein G agarose beads (Ig, Lane 5), or the kinase assay carried out without the addition of enolase, (En, Lane 7). RIPA alone instead of cartilage lysate was also included in the assay (L, Lane 6). This is representative of 3 different experiments.

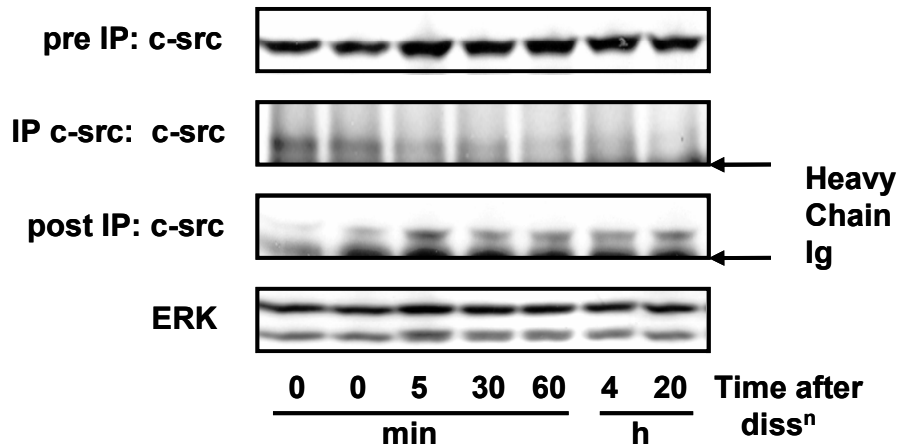
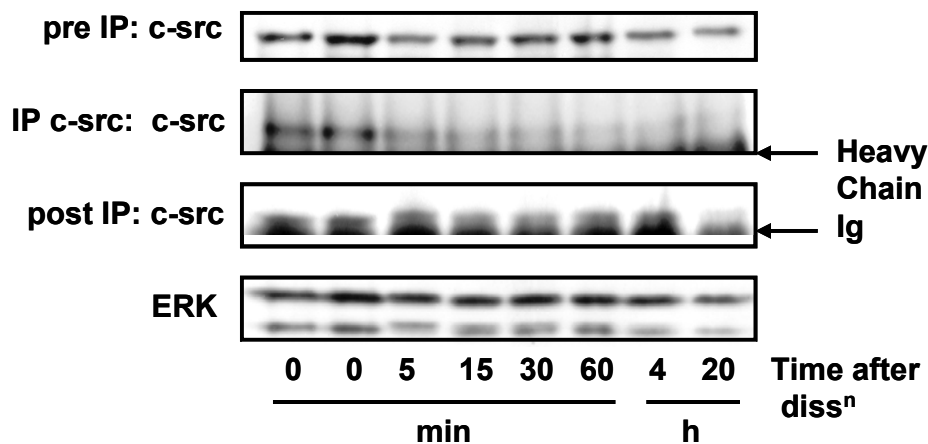
Expt 1**Expt 2**

Figure 5.15. The effect of dissection and culture on c-src in cartilage explants.

Trotters were equilibrated at 37°C. ~0.5g cartilage was dissected into serum-free medium and cultured for increasing times prior to lysis. Lysates were pre-cleared by incubation with protein G agarose beads for 30min. 150µl pre-cleared lysate was then assayed for c-src by western blotting (pre-IP). The remaining lysate was incubated with antibody to c-src and protein G agarose. The immunoprecipitates were assayed for c-src by western blotting (IP c-src). The supernatant recovered from the immunoprecipitates was also assayed for c-src content by western blotting (post-IP). The 'pre-IP' membrane was stripped and re-probed for ERK to ensure equal cellular protein loading. 2 different experiments are shown.

5.4.3 The effect of PP2 on MAPK and IKK activation

The src inhibitor PP2 had been previously noted to suppress induction of bands at 60 and 120 kDa on 4G10 blotting (Ch 4, Fig 4.5B). Whether PP2 suppressed FAK (and paxillin) phosphorylation on explantation was checked. Porcine MCP joints were pre-injected with medium plus DMSO or increasing concentrations of PP2. Cartilage was then dissected either onto dry ice, or into medium alone, or into medium containing DMSO or PP2, and cultured for 10min. Lysates were assayed for phospho-tyr 576 FAK (the src-dependent FAK phosphorylation) and phospho-paxillin by western blotting. Phosphorylation of paxillin followed dissection of cartilage. 10 μ M PP2 reduced this phosphorylation to baseline levels (Fig 5.16). The staining of phospho-FAK in this experiment was disappointing (there was some batch variation in the antibody). However, there was suppression of FAK phosphorylation at 10 μ M PP2. Lower concentrations of PP2 (0.1 or 1 μ M) did not appear to substantially reduce phosphorylation of either FAK or paxillin following explantation, and for this reason 10 μ M PP2 was used in further experiments. Western blotting of phospho-tyr 576 FAK or phospho-paxillin was used as an internal control for subsequent experiments with PP2.

It appeared that src was required for phosphorylation of both FAK and paxillin after cartilage injury. Whether src activation was required for other inflammatory signalling such as MAPK activation following cartilage injury was investigated. Porcine MCPJs were injected with medium containing either DMSO or 10 μ M PP2. Cartilage was then dissected either directly into a tube on dry ice, or into medium with, or without, the inhibitor for 10min prior to lysis. Following immunoprecipitation with 4G10, eluates were assayed by western blotting.

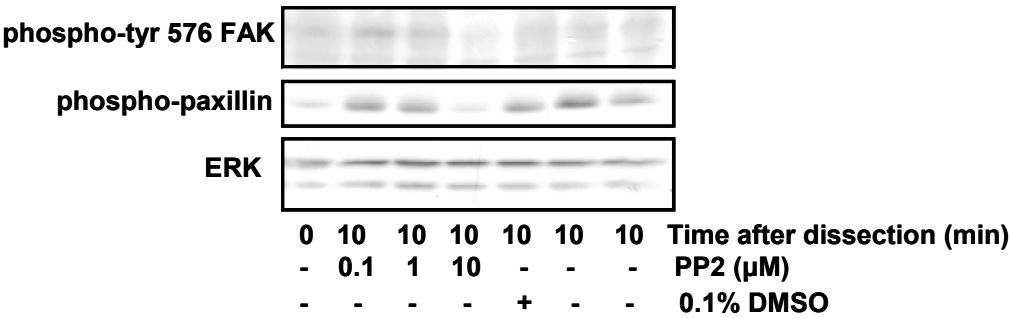


Figure 5.16. PP2 suppresses phosphorylation of FAK and paxillin after cartilage injury.
Porcine MCP joints were injected with medium plus the vehicle (0.1% DMSO) or medium containing increasing concentrations of PP2, or not injected and equilibrated at 37°C for 1h. Then, ~0.5g cartilage was dissected either directly into a tube on dry ice (0), or into medium containing 0.1% DMSO or appropriate concentrations of the inhibitor, or into medium alone and cultured for 10min prior to snap freezing. Lysates were assayed for phospho-tyr 576 FAK and phospho-paxillin by western blotting. The membrane was stripped and re-probed for ERK, to ensure equal cellular protein loading.

The induction of phosphorylation of FAK and paxillin by dissection was inhibited almost to baseline levels by the presence of PP2 (upper panels, Fig 5.17A). Lysates from the same experiment were electrophoresed and assayed for the 3 phospho-MAPKs by western blotting. Increased phosphorylation of all 3 MAPKs was seen 10min after dissection. In the presence of PP2, no difference was seen in the activation of any of the MAPKs (lower panels, Fig 5.17A). The injection of joints with the vehicle 0.1% DMSO in medium appeared to reduce the subsequent phosphorylation of ATF-2 in some experiments, as seen here. Therefore experiments were carried out without prior injection. Cartilage was instead dissected directly into the inhibitor, medium containing DMSO, or medium alone. Again, there was suppression of FAK and paxillin phosphorylation, but no inhibition of MAPK signalling in the presence of PP2 (Fig 5.17B). These experiments both suggested that src was not required for MAPK activation following explanation.

Whether PP2 had any effect on IKK activity seen following cartilage injury was also investigated. Porcine MCPJs were injected with 10 μ M PP2, the vehicle 0.1% DMSO, or not injected and equilibrated at 37°C. Cartilage was dissected and assayed for IKK activity as described previously. The phosphorylation of I κ B α was quantified by phosphorimaging and the membrane was re-probed for IKK α to check for equal protein loading. Activation of IKK was seen upon dissection and maximal by 5min, as had been shown previously (Fig 5.18 & Ch 3, Fig 3.4). The presence of PP2 or DMSO did not affect the activation of IKK. It was concluded that whilst src (and possibly FAK) were activated by explantation, they were unlikely to be responsible for the activation of the MAPKs and IKK.

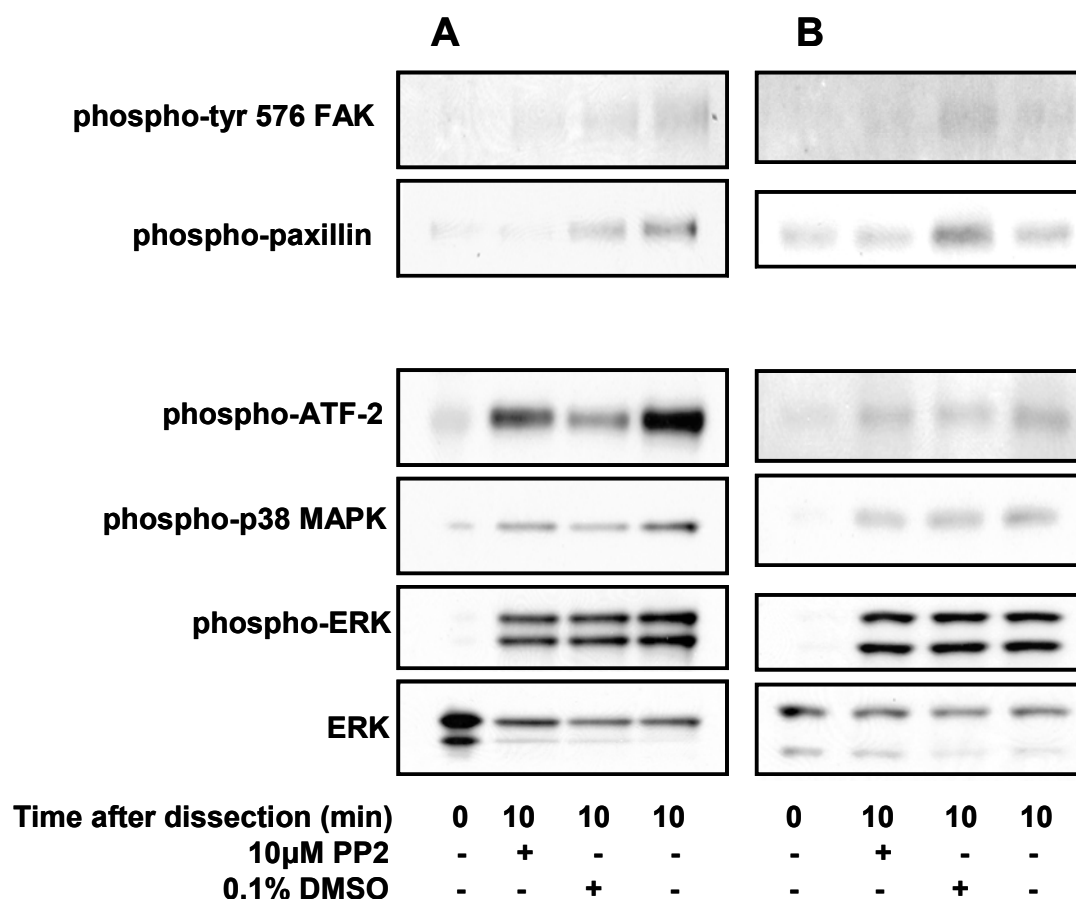


Figure 5.17. PP2 has no effect on the induction of MAPK signalling following explantation of cartilage, either with (A), or without (B), prior intra-articular injection of inhibitor.

A. Porcine MCPJs were equilibrated at 37°C and injected with medium containing either the vehicle 0.1% DMSO or 10μM PP2 for the last hour, or not injected. ~0.2g cartilage was then dissected from each of 2 joints either directly into a tube on dry ice, or into medium alone, or medium containing either 0.1% DMSO or 10μM PP2 for 10min prior to lysis.

B. Porcine MCPJs were equilibrated at 37°C. ~0.2g cartilage was then dissected from each of two joints either directly into a tube on dry ice, or into medium alone, or medium containing either 0.1% DMSO or 10μM PP2 for 10min prior to lysis.

For both **A.** & **B.**, 4G10 immunoprecipitation was carried out from pooled lysates, and eluted material assayed for phospho-FAK and phospho-paxillin by western blotting (upper 2 panels). Lysates from the same experiment were electrophoresed and assayed for phospho-ATF-2, phospho-p38 MAPK, phospho-ERK and total ERK by western blotting (lower 4 panels).

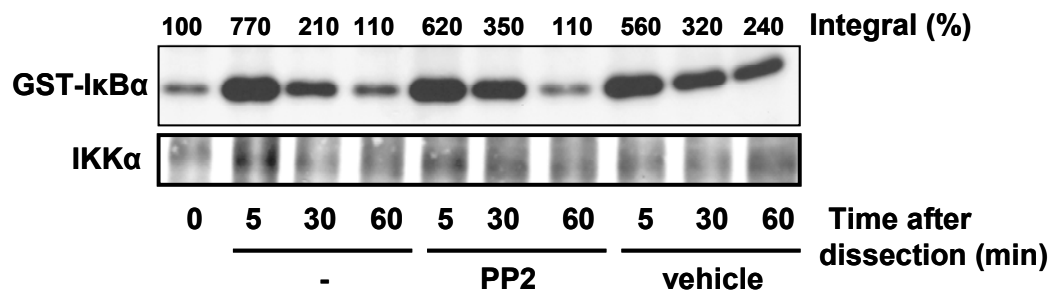


Figure 5.18. Testing the effect of PP2 on IKK activation caused by explantation of articular cartilage.

Porcine MCPJs were equilibrated at 37°C and then injected with medium containing 10μM PP2 (PP2), or medium containing 0.1% DMSO (vehicle), or not injected (-). After 1h, ~0.2g cartilage was dissected from each of 2 trotters either directly into kinase assay lysis buffer (0), or into serum-free medium alone (-), or medium containing 10 μM PP2 (PP2), or into medium containing 0.1% DMSO (vehicle) and cultured for increasing times indicated prior to lysis. Pooled lysates were incubated with antibody to IKK, and the ability of the immunoprecipitates to phosphorylate the substrate IκBα assessed by kinase assay. Quantitation of phosphorylation of the substrate is shown as a % of the 0 measurement. The membrane was stripped and re-probed for IKKα to ensure equal immunoprecipitation of the IKK complex from samples. This is representative of 3 experiments.

5.5 The effect of the FGF receptor tyrosine kinase inhibitor, PD173074 on MAPK and IKK signalling after cartilage explantation

The tyrosine phosphorylation seen following explantation appeared to be largely src-dependent (Ch 4, Fig 4.5B). Inhibition of the FGF receptor had previously been shown to cause partial suppression of paxillin phosphorylation, but FAK and general tyrosine phosphorylation were largely unaffected (Fig 5.9 & also Ch 4, Fig 4.5A). This suggested that src activity was probably independent of FGF. The FGF receptor inhibitor PD173074 suppressed activation of the ERK pathway after explantation (Ch 3, Fig 3.18). The effects of this agent on the other MAPK pathways were therefore explored.

In similar experiments to Fig 5.17A, trotters were equilibrated and then injected with medium containing 250nM FGF receptor inhibitor PD173074, or the vehicle medium alone, or not injected. Cartilage was then dissected and lysate was examined for the 3 phosphorylated forms of the MAPKs, and also for phospho-Akt. The membrane was stripped and re-probed for ERK to ensure equal cellular protein loading. Two separate experiments are shown. In both cases, there was suppression of phospho-ATF-2 and phospho-p38 as well as phospho-ERK in the presence of the FGF receptor inhibitor (Fig 5.19). The results with phospho-Akt were less clear, with one experiment suggesting suppression, and the other being equivocal. These results were unexpected. They suggested that FGF signalling was at least partially responsible for the activation of all 3 MAPK pathways after injury, not just ERK.

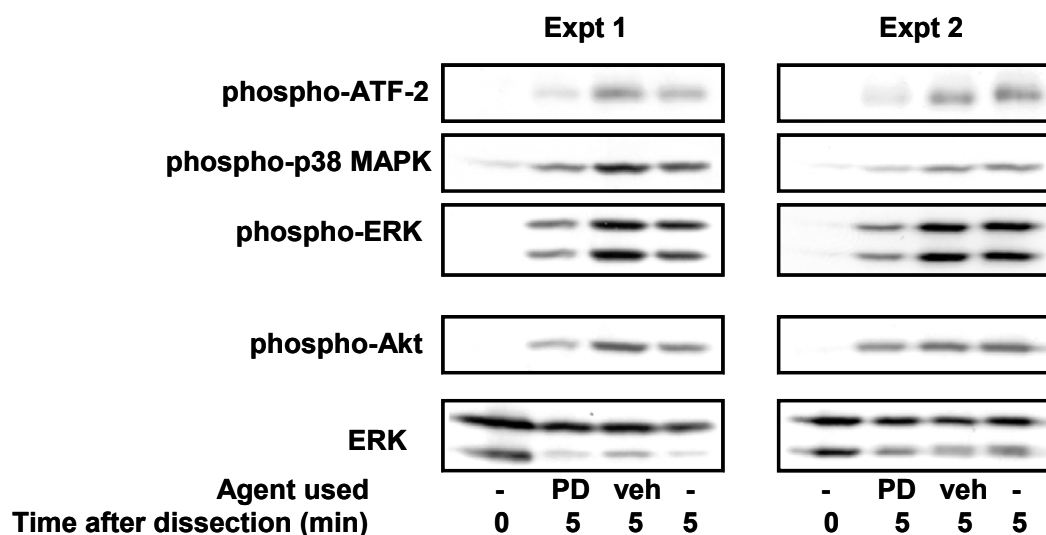


Figure 5.19. The effect of PD173074 on activation of MAPK and PI3 kinase after explantation of articular cartilage.

Porcine MCPJs were equilibrated at 37°C and then injected with medium containing 250nM PD173074 (PD) or medium alone (vehicle), or not injected (-). After 1h, ~0.2g cartilage was dissected from each of 2 trotters either directly into kinase assay lysis buffer (0), or into serum-free medium alone (-, veh), or medium containing 250nM PD173074 (PD) and cultured for 5min prior to lysis. Cartilage lysates were assayed for phospho-ATF-2 and phospho-Akt by western blotting. The membranes were stripped and re-probed for phospho-p38 MAPK, phospho-ERK and ERK, the latter to check for equal cellular protein loading. Two separate experiments are shown.

In view of the unexpected apparent FGF-dependence of JNK activity, the effect of the FGF receptor inhibitor upon activation of IKK was investigated. Lysates from the 2 experiments in Fig 5.19 were assayed for activity of IKK. Injection of the vehicle alone had little effect on IKK signalling (Fig 5.20A). Figure 5.20B shows the combined results. In the two experiments, the IKK activation was about 50% reduced by the FGF receptor inhibitor PD173074 (Fig 5.20A&B). These results suggested that FGF signalling was involved in the activation of all 3 MAPKs and IKK following cartilage injury.

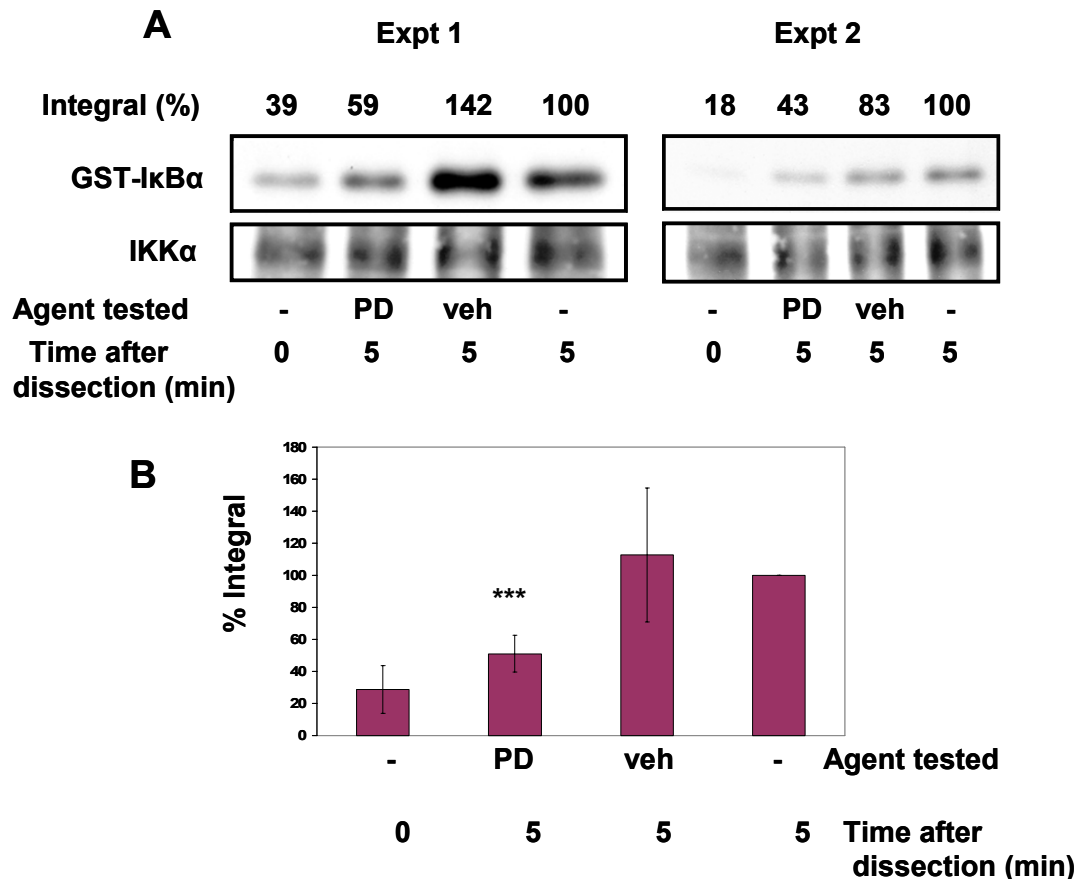


Figure 5.20. The effect of PD173074 on IKK activity caused by dissection of articular cartilage.

A. Porcine MCPJs were equilibrated at 37°C and then injected with medium containing 250nM PD173074 (PD) or medium alone (vehicle), or not injected (-). After 1h, ~0.2g cartilage was dissected from each of 2 trotters either directly into kinase assay lysis buffer (0), or into serum-free medium alone (-, veh), or medium containing 250nM PD173074 (PD) and cultured for 5min prior to lysis. Pooled lysates were incubated with antibody to IKK, and the ability of the immunoprecipitates to phosphorylate the substrate IkB α assessed by kinase assay. Quantitation of phosphorylation of the substrate is shown as a % of the 5 min un-injected measurement. The membrane was stripped and re-probed for IKK α to ensure equal immunoprecipitation of the IKK complex from samples. 2 separate experiments are shown.

B. The results from the 2 experiments were pooled. The mean of IKK activity from 5min uninjected points from individual experiments was normalised to 100%, and quantitation of IKK activity of other samples expressed as a % of this. The bars show the mean % integral and the error bar shows the standard deviation of these measurements. ***P=0.002 vs. untreated (-), by unpaired *t*-test.

5.6 Discussion

The identification by mass spectrometry of FAK and one of its substrates paxillin as proteins phosphorylated upon injury was confirmed. By using immunoprecipitation with 4G10 followed by western blot with either antibodies to the proteins or to their known phosphorylation sites, their phosphorylation was shown to be regulated by injury. On electrophoresis, they aligned with 2 of the areas of interest on the 4G10 staining pattern: FAK aligned with the upper end of the 100-120 kD complex and paxillin aligned with the 60kD band.

The phospho-FAK antibodies were disappointing. There was high background staining on western blotting and variable staining of the antigen between batches of antibodies. Their specificity for phosphorylated FAK was uncertain, particularly in the case of phospho-tyr 397 antibodies, but to test this would have meant synthesising the un-phosphorylated and phosphorylated forms of the antigen peptides. If only a small percentage of the FAK is phosphorylated on tyr 397 at any given time and the antibody has some affinity for the non-phosphorylated protein, a change in the FAK band on cell activation may not be detectable. The Pfizer FAK inhibitor did not prove useful in experiments with chondrocytes or cartilage. It gave variable and unconvincing results and had paradoxical effects at higher concentrations. This meant that the role of FAK in the injury response could not be investigated further pharmacologically.

The identification of cortactin as a further injury-induced phosphorylation which accounted for the 80 kD doublet on 4G10 blotting was further evidence for src activation, since it is a well-characterised substrate of src family kinases: tyrs 421, 466 and 482 on cortactin are targeted by srcs (Daly, 2004; Huang et al., 1998). Cortactin has also been reported to be phosphorylated by other tyrosine kinases

including syk and fer (Daly, 2004). The 190 kD band and the lower end of the 100-120 kD complex were not identified. It is possible that the proteins identified may simply overlie other phosphoproteins of the same size. This is always a potential pitfall with mass spectrometric identification of proteins from 1D gels.

It seems likely that srcs, or src-like kinases are activated following explantation. They play a role in regulating expression of activin A after cartilage injury. Three known src substrates are phosphorylated in the response, and the specific src kinase family inhibitor PP2 suppresses these phosphorylations. I was unable to directly show any activation of srcs following cartilage injury. Whilst there was no detectable change in src phosphorylation following injury, a change in the immunoprecipitability of src was found. This suggested that the protein may have been involved in interactions or have undergone a conformational change, masking an epitope. Such events could be associated with change in location or function of the enzyme in the cell. It is possible that other src family members such as fyn, yes or hck, which are all expressed in porcine chondrocytes, were involved in the increase in protein tyrosine phosphorylation. It would be interesting to assay their activity following cartilage injury. Pharmacological suppression of src signalling had no effect on the activation of MAPKs or IKK following explantation. This suggests that any src signalling that occurs is in parallel to MAPK and IKK activation. The effects of PP2 on other gene expression in cartilage following injury should be investigated.

MAPK and IKK activation following explantation is suppressed by approximately 50% by the FGF receptor inhibitor PD173074. It was known that FGF was likely to be responsible for ERK activation following explantation, but the effects on the other pathways were unexpected (Vincent et al., 2002). Growth factors such as FGF are not usually considered to be activators of inflammatory signalling pathways. In resting

cartilage explants, FGF did not activate IKK. In previous experiments on chondrocytes and resting explants FGF did not cause JNK phosphorylation (A. Didangelos, Ph.D. thesis, 2008). The fact that the inhibition was only around 50% suggests that either it was inefficient or there was an additional mechanism activating signalling which FGF-2 augmented.

The FGF receptor inhibitor did not suppress the tyrosine phosphorylation pattern seen on 4G10 blotting. Thus, in general the tyrosine phosphorylation following injury appeared to be src-dependent, rather than FGF-dependent.

I have illustrated the possible events that my experiments suggest follow injury in Figure 5.21. It is likely that some unknown event, X initiates IKK and MAPK activation and that this is amplified by FGF-2. The src activation appears to be largely independent of FGF-2 so I have shown this as being initiated by another event, Y (although X and Y could of course be the same). FGF-2 may contribute to the src activation (e.g. paxillin phosphorylation), so I have indicated the possible contribution with the broken line. The src inhibitor affects activin A expression, so src may be necessary for optimum gene expression.

There is always a concern that pharmacological inhibitors induce non-specific effects, particularly at the higher concentrations used on tissue. Previous testing on chondrocytes suggested that PD173074 did not target other growth factor-induced ERK signalling (T. Vincent, Ph.D. thesis, 2002). It was designed as an FGFR1 inhibitor (Mohammadi et al., 1998). Given the high degree of homology of FGFRs, it is likely that this compound has effects on other receptors in the family, although no systematic studies have been carried out in this regard (Ezzat et al., 2005).

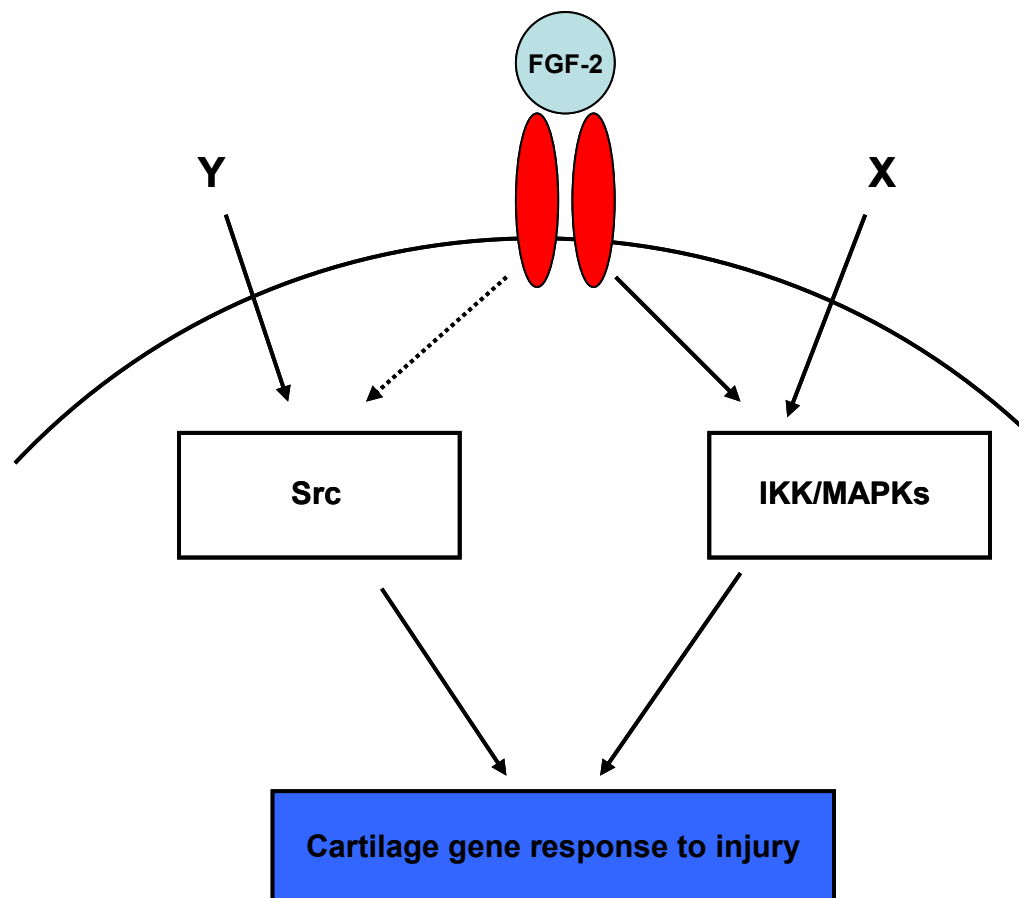


Figure 5.21. Scheme of the signalling response to cartilage injury.

Stimuli X and Y could be the same or separate, and remain unidentified. Following injury, X and Y occur and cause activation of IKK, MAPKs and src family kinases. All of these pathways have been implicated in the net gene response to cartilage injury. FGF signalling contributes to 50% of IKK and MAPK signalling seen following injury. It is unclear if it has any effect on src. To exert this effect, FGF might augment signal X, or act directly on IKK and MAPKs, or in the higher control of these pathways.

To clarify the role of FGF-2 in injury-induced MAPK and IKK signalling, experiments could be carried out in FGF-2 null mice. In the light of my findings, reduction in the activation of these pathways in response to wounding of cartilage would be expected.

6 The response of synovium to injury

6.1 Introduction

I wanted to find out whether the intracellular signalling caused by sharp injury was a response unique to articular cartilage, or whether it occurred in other connective tissues upon injury. Synovium is a soft vascular connective tissue which lines joints. It comprises a lining layer of cells classified as type A (macrophage-like) and B (fibrocyte-like). These rest on a loose connective tissue of variable thickness outside which is the joint capsule, a dense fibrous tissue.

6.2 Signalling in synovium in response to injury

Synovium from a porcine trotter (~0.2g) was quickly dissected from the capsule either directly into a tube on dry ice or into serum-free culture medium. Tissue was left in medium for 10min, then rapidly washed. It was lysed in RIPA buffer and western blotting for phospho-ATF-2, phospho-p38 MAPK and phospho-ERK was carried out. The membrane was re-probed with antibodies to ERK as a loading control. As in cartilage, the MAPKs were seen to be strongly activated in synovium by dissection and culture (Fig 6.1).

Synovial lysates were examined for phosphotyrosine content by western blotting with the 4G10 antibody. Induction of many phosphotyrosine bands was seen in response to dissection (Fig 6.2A). The major features of the pattern were similar to those seen in the lysates of injured articular cartilage (Fig 6.2B). There was very striking increase in tyrosine phosphorylation of a 60 kD protein in synovium. The membrane was stripped and re-probed with ERK antibody. In synovium, ERK 1 appears more abundant than ERK 2. (The converse is true for cartilage).

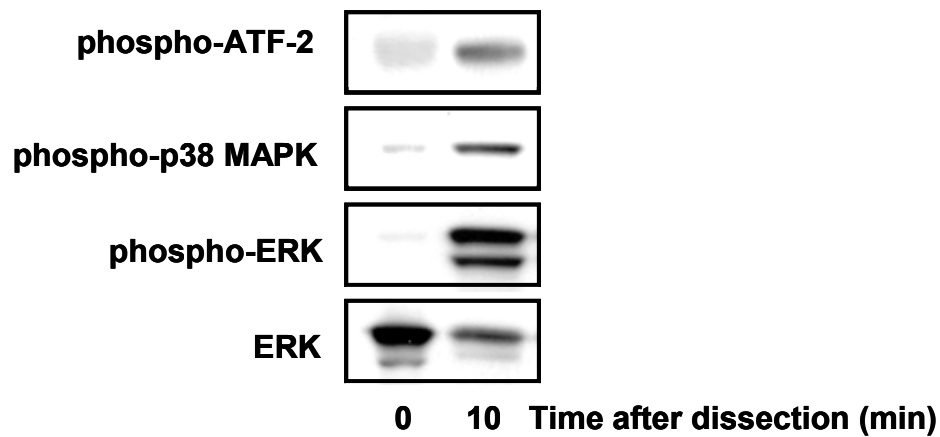


Figure 6.1. MAPKs are activated following dissection of synovium into culture medium.

~0.2g synovium was dissected either into lysis buffer (0) or into serum-free medium and cultured for 10min prior to lysis. Western blotting of lysates for phospho-ATF-2, phospho-p38 MAPK and phospho-ERK was carried out. The membrane was stripped and re-probed for ERK to ensure equal cellular protein loading.

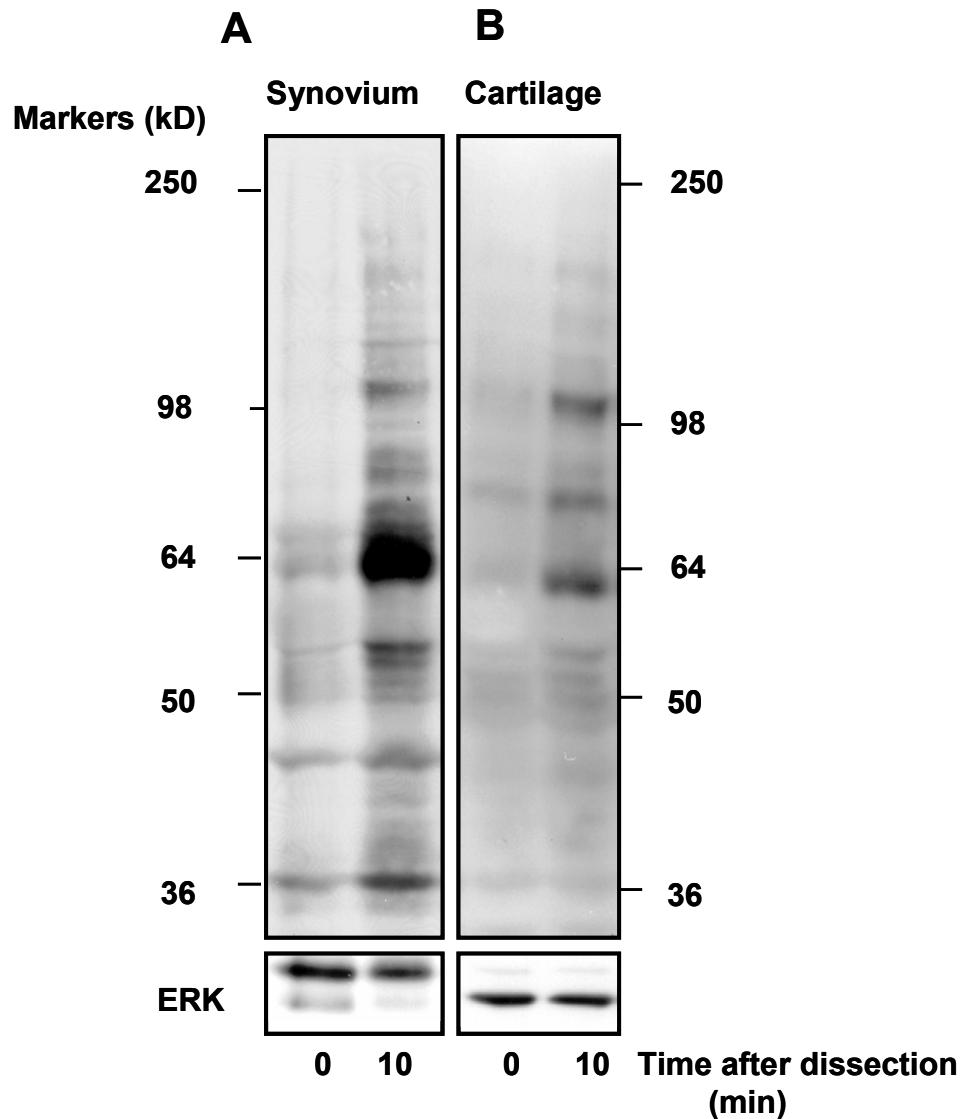


Figure 6.2. Tyrosine phosphorylation in (A) synovium and (B) cartilage in response to dissection into culture medium.

In separate experiments, ~0.1g synovium (A) or ~0.5g cartilage (B) was dissected from a porcine MCP joint either immediately into a tube on dry ice (0) or into serum-free medium and cultured for 10min prior to lysis. Western blotting with 4G10 antibody was carried out. The membranes were re-probed for ERK. **A.** and **B.** are separate experiments, but are aligned for comparison.

The time course of the phosphorylation, and its possible dependence on contact of the tissue with culture medium was examined. Synovium was dissected either immediately into a tube on dry ice, or dissected into culture medium for increasing times, or into a dry tube for 10min prior to snap freezing, lysis and western blotting with 4G10 antibody. The tyrosine phosphorylation seen in synovium following dissection was, as in the case of cartilage, rapid and not dependent on culture medium (Fig 6.3A). Many bands including some at 55 kD, 60 kD, and 100 kD were seen to be induced in synovium following injury, whether or not culture medium was present. Again, these aligned with inducible bands of the same size in lysates of injured cartilage which were run on the same gel for comparison (Fig 6.3B). 3 times as much material from cartilage as synovium was loaded: the cartilage and synovial samples loaded thus had a similar amount of ERK, suggesting a possibly similar content of cellular protein was present. Tyrosine phosphorylation was far stronger in synovium however (Fig 6.3 shows the same exposure of the membrane for all samples).

4G10 immunoprecipitation was employed to investigate further the protein tyrosine phosphorylation in synovium. The pattern of phosphotyrosine bands in synovial and cartilage 4G10 immunoprecipitates was very similar (Fig 6.4).

To test whether FAK, paxillin and cortactin accounted for the phosphorylated proteins at 100, 60 and 80 kD, 4G10 immunoprecipitation of synovial lysates was carried out and eluates were western blotted with antibodies to phospho-tyr 576 FAK. The membrane was re-probed with phospho-paxillin antibodies and cortactin antibody. Dissection induced a doublet that stained with phospho-FAK antibodies which aligned with the top of the 100-120 kD cluster on 4G10 blotting (Fig 6.5).

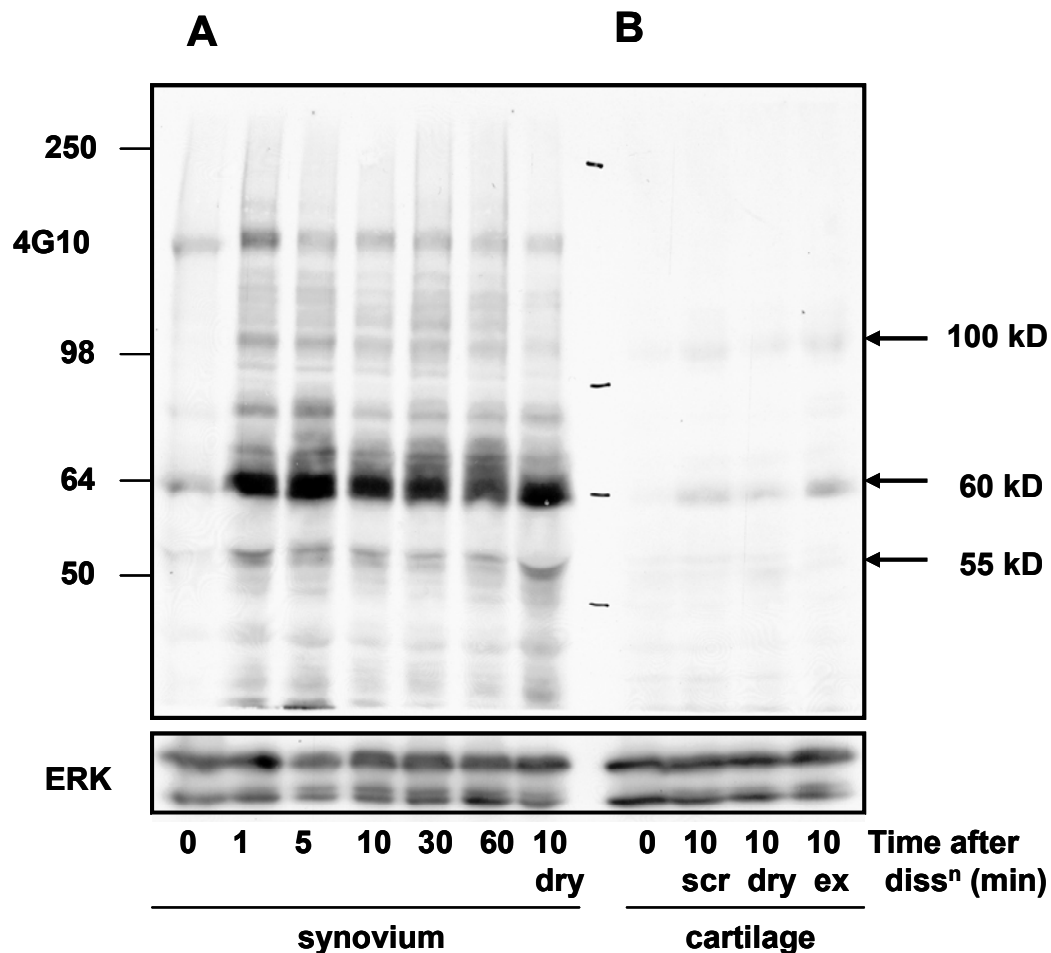


Figure 6.3. Dissection of synovium rapidly induces tyrosine phosphorylation which is not dependent on contact with culture medium.

A. ~0.1g synovium was dissected from each porcine MCP joint either into ice cold lysis buffer (0) or into culture medium for increasing times prior to lysis, or into a dry tube for 10min at 37°C prior to lysis (10 dry).

B. Joints were opened and equilibrated at 37°C. ~0.5g cartilage was either dissected immediately into lysis buffer (0), or into a dry tube (10 dry) or culture medium (10 ex) for 10min prior to lysis, or the surface was scored (see Materials & Methods for details) and the trotter returned to the incubator for 10min, prior to dissecting the scored cartilage rapidly into lysis buffer (10 scr).

For both A. & B., lysates were run on the same gel for comparison. (3 times as much estimated protein from cartilage as synovium was electrophoresed). Western blotting with 4G10 antibody was carried out. The membrane was re-probed with ERK as a loading control.

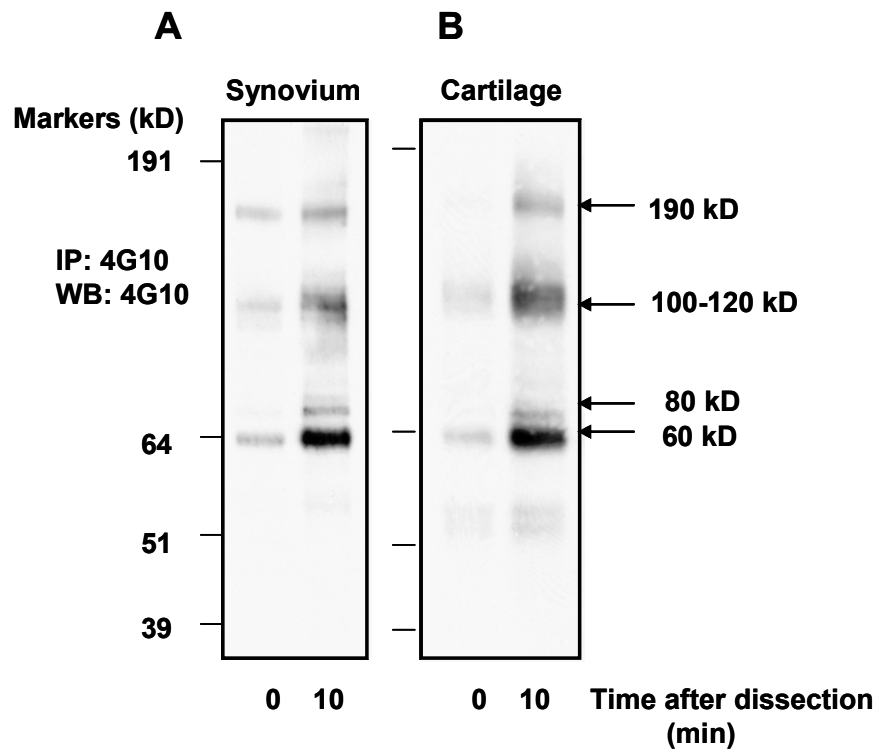


Figure 6.4. A similar pattern of tyrosine-phosphorylated proteins is precipitated by 4G10 antibody from dissected synovium (A) and cartilage (B).

A. ~0.2g synovium was dissected either directly into ice cold lysis buffer (0) or into culture medium for 10min (10) prior to lysis in RIPA.

B. ~0.5g cartilage was dissected either directly into ice cold lysis buffer (0) or into culture medium for 10min (10) prior to lysis in RIPA.

For both A. and B., lysates were incubated with 4G10 beads overnight prior to washing the beads 5 times in RIPA, eluting in phenyl phosphate and boiling the eluate with SDS sample buffer. Western blotting with 4G10 antibody was carried out.

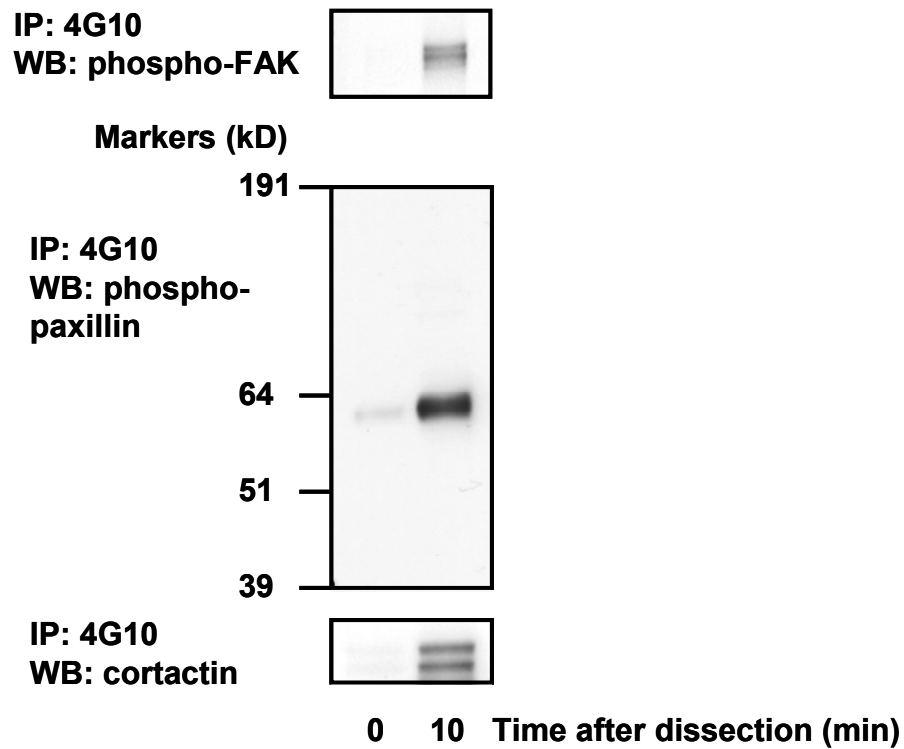


Figure 6.5. Phosphorylation of FAK, paxillin and cortactin is induced by synovial injury.

~0.2g synovium was dissected either directly into lysis buffer (0), or into medium and cultured for 10min (10), prior to lysis and 4G10 immunoprecipitation. Beads were eluted with phenyl phosphate. Western blotting of eluates for phospho-tyr 576 FAK, phospho-paxillin and cortactin was carried out.

Strong induction of phospho-paxillin was also seen which aligned with the 60 kD band on 4G10 blotting. The cortactin antibody stained a doublet at 80 kD (Fig 6.5). This doublet also aligned with the doublet seen on 4G10 western blotting (see Fig 6.4 for position of this doublet).

All of this work was performed on synovium from the MCP joints of pigs, but similar tyrosine phosphorylation was also seen in injured cartilage and synovium from porcine knees (data not shown).

6.3 Attempts at phosphoprotein identification from synovium

Given that synovium is far more cellular than cartilage, and the strength of the phosphorylation in response to injury, this tissue seemed a promising source for further scale-up and mass spectrometric identification of more tyrosine-phosphorylated proteins. It was possible that a src or src-like kinase might be found, and also the 190 kD protein identified (this appeared to be phosphorylated in both tissues).

Conditions for 4G10 immunoprecipitation were optimised for synovium in the same way as had previously been shown for cartilage. Equal amounts of synovium were dissected into RIPA buffer and lysed for increasing times prior to clarification and boiling with sample buffer. These experiments suggested that a shorter lysis time for synovium of 90min gave optimal extraction of phosphorylated proteins (Fig 6.6). Optimal conditions, such as length of immunoprecipitation with 4G10 beads were otherwise found to be similar to those used for cartilage (data not shown, see Materials & Methods, Fig 2.3 for summary of 4G10 immunoprecipitation protocol for synovium).

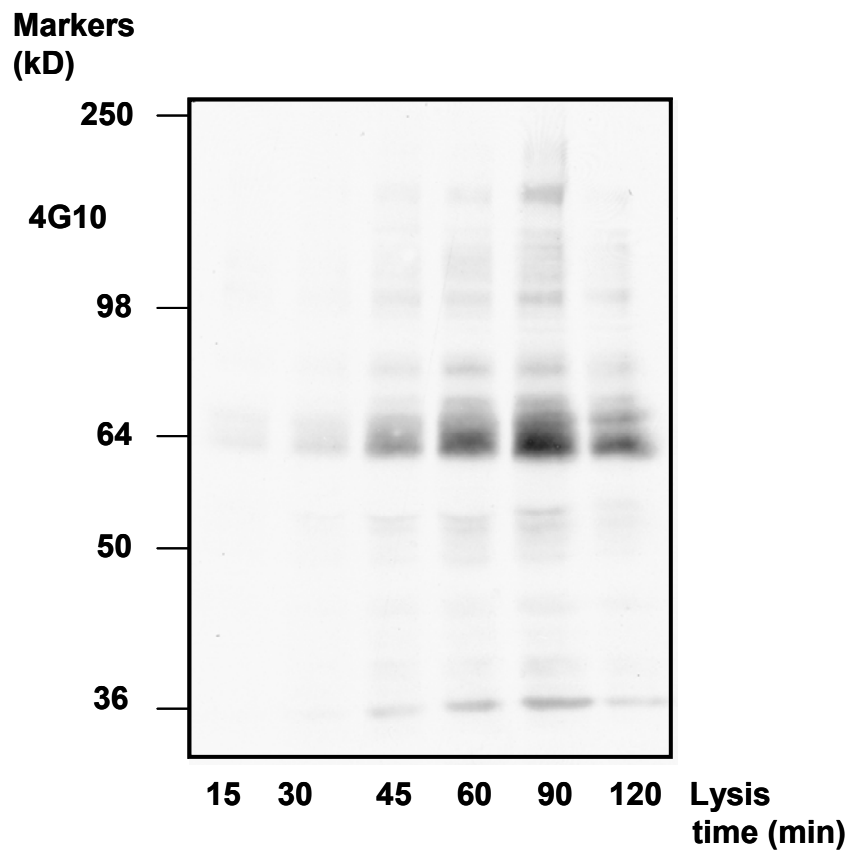


Figure 6.6. Optimal duration of lysis for phosphotyrosine immunoprecipitation from synovial lysates.

~0.1g synovium was dissected into culture medium for 10min prior to lysis in RIPA buffer, shaking at 4°C for the increasing times indicated. Lysis buffer was removed, clarified and western blotting of the lysates with 4G10 antibody to assay the phosphotyrosine content was carried out.

The phosphorylation pattern was preserved on phospho-western blotting after concentration of synovial eluates with the GE kit. However, the silver stain of these samples was dominated by many bands which were identified by mass spectrometry as mainly porcine immunoglobulin and albumin (data not shown). In further experiments, synovium was extensively washed prior to lysis. This depleted serum protein bands when stained with silver or Coomassie Brilliant Blue (data not shown). Increased pre-clearing of lysates was also carried out. Despite these measures, silver staining patterns of control and dissected tissue samples were indistinguishable and complex (Fig 6.7): no injury-dependent bands were seen. The lanes of the gels were sliced and analysed by mass spectrometry but no peptides were found restricted to the injury lane samples. There was heavy contamination with serum protein and some abundant cellular proteins such as actin were found when samples were concentrated. Given the low amounts of tyrosine-phosphorylated proteins in the samples compared to the non-specific contaminants, further attempts at purification from synovial preparations were abandoned.

6.4 The effect of pharmacological inhibitors on intracellular signalling in injured synovium

One uncertainty in testing pharmacological inhibitors on cartilage was their penetration into the tissue. The protein kinase inhibitors needed to be injected into the joint prior to dissection of cartilage. Some unpredictable effects were seen with this approach, such as the effect of 0.1% DMSO on phosphorylation of ATF-2 (Ch 5, Fig 5.17, Expt 1). Because pieces of synovium were far smaller and probably more permeable to small molecule inhibitors than cartilage, I avoided prior injection.

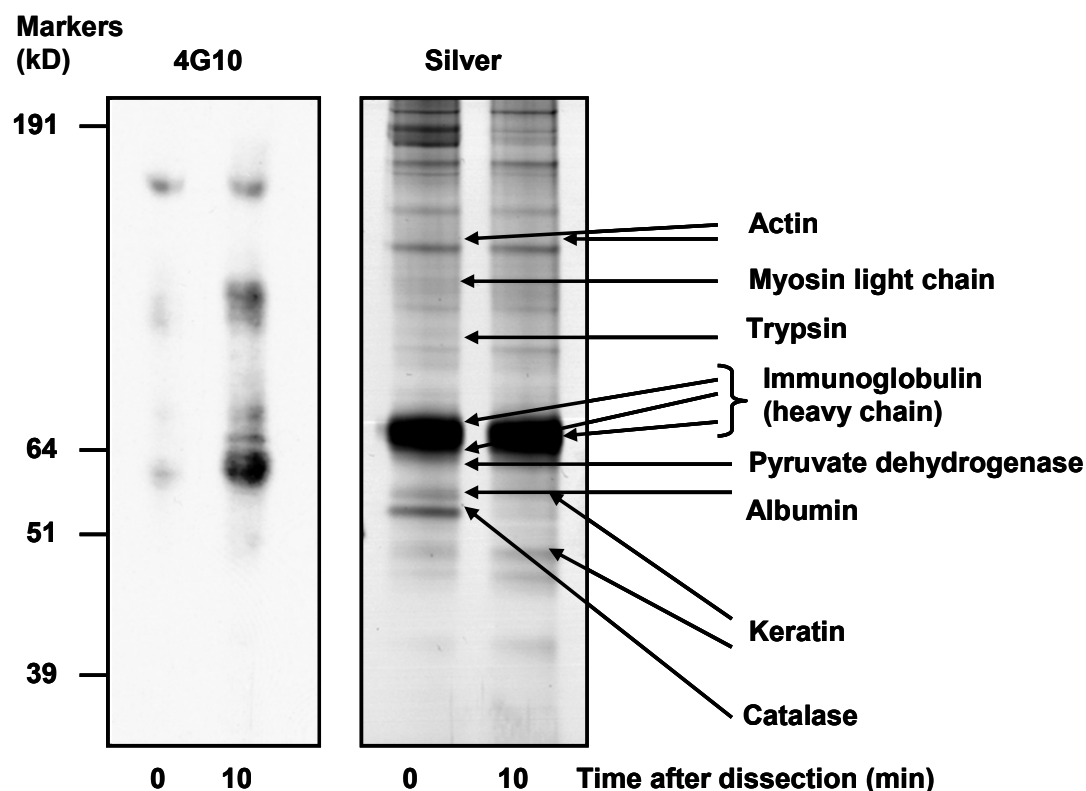


Figure 6.7. Scale-up: attempts to identify tyrosine-phosphorylated proteins in injured synovium.

~1g (0.12g x 8 samples) of porcine synovium was dissected from the MCP joint either directly into tubes on dry ice (0) or into serum-free medium and cultured for 10min (10). All samples were washed and vortexed 5 times, and then washed for a further 3 x 10min rotating at 4°C prior to lysis. Samples were lysed separately, pre-cleared with 100µl protein A agarose and 100µl protein G agarose twice for 30min and then standard 4G10 immunoprecipitation was carried out. Phenyl phosphate eluates were pooled and concentrated with the clean up kit (GE) prior to boiling samples with SDS sample buffer. 1/6 of the concentrated material for each sample was electrophoresed and assayed for phosphotyrosine content by western blotting with 4G10 antibody (4G10). 5/6 of the concentrated material was electrophoresed on the same gel, the gel divided, and silver staining carried out. The figure shows alignment of the two pieces of gel. Bands from the silver stained gel were cut out, digested with trypsin overnight, and analysed by mass spectrometry.

Although similar intracellular signalling pathways were activated in synovium in response to injury, their regulation may be different to cartilage. Experiments testing the effects of the same inhibitors on synovium were therefore carried out.

It was possible that blocking src signalling in synovium would have different effects to those seen in cartilage. ~0.2g synovium was dissected either directly into a tube on dry ice, or into medium with or without 10 μ M PP2 for 10min prior to lysis. Lysates were electrophoresed and western blotting for phospho-paxillin carried out. (This was sometimes used alone, as a single read-out of FAK/src signalling). The induction of phospho-paxillin upon dissection was largely prevented by PP2, as seen previously in cartilage (Fig 6.8). The lysates were subsequently assayed for phospho-ATF-2, phospho-p38 and phospho-ERK by western blotting. PP2 had no effect on the induction of any of these pathways after synovial injury (Fig 6.8). The synovium behaved similarly to cartilage in that src (and possibly FAK) activity did not cause the MAPK activation seen on injury.

The FGF receptor inhibitor reduced both MAPK and IKK signalling after explantation of cartilage. Cartilage contains a pool of FGF-2 in the pericellular matrix, which is released upon injury and contributes to cell activation (Vincent et al., 2002; Vincent et al., 2007). Whether synovium has extracellular FGF-2 in similar quantities and would respond in the same way was unknown. I therefore tested the FGF receptor inhibitor on the synovial tissue. In a similar way to that shown in Fig 6.8, synovium was either dissected into a tube on dry ice, or into medium with, or without, 250nM PD173074 for 10min prior to lysis. Western blotting of lysates for the 3 phospho-MAPKs was carried out.

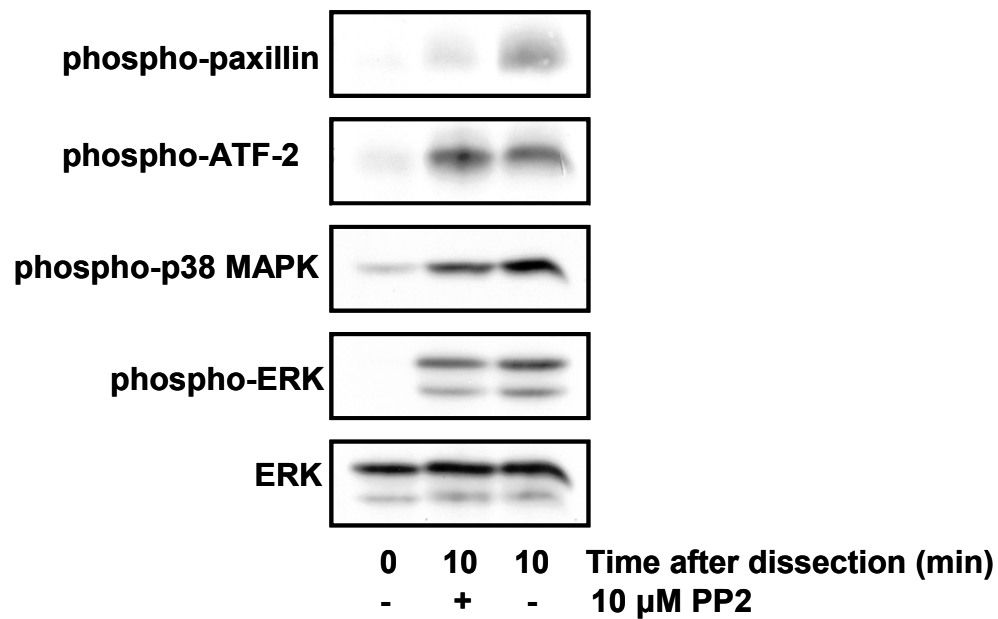


Figure 6.8. PP2 has no effect on the induction of MAPK signalling following dissection and culture of synovium.

~0.2g synovium was dissected either directly into a tube on dry ice (0), or into medium with or without 10μM PP2 for 10min prior to lysis. Lysates were electrophoresed and western blotting for phospho-paxillin, phospho-ATF-2, phospho-p38 and phospho-ERK was carried out. The membrane was stripped and re-probed with ERK to ensure equal cellular protein loading. This result is representative of 2 similar experiments.

Induction of all 3 MAPK pathways was again seen following synovial injury, and the FGF receptor inhibitor did not prevent this (Fig 6.9, upper panels). On re-probing the membrane with ERK antibodies, it was apparent that the sample treated with PD173074 was somewhat overloaded in this experiment. However, the western blot for phospho-ERK mirrored this pattern, suggesting that the inhibitor was not reducing MAPK activation in this tissue. The lysates were also assayed for IKK activity. Phosphorylation of the substrate I κ B α was increased following synovial dissection, showing that in addition to activation of the MAPK pathways, IKK signalling was also induced (Fig 6.9, lower panel). Again, the presence of the FGF receptor inhibitor did not affect the activation of IKK in synovium, in contrast to its effects in cartilage (Ch 5, Fig 5.20). This experiment needs to be repeated.

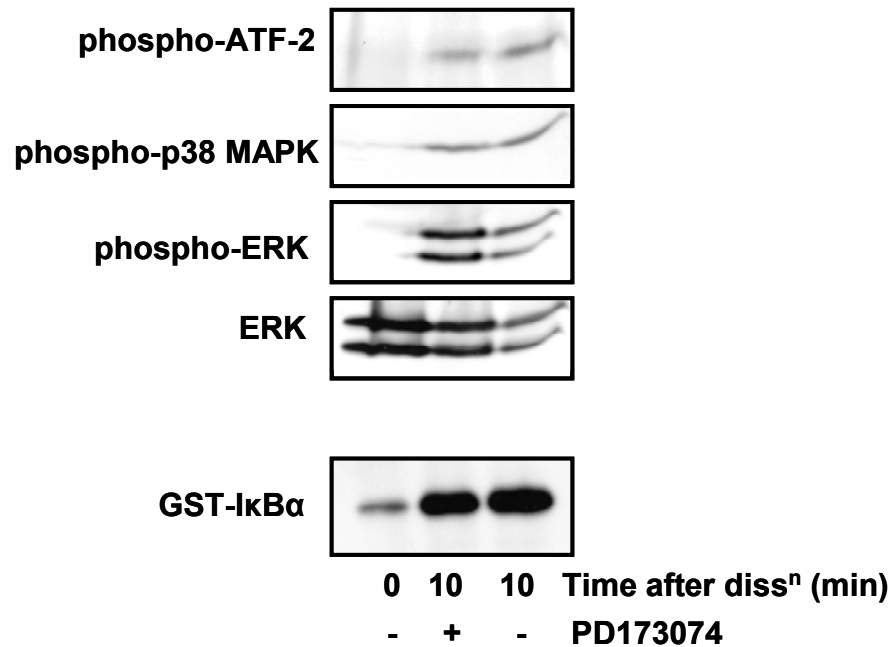


Figure 6.9. PD173074 has no effect on the induction of MAPK or IKK signalling following dissection and culture of synovium.

~0.2g synovium was dissected either directly into a tube on dry ice (0), or into medium with or without 250nM PD173074 for 10min prior to lysis. Lysates were electrophoresed and western blotting for phospho-ATF-2, phospho-p38 and phospho-ERK carried out. The membrane was stripped and re-probed with ERK (upper panels). IKK activity in the lysates was also measured by kinase assay (lower panel).

6.5 Immunolocalisation of phospho-paxillin in injured synovium

The only cell type in cartilage is the chondrocyte. In contrast, there are many different cell types in synovium. I investigated the site of cellular activation in dissected synovium. For immunohistochemistry, phospho-paxillin antibodies were chosen. These detected a single strong band in synovial lysates at 60 kD, which aligned with the striking band on 4G10 staining (Figs 6.5 & 6.2). The phospho-FAK antibodies were unsuitable because they gave a high background. Synovium was dissected either immediately into formaldehyde (which had been equilibrated at 37°C to improve its tissue penetration), or dissected into medium with, or without, sodium pervanadate for 10min prior to fixation in warm formaldehyde. Tissues were then embedded in paraffin, sectioned and immunohistochemistry carried out, with incubation of the sections in 1:50 phospho-paxillin antibody at room temperature for 1h (see Materials & Methods for details, section 2.10).

There was little phospho-paxillin staining in *ex vivo* tissue (Fig 6.10). In contrast, synovium cultured in sodium pervanadate had small areas of strong phospho-paxillin staining throughout the tissue. These appeared to be small blood vessels. Synovium which had been dissected and left in serum-free medium alone also showed cellular staining with the antibodies. This was weaker than that seen with pervanadate, and appeared proportionate to the relative strength of staining on western blot with phospho-paxillin antibodies in response to these 2 stimuli (Ch 5, Fig 5.6). The staining was seen most clearly at x100 magnification. The phospho-paxillin staining on synovial injury appeared to be throughout the tissue, rather than just at the cut edge (see middle panels, x100 magnification). There was no staining of the sections when incubated with a non-immune rabbit IgG, as a control for the procedure (lower panels,

Fig 6.10). Sections of synovium dissected and cultured for shorter times were examined, but a clear temporal or spatial pattern of cell activation could not be defined (data not shown).

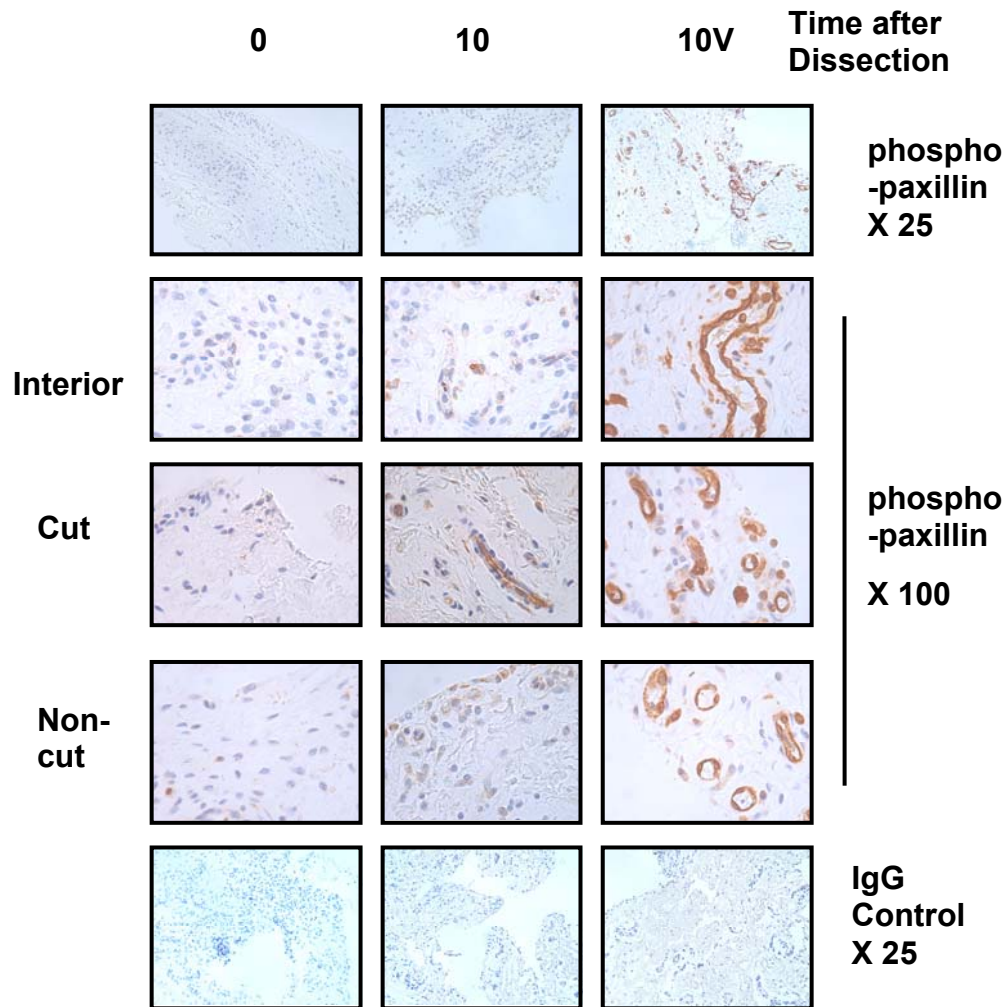


Figure 6.10. Immunolocalisation of phospho-paxillin in synovium after dissection.

Synovium was dissected either immediately into formaldehyde (which had been equilibrated at 37°C), or dissected into medium alone (10) or into medium containing 10mM sodium pervanadate (10V) for 10min, prior to fixation in warm formaldehyde. Tissues were then embedded in paraffin, sectioned and standard immunohistochemistry protocol carried out with incubation of the sections with 1:50 phospho-paxillin antibody at room temperature for 1h. As a control, other sections were incubated with a non-specific immunoglobulin instead (IgG control). All sections were stained with diaminobenzidine. Nuclei are stained blue and phospho-paxillin brown.

6.6 Discussion

Connective tissues such as synovium and cartilage appear to have similar initial responses to injury. MAPKs, IKK and src-like signalling are induced in both tissues. This may represent a ubiquitous response of connective tissue to injury. It would be interesting to investigate whether other tissues such as skin which contain connective tissue elements respond in a similar manner.

The mechanism of activation of the intracellular pathways in the two tissues showed one major difference. The FGF receptor inhibitor did not prevent the activation of MAPK signalling and IKK in synovium, whereas it caused partial suppression of activation of these pathways in cartilage. Synovial and cartilage extracellular matrix architecture is very different. It may be that there is no perlecan or FGF-2 in synovial matrix as there is in cartilage. These findings would support the hypothesis that connective tissues, and perhaps all tissues, respond to injury by activation of inflammatory signalling pathways, via an unknown mechanism. In the case of cartilage, the simultaneous release of FGF-2 from the tissue enhances the signalling. This secondary augmentation does not appear to occur in synovium. (It may have been prudent to inject the joints with inhibitor to check that this did not make a difference). It would be interesting to investigate this further by looking for FGF-2 in synovium, by ELISA of conditioned medium, or western blotting of lysates. The differing findings in these two tissues also suggest that the observation in cartilage is not simply due to toxicity or non-specific effects of the FGF receptor inhibitor on cells.

The effect of inhibition of src was similar in both cartilage and synovium. This lends support to the findings in cartilage. These kinases appear to be activated in parallel to the MAPK pathways. The mechanism of their activation and the subsequent effect on

cartilage physiology remain unknown. They do not appear in either tissue to be responsible for MAPK activation. Their effects on IKK signalling in synovium were not assessed, but seem likely to be similar to cartilage.

That further phospho-proteins could not be identified by preparing and immunoprecipitating larger quantities of synovial lysates was disappointing. Contamination with serum proteins was probably due to a combination of synovial fluid contamination and serum proteins which were present in the tissue itself. Depletion of serum proteins via a binding column was considered, but this would not have addressed contamination with other cellular proteins. These columns are also designed to bind human or mouse protein, and we were unsure how they would perform with porcine protein. Given that there were some differences in the initial appearance of 4G10 western blotting of synovial lysates compared with cartilage lysates, it was perhaps surprising that the 4G10 immunoprecipitates of the two tissues appeared so similar. As discussed previously in Chapter 4, it was possible that 4G10 was not immunoprecipitating all tyrosine-phosphorylated proteins. However, even if further tyrosine kinases were identified via this approach, the lack of effect of PP2 on our pathways of interest meant that this was unlikely to identify a key pathway in the initiation of MAPK signalling.

The results from the histology were interesting. It will be important to establish which cells in synovium are responding to injury, and this could be investigated by the use of specific immuno-labelling for different cell populations. It would have been interesting to examine the response of cartilage by immunohistochemistry. However, technical issues (fixed sections falling off slides, non-specific staining in frozen sections) prevented this.

7 Final discussion

Explantation of articular cartilage activates the NF κ B pathway in addition to MAPKs and src-dependent signalling (Summary schematic, see Fig 7.1). All of these pathways appear to contribute to the gene response to injury. The most upstream kinase shown to be activated in response to injury is IKK. This is the beginning of the canonical NF κ B pathway since the kinase assay detects the trimeric IKK complex. Further questions include whether or not IKK α and/or β are activated, what controls IKK activation and whether other pathways leading to activation of NF κ B are activated by injury. TAK-1 is the best characterised enzyme known to activate IKK. It phosphorylates specific serine residues on IKK α and β (Ch 1, Fig 1.4). TAK-1 deficient cells have severe defects of both IL-1 and TNF α induction of inflammatory signalling pathways (Shim et al., 2005; Sato et al. 2005). However, a preliminary experiment which needs to be repeated suggests that TAK-1 is not activated by cartilage explantation.

If the activation of IKK is not TAK-1 dependent, the role of other MKKKs would need to be explored. NIK and MKKKs (MEKKs)-1,-2 and -3, when overexpressed, are reported to activate NF κ B via IKK (Hacker and Karin, 2006; Malinin et al., 1997; Yang et al., 2001). However, whether they are important for *in vivo* activation of IKK is less clear. MEKK3 is required in addition to TAK-1 for TNF α -induced IKK activation in embryonic fibroblasts (Blonska et al., 2005; Yang et al., 2001). NIK and MEKK1 knockout mice do not have defects in TNF or IL-1-mediated IKK activation, although interestingly the latter do have abnormal wound healing (Xiao et al., 2001; Yujiri et al., 2000). Ultimately, the most efficient way of dissecting the higher control of IKK activation would be to examine the responses to injury of mouse cartilage. This would enable the investigation of the role of key signalling molecules by the use of knock-out animals. These would include the higher kinases TAK-1, NIK, and

MEKK1-3. Use of MyD88 and TRAF-2 deficient mice would allow us to understand whether TIR or TNF signalling respectively are required for the injury response (Ch 4, Fig 1.4).

My experimental evidence is consistent with the activation of srcs by injury. Which family member(s) is activated, and the mechanism by which this occurs are unknown. Although growth factors such as PDGF and FGF are typically thought to activate src, I found little evidence that FGF-2, while activating the cartilage on injury, was contributing significantly to src activation. The only src substrate whose phosphorylation was reduced by the FGFR inhibitor was paxillin. Quantitation of relative amounts of src family members in chondrocytes by RT-PCR could indicate which are likely candidates. Kinase assays for possible candidates (e.g. yes and fyn) could then be carried out in the same way as described for c-src. It may be that the apparent src activation is not due to a change in intrinsic activity following injury, but to interactions with other proteins affecting localisation or access to substrates within the cell. Although src-like activity appeared to contribute to gene induction upon injury, it was not responsible for IKK or MAPK activation. Whether srcs are activated by the same mechanism as IKK and MAPKs following injury, or by a different mechanism remains unclear. There is extensive and persistent tyrosine phosphorylation in cartilage explants following injury. It may be this continuing response which makes cultured cartilage behave differently from the intact articular surface.

FGF-2 augments MAPK and IKK signalling following injury, and this may be a particular feature of cartilage. In contrast, the FGFR inhibitor had no effect on activation of protein kinases when synovium was injured. Thus, some other mechanism accounts for IKK and MAPK activation following injury. In cartilage,

FGF enhances this signalling by around 50%. This data was gained with the use of a pharmacological inhibitor. Preliminary murine experiments on MAPK signalling in the FGF knock-out are under way (K. Chong & T. Vincent). How, and at what level, FGF exerts this effect is currently a difficult question to address, given our lack of knowledge of control of higher signalling in this system. It is interesting that FGF is suppressive of IL-1-induced catabolic processes *in vitro* and appears to be chondroprotective in surgically-induced OA in the mouse and yet it promotes inflammatory signalling upon injury (Sawaji et al., 2008; Chia et al., 2009). These observations perhaps illustrate the difference in response between cultured cells or tissues and *ex vivo/in vivo* tissue. This difference has been evident throughout this work and should inform our approach to further studies of how resting intact tissues detect wounding.

Immunolocalisation experiments in synovium suggest that a mechanical stimulus may somehow activate cells distant to the site of an injury. This suggests a novel signalling mechanism in connective tissues which is not understood. This could be explored by further experiments immunolocalising other signalling molecules in injured cartilage, synovium or other connective tissues. Such a mechanism would potentially have far-reaching implications for our understanding of the response of tissues to injury in general.

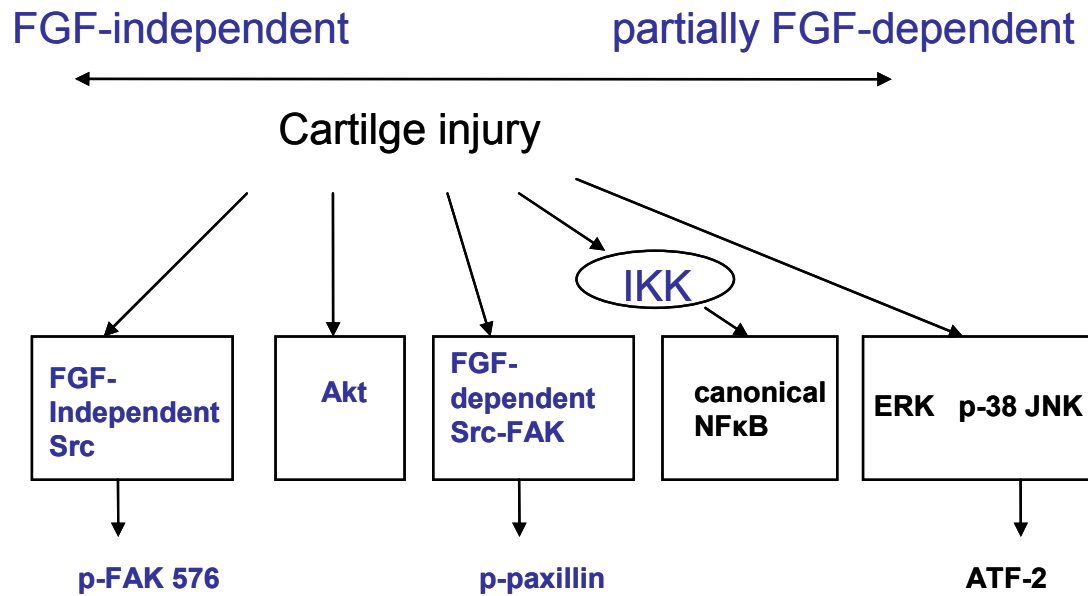


Figure 7.1. Signalling activated by cartilage injury.

A schematic of the signalling shown to be activated by immediate injury to the intact articular surface. Some pathways appear to be regulated in part by the release of FGF-2 (e.g. MAPKs) whereas some appear to be FGF-independent (e.g. the phosphorylation of FAK). Blue typeface shows the pathways that the experiments presented in this thesis have shown to be activated by signalling after injury and the influence of FGF on these pathways.

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APPENDIX

Activin A Is an Anticatabolic Autocrine Cytokine in Articular Cartilage Whose Production Is Controlled by Fibroblast Growth Factor 2 and NF- κ B

Susan Alexander, Fiona Watt, Yasunobu Sawaji, Monika Hermansson, and Jeremy Saklatvala

Objective. Proteomic analysis has previously shown that activin A, a member of the transforming growth factor β family, is produced by human articular cartilage. This study was undertaken to investigate whether activin A affects cartilage matrix catabolism and how its production is regulated.

Methods. The effect of exogenous activin A on interleukin-1-induced aggrecanase-generated neopeptide production was assessed by Western blotting, using medium from human cartilage explants. Levels of activin A production were determined by enzyme-linked immunosorbent assay. For genes of interest, messenger RNA (mRNA) induction in cartilage explants or primary chondrocyte monolayers was assessed by reverse transcriptase-polymerase chain reaction. Activin A activity in cartilage explant medium was measured by incubating it with human dermal fibroblasts and determining the increase in phospho-Smad2 by Western blotting.

Results. Activin A (1–10 ng/ml) suppressed aggrecanase-mediated cleavage of aggrecan in human articular cartilage. Activin A mRNA and protein secretion were induced by dissection and culture of human or porcine articular cartilage. This activin A was bio-

logically active. Its production was due to an active cellular process and was enhanced in osteoarthritic (OA) tissue. Activin A production on dissection was reduced by 80% by the fibroblast growth factor (FGF) receptor inhibitor PD173074 and by 70% by the IKK inhibitor BMS345541.

Conclusion. Activin A is potentially an anticatabolic molecule in articular cartilage. Its expression is induced by wounding in an FGF-2- and NF- κ B-dependent manner. OA cartilage produced more activin A than did normal cartilage in vitro.

Proteomic analysis of the proteins secreted by human articular cartilage in vitro has revealed activin A as a potential regulatory molecule of the tissue (1). Activin A is a member of the transforming growth factor β (TGF β) family (2,3) and is a homodimer of proteolytically processed inhibin β A chains. Inhibin β -chains also heterodimerize with the inhibin α -chain to form inhibins. Activins bind and activate activin receptors on the cell surface. Inhibin binds to the receptors but does not activate them.

There are 4 genetically different mammalian inhibin β -chains: A, B, C, and E. These potentially form either homodimers or heterodimers, giving rise to activins A, B, AB, and so on. Proteomic analysis of cartilage has not revealed any inhibin α -chains, suggesting that activin, rather than inhibin, is the likely product (1). Both pro-inhibin β A (35-kd) and processed inhibin β A (14-kd) forms have been found, but no inhibin β B or β C chains have been identified. Activins, besides binding to cell surface receptors, also bind to follistatin or follistatin-related protein, which act as inhibitors by preventing binding to the receptors.

Activin was originally discovered as a molecule in ovarian follicular fluid that caused pituitary cells in culture to secrete follicle-stimulating hormone, while inhibin was found to be an inhibitor of this process (2,3).

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Inhibin β A-knockout mice have severe craniofacial abnormalities, suggesting that activin A or inhibin is important for intramembranous development of bone (4). Activin A is produced at sites of inflammation and is induced in cultured cells by inflammatory stimuli, such as interleukin-1 (IL-1) or bacterial lipopolysaccharide, and by growth factors (5). It is strongly expressed in injured skin (6), in inflamed gastrointestinal mucosa (7,8), and in the lung in conditions associated with pulmonary fibrosis (9). It is also found in synovial fluid in inflammatory arthritis (10).

Overexpression of follistatin in the basal epidermis of the skin was found to delay wound healing in mice, while overexpression of activin A increased the amount of granulation tissue formed and subsequent scarring, suggesting that activins play a physiologic role in the healing process (11). The function of activin A in inflammation is uncertain. Its relationship to TGF β makes it likely to be a regulatory molecule, but its diverse effects on gene expression in different cell types are consistent with both proinflammatory and antiinflammatory roles (3).

The activin receptors are related to those of TGF β (12). Activins and inhibins bind to the activin type II or IIB receptors. The type II receptors are constitutively active serine/threonine protein kinases which, when engaged, bind, phosphorylate, and activate the type I receptor, activin receptor-like kinase 4 (ALK-4), which is also a serine/threonine protein kinase. ALK-4 then phosphorylates the transcription factors Smad2 and Smad3. Smad2 and Smad3 are also activated by the TGF β receptor. TGF β is a pleiotropic cytokine. It is immunomodulatory, chemotactic for leukocytes, stimulates deposition of collagen, and may inhibit expression of inflammatory response proteins (13). It can reduce the IL-1-induced degradation of cartilage proteoglycan (14–16).

Because activin A shares a postreceptor mechanism with TGF β , we investigated the effect of activin A on articular cartilage. It physiologically antagonized aggrecanolytic stimulation by IL-1. Osteoarthritic (OA) cartilage explants appeared to secrete more activin A than normal tissue, so we therefore explored how this potentially autocrine anticatabolic cytokine might act and how its production in cartilage is controlled.

MATERIALS AND METHODS

Cartilage. Porcine articular cartilage was dissected from the metacarpophalangeal (MCP) joints of freshly slaughtered 3–6-month-old pigs obtained from a local abattoir. Normal and OA human femoral head cartilage and OA knee cartilage were obtained from patients at Charing Cross Hospital (London, UK) who were undergoing joint replacement

surgery for either fractured femoral neck or OA. Normal knee cartilage was obtained from patients at the Royal National Orthopaedic Hospital (RNOH; Stanmore, Middlesex, UK) who were undergoing surgery for tumors involving the lower leg (not involving the knee). All human samples were used with consent from the local ethics committees (Riverside Research Ethics Committee and Joint RNOH/Institute of Orthopaedics and Musculoskeletal Science Research Ethics Committee), and written informed consent was obtained from all patients. Human dermal fibroblasts were obtained from infant foreskin and were used at passages 7–16.

Reagents. Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) (both from BioWhittaker, Verviers, Belgium) were supplemented with 25 mM HEPES, 1.25 units/ml penicillin, 100 μ g/ml streptomycin, and 2 μ g/ml amphotericin B. The DuoSet enzyme-linked immunosorbent assay (ELISA) kit for activin A was obtained from R&D Systems (Minneapolis, MN). Recombinant human activin A, TGF β , follistatin, and neutralizing mouse monoclonal antibodies (mAb) to activin A and TGF β were also from R&D Systems. Rabbit polyclonal antibody to phosphorylated Smad2 was obtained from Cell Signaling Technology (Beverly, MA), and murine mAb to phosphorylated ERK was from Sigma (Poole, UK). Rabbit polyclonal antibody to ERK was from Santa Cruz Biotechnology (Santa Cruz, CA). Chondroitinase ABC and keratanase were obtained from Seikagaku Kogyo (Tokyo, Japan). Mouse mAb to aggrecan neoepitope ARGSV was provided by Bruce Caterson (University of Cardiff, Cardiff, UK). Sheep tissue inhibitor of metalloproteinases 1 (TIMP-1) antibody was provided by Hideaki Nagase (Kennedy Institute, London, UK), and mAb to TIMP-3 was obtained from Fuji Chemical (Takaoka, Japan). Horseradish peroxidase (HRP)-linked anti-mouse IgG or anti-rabbit IgG was from Dako (Glostrup, Denmark). The electrochemiluminescence Western blot detection system was from Amersham Biosciences (Little Chalfont, UK).

The fibroblast growth factor receptor (FGFR) kinase inhibitor PD173074, also called SB402451, was provided by Stephen Skaper (GlaxoSmithKline, London, UK). U0126 was from Promega (Madison, WI), and MG-132 was from Biomol (Plymouth Meeting, PA). SB202190, LY294002, BMS345541, PP1, PP2, G6983, and G6976 were from Calbiochem (La Jolla, CA).

Preparation and culture of cartilage explants. Cartilage was dissected into DMEM (supplemented as indicated above) and washed briefly to remove synovial fluid. Cartilage was cut into pieces $\sim$ 3–4 mm³. It was then cultured at 0.5 gm/ml, 1 gm/ml, or as indicated, in DMEM for up to 20 hours.

Assay for aggrecanase-generated neoepitope. Three pieces of cartilage were cultured in 1 well of a 48-well plate in 250 μ l of medium for 24 hours in the absence or presence of IL-1 (10 ng/ml). For each assay, 3 culture wells were used (i.e., 9 explants). The medium samples were deglycosylated at 37°C overnight with chondroitinase ABC and keratanase in 50 mM Tris HCl (pH 7.5), 50 mM sodium acetate, and 10 mM EDTA. Deglycosylated proteoglycan from the medium was precipitated with ice-cold acetone, air-dried, and resuspended in sodium dodecyl sulfate (SDS) sample buffer. Samples were loaded onto an 8% Tris-glycine gel and separated by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Protein was electrophoretically transferred to poly-

vinylidene difluoride (PVDF) membrane, and aggrecan fragments were detected with the BC-3 antibody to the ARGSV neoepitope. After reaction with HRP-linked anti-mouse IgG antibody, proteins were detected by electrochemiluminescence.

Western blotting for determination of phospho-ERK and phospho-Smad2 content. Cartilage tissue was snap-frozen and kept at -70°C until used. Cellular protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% [volume/volume] Triton X-100, 0.1% [weight/volume] SDS, 1% [w/v] sodium deoxycholate, 0.5% [v/v] Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 10 μM E64, 1 $\mu\text{g}/\text{ml}$ pepstatin A, and 10 $\mu\text{g}/\text{ml}$ aprotinin) at 4°C for 2 hours with agitation. Extracts were clarified by centrifugation (at 13,000 revolutions per minute for 20 minutes) and mixed with reducing SDS sample buffer. Samples were boiled, subjected to SDS-PAGE on a 10% gel, transferred to PVDF membrane, and immunostained, and proteins were visualized by electrochemiluminescence, all as described above. For cells, fibroblasts were lysed in ice-cold RIPA buffer and left on ice for 10 minutes. Lysates were scraped and aspirated from the culture surface, clarified by centrifugation, and mixed with reducing SDS sample buffer. Samples were boiled, subjected to SDS-PAGE on a 12.5% gel, and immunostained for phospho-Smad2, which was visualized by electrochemiluminescence as described above.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Approximately 200 mg of cartilage was homogenized with a Polytron rotor-stator homogenizer in 3 ml of TRI Reagent (Helena Biosciences, Gateshead, UK), then extracted with chloroform. The aqueous phase was mixed with an equal volume of 70% (v/v) ethanol, and RNA was extracted with the QIAamp RNA Blood Mini kit (Qiagen, Chatsworth, CA). Total RNA (1 μg) was reverse-transcribed using Superscript II (Invitrogen, San Diego, CA). PCR was performed using Ready-to-Go PCR beads (Amersham Biosciences) with the appropriate primers. Amplified DNA was analyzed on a 1% (w/v) agarose gel and then visualized by ethidium bromide staining.

RT-PCR primers (MWG Biotech, Ebersberg, Germany) and conditions were as follows: for ADAMTS-4, sense 5'-ACCACTTTGACACAGCCATTCTG-3' and antisense 5'-ACCCCCACAGGTCCGAGAGCAG-3' (annealing at 63°C , 27 cycles); for ADAMTS-5, sense 5'-TGTGCTGTGATTGAAGACGAT-3' and antisense 5'-GACTGCAGGAGCGGTGAGTGG-3' (annealing at 60°C , 24 cycles); for inhibin βA (17), sense 5'-GACATCCGGACTGCCTGCGAGCAG-3' and antisense 5'-GTAGCCGGACGGAGCGATTAGCCAGTC-3' (annealing at 65°C , 30 cycles); and for GAPDH, sense 5'-CATGGAGAAGGCTGGGGCTC-3' and antisense 5'-ATGAGGTCCACCACCCTGTT-3' (annealing at 60°C , 23 cycles).

RESULTS

Physiologic antagonism of the aggrecanase-mediated cleavage of aggrecan in articular cartilage by activin A. Explants of normal human femoral condylar cartilage were rested for 24 hours after dissection into culture medium. They were then stimulated with IL-1 or

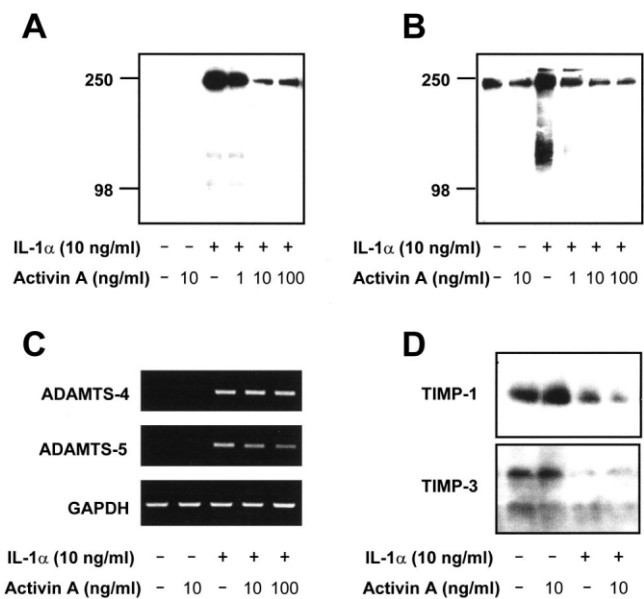


Figure 1. Activin A inhibition of aggrecan cleavage in human articular cartilage stimulated with interleukin-1 α (IL-1 α). **A** and **B**, Normal human femoral condylar cartilage was dissected from fresh surgical specimens from a 16-year-old girl (**A**) and a 51-year-old woman (**B**). Explants were washed and rested overnight in serum-free Dulbecco's modified Eagle's medium (DMEM), preincubated for 30 minutes in the presence or absence of activin A, and then left untreated or treated with 10 ng/ml IL-1 α for 24 hours. Aggrecan fragments released into conditioned medium were detected by Western blotting with the antibody to the ARGSV neoepitope as described in Materials and Methods. **C**, Confluent human chondrocytes (from a 43-year-old man) in primary culture were serum-starved, preincubated for 30 minutes in the presence or absence of activin A, and then left untreated or treated with 10 ng/ml IL-1 α for 4 hours. RNA was extracted and subjected to reverse transcriptase-polymerase chain reaction for ADAMTS-4, ADAMTS-5, and GAPDH. **D**, Human cartilage explants (from a 51-year-old woman) were rested overnight in serum-free DMEM, preincubated for 30 minutes in the presence or absence of activin A, and then left untreated or treated with 10 ng/ml IL-1 α for 24 hours. Secreted tissue inhibitor of metalloproteinases 1 (TIMP-1) and TIMP-3 were detected by Western blotting. In experiments for TIMP-3, heparin was added to the medium before addition of activin A, to trap the protein.

were left resting for a further 24 hours, after which the culture medium was analyzed by Western blotting for released aggrecan fragments containing the neoepitope ARGSV. This sequence forms the new N-terminal sequence generated by aggrecanase cleavage of the major cartilage proteoglycan aggrecan between E³⁷³ and A³⁷⁴. The likely potential aggrecanases in cartilage are ADAMTS-4 and ADAMTS-5 (18–20).

IL-1 stimulation caused the expected release of ARGSV-bearing fragments from the cartilage (Figure 1). Inclusion of recombinant human activin A in the

culture, which was added 1 hour before the addition of IL-1, suppressed the release of the proteoglycan fragment in a dose-dependent manner. The results shown were obtained with cartilage from a 16-year-old girl (Figure 1A) and a 51-year-old woman (Figure 1B). Suppression occurred with 1–10 ng/ml activin A. The steady-state levels of both ADAMTS-4 and ADAMTS-5 mRNA were increased by IL-1 stimulation of isolated human chondrocytes, but these were unaffected by activin A at 10 ng/ml (Figure 1C). Some suppression of ADAMTS-5 mRNA was seen at the high concentration of 100 ng/ml. We have been unable to detect either ADAMTS-4 or ADAMTS-5 proteins by Western blotting with a number of different antibodies.

Aggrecanases are inhibited by TIMP-3 (21). We examined the effect of activin A on the expression of TIMP-3 and, because of the relationship of activin A to TGF β , on the expression of TIMP-1 (22). Activin A increased the amount of TIMP-1 in the cartilage explant medium, while IL-1 decreased it (Figure 1D). However, activin A did not increase the amount of TIMP-1 in the presence of IL-1. At the mRNA level, the amount of TIMP-1 was unaffected by IL-1 stimulation, but the modest 2-fold increase caused by activin A (similar to that shown for protein in Figure 1D) was prevented (results not shown). IL-1 stimulation caused a reduction in the amount of TIMP-3 in the medium, and this was also unaffected by the presence of activin A (Figure 1D). At the mRNA level, neither IL-1 nor activin A had any consistent marked effect on the levels of TIMP-3 expression (results not shown).

Activin A induction in cartilage by dissection and culture. The fact that activin A inhibits the aggrecanase cleavage induced by IL-1 suggests that it could be an autocrine anticatabolic molecule. It was therefore important to establish how the production of activin A was controlled. Proteomic analysis showed that both large and small forms of inhibin β A chain were produced by cartilage. These corresponded to the precursor pro-inhibin β A, and the mature processed inhibin β A, which homodimerizes to form activin A. We used ELISA to measure the levels of production of activin A.

Articular cartilage was dissected from porcine MCP joints into DMEM and cultured for 16 hours, and the activin A content in the medium was determined (Figure 2A). To establish the extent to which activin A was newly synthesized or was preformed in the tissue, explants were cultured with the protein synthesis inhibitor cycloheximide (CHX) or were rapidly frozen and thawed 3 times upon dissection. These explants released little detectable activin A, which suggested that the

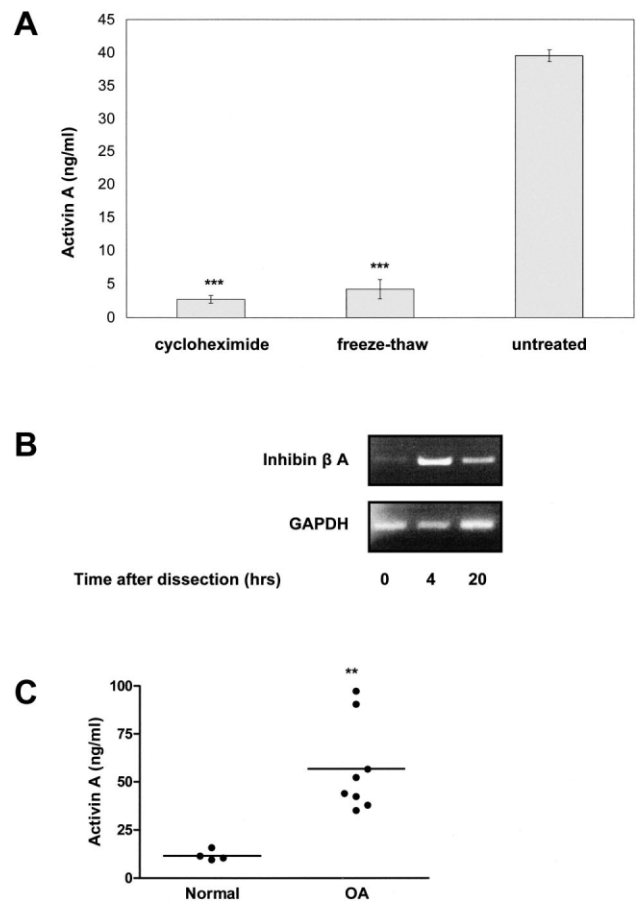


Figure 2. A, Secretion of activin A by freshly dissected cartilage. Porcine cartilage was dissected and cultured for 16 hours in 1 gm/ml serum-free Dulbecco's modified Eagle's medium (DMEM). Some explants were cultured with 10 μ g/ml cycloheximide, some were frozen and thawed 3 times before culture, and some were left untreated. Activin A content in medium was determined by enzyme-linked immunosorbent assay (ELISA). Bars show the mean $\pm$ SD of triplicate cultures. *** = $P < 0.0001$ versus untreated cultures, by unpaired t -test. B, Induction of inhibin β A mRNA by dissection. Porcine articular cartilage was dissected directly into ice-cold TRI Reagent and homogenized prior to RNA extraction (0 hours), or was cultured in serum-free DMEM for 4 hours or 20 hours before homogenization and extraction of RNA. Reverse transcriptase–polymerase chain reaction for determination of inhibin β A and GAPDH levels was carried out as described in Materials and Methods. C, Levels of activin A production in normal and osteoarthritic (OA) cartilage. Human articular cartilage was dissected from normal-appearing femoral heads of 4 patients (3 women and 1 man; mean age 83 years [range 78–86 years]) who were undergoing surgery for femoral neck fracture (normal) or from the joints of 8 patients (5 women and 3 men; mean age 70 years [range 54–84 years]) who were undergoing joint replacement for OA. Cartilage explants were cultured for 16 hours in serum-free DMEM, and explants were then incubated for a further 5 hours in 1 gm/ml fresh serum-free DMEM. Medium was collected, and activin A content was determined by ELISA. Horizontal bars show the mean. ** = $P = 0.0043$ versus normal cartilage, by unpaired t -test.

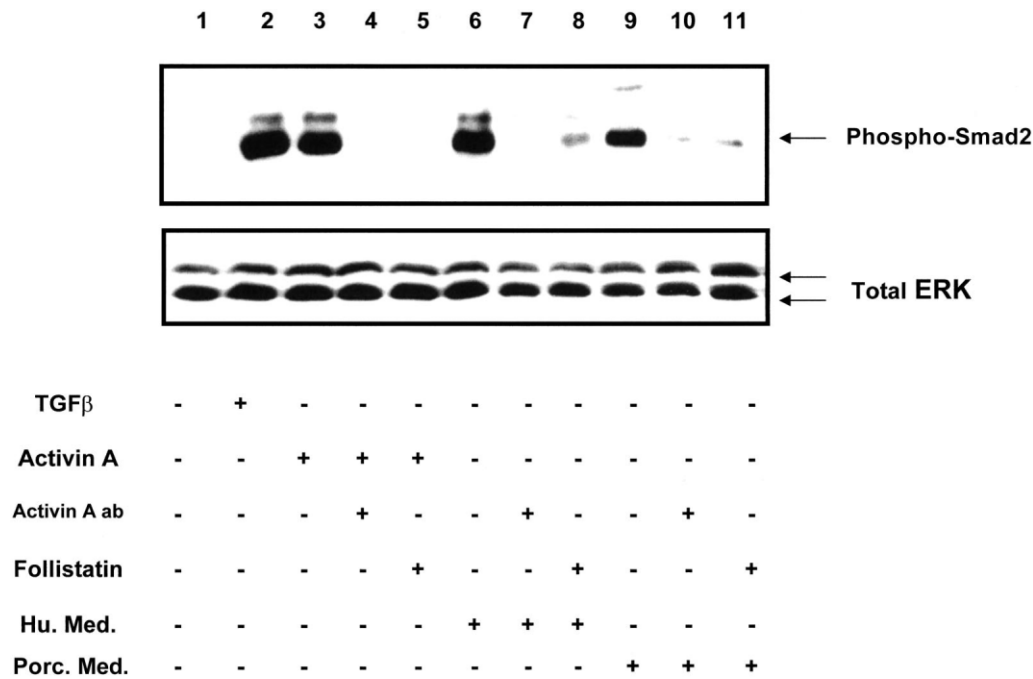


Figure 3. Phosphorylation of Smad2 in human fibroblasts due to the presence of activin A in medium conditioned with live cartilage explants. Conditioned medium from both human and porcine cartilage explant cultures was collected and diluted 1:4 with fresh Dulbecco's modified Eagle's medium (DMEM). These diluted medium samples were preincubated for 2 hours alone or with either a neutralizing antibody (ab) to activin A (5 μ g/ml) or recombinant follistatin (2.5 μ g/ml). Confluent human dermal fibroblasts were kept overnight in serum-free DMEM and then stimulated for 45 minutes with the test samples in serum-free DMEM. Cells were washed and lysed. The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted for phosphorylated Smad2. Samples were also blotted for ERK protein to show even loading of the lysates. Samples tested contained either no stimulus, 10 ng/ml transforming growth factor β (TGF β), 50 ng/ml activin A, medium conditioned with human cartilage (Hu. Med.), or medium conditioned with porcine cartilage (Porc. Med.), with or without antibody to activin A or recombinant follistatin as indicated.

cytokine from the live explants was synthesized in the culture. It is not possible to measure the synthesis of activin A protein *in vivo*; however, inhibin β A mRNA was barely detectable in cartilage *ex vivo*, but was strongly induced after dissection and culture (Figure 2B). Higher levels of mRNA were present at 4 hours after injury than at 20 hours after injury. Thus, activin A expression is strongly induced by the physical injury of dissection. Interestingly, explants of human OA hip and knee cartilage secreted more of the cytokine than did tissue from joints with an apparently normal surface (Figure 2C).

Production of biologically active activin A by cartilage. It was important to show that the cartilage protein was biologically active. It might be inactive, or its activity might be masked by either follistatin or inhibin. Phosphorylation of Smad2 in human skin fibroblasts was

used as an indicator of activity of the activin A in cartilage conditioned medium. Fibroblasts were used as a readily available human target cell. They were serum-starved, then treated for 45 minutes with the stimulus, harvested, and subjected to Western blotting for determination of phospho-Smad2 content (Figure 3). Blots were also stained for ERK to check for even loading. The strongly induced phosphorylated band caused by adding TGF β (Figure 3, lane 2) or activin A (lane 3) was identified as phospho-Smad2. The faint retarded band was unidentified.

The effect of activin A was prevented by preincubating it with either a neutralizing antibody (Figure 3, lane 4) or with follistatin (lane 5). Culture medium conditioned with either human cartilage (lane 6) or porcine cartilage (lane 9) increased the level of phosphorylation of Smad2 as compared with that seen in unstimulated cells (lane 1). Both human and porcine

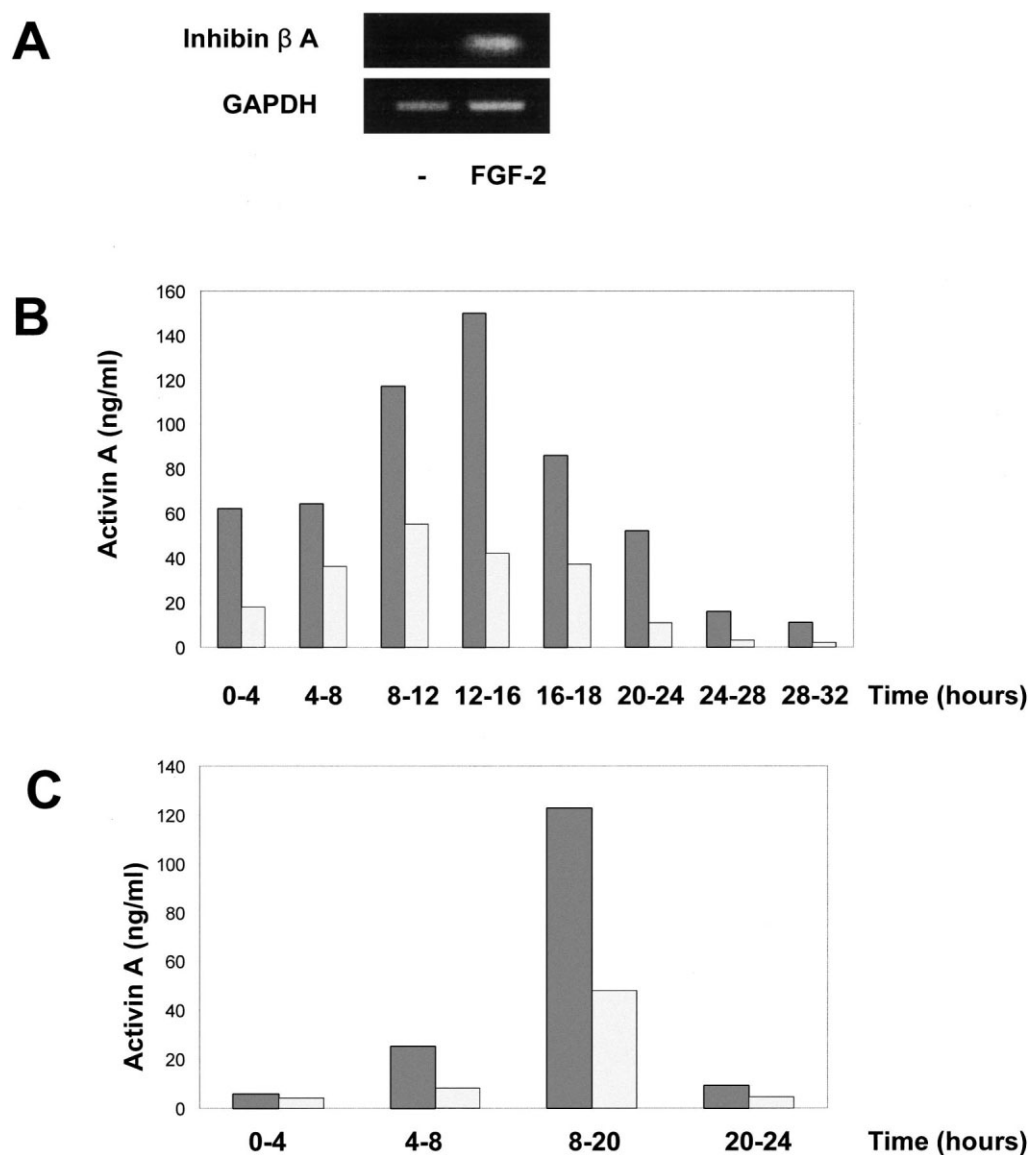


Figure 4. Dependence of activin A expression on fibroblast growth factor 2 (FGF-2). **A**, FGF-2 induction of inhibin β A mRNA in rested cartilage explants. Explants of porcine cartilage were rested for 24 hours in serum-free Dulbecco's modified Eagle's medium (DMEM) and then left untreated or treated overnight with 20 ng/ml FGF-2. RNA was extracted and subjected to reverse transcriptase-polymerase chain reaction for inhibin β A and GAPDH. Results of 1 of 2 experiments are shown. **B** and **C**, Inhibition of activin A production in freshly dissected porcine (**B**) and human (**C**) articular cartilage by FGF receptor inhibitor (PD173074). Articular cartilage was dissected into 1 gm/ml of serum-free DMEM containing vehicle (darkly shaded bars) or 250 nM FGF receptor inhibitor (PD173074) (lightly shaded bars). Medium was collected and replaced with fresh DMEM in the presence or absence of the inhibitor at the times shown, and the activin A content was measured. Experiments were performed in triplicate; bars show the results of 1 representative experiment.

conditioned medium induced additional fainter retarded bands, which were unidentified. Adding the activin A neutralizing antibody nullified the effect of both the human (lane 7 versus lane 6) and porcine (lane 10 versus

lane 9) cartilage conditioned medium. Follistatin was also added to cartilage conditioned medium (lanes 8 and 11), and it suppressed Smad2 phosphorylation to the same extent as did antibody to activin A. These results

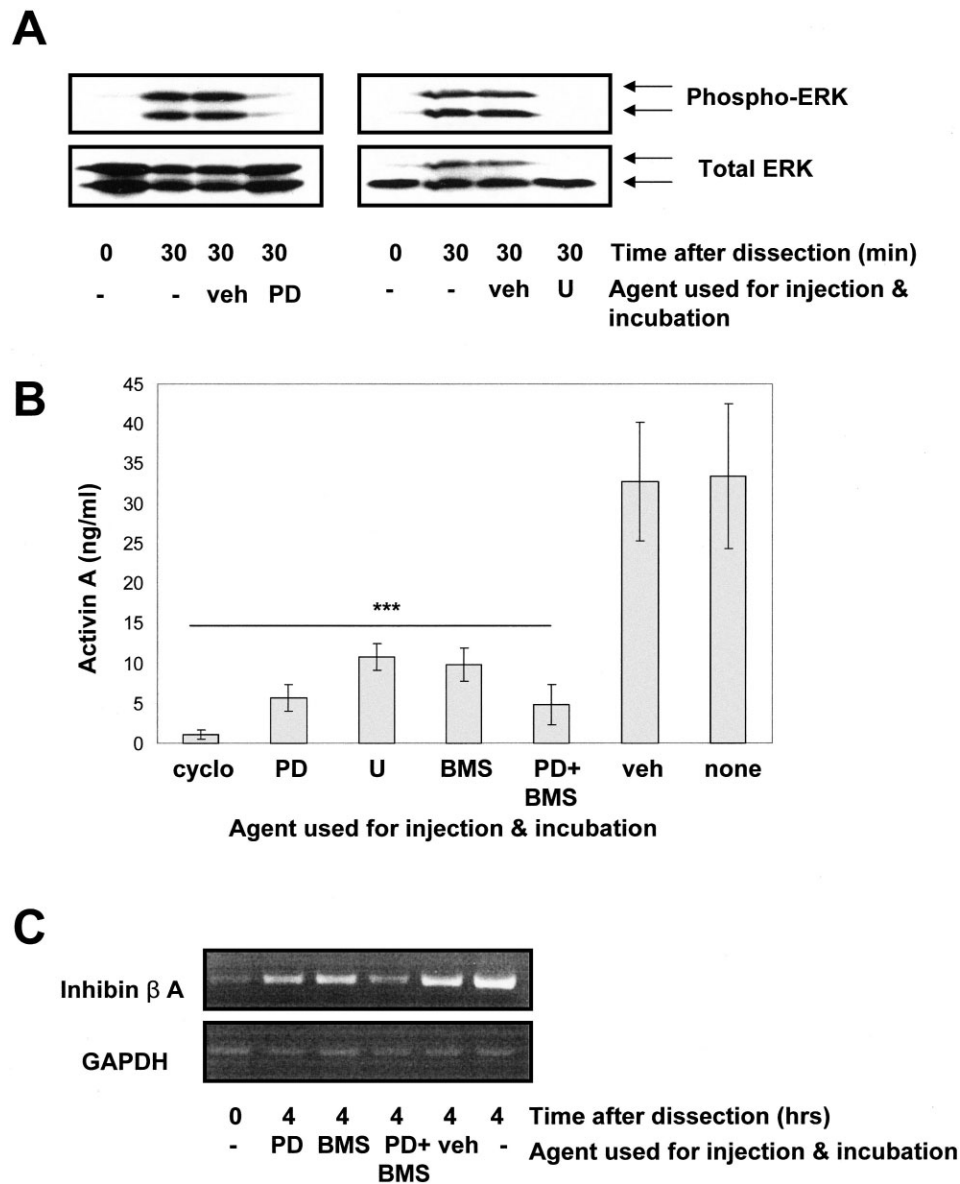


Figure 5. Efficient blocking of signaling and activin A expression induced by dissection and culture of articular cartilage, by intraarticular injection of inhibitors. Porcine metacarpophalangeal joints were initially injected with 3 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) containing vehicle (veh) (0.1% DMSO), 250 nM fibroblast growth factor receptor inhibitor PD173074 (PD), 20 μ M MEK-1 inhibitor U0126 (U), 10 μ M of IKK-2 inhibitor BMS345541 (BMS), a combination of PD173074 and BMS345541, or 10 μ g/ml cycloheximide (cyclo) or were left untreated (none). Following equilibration at 37°C for 2 hours, cartilage was dissected. **A**, Cartilage was dissected directly into ice-cold lysis buffer or was cultured for 30 minutes in serum-free DMEM in the presence or absence of inhibitor prior to lysis. Phospho-ERK content in cartilage lysates was determined by Western blotting. The membrane was stripped and reprobed for determination of total ERK content. **B**, Cartilage was dissected into 1 gm/ml of DMEM in the presence or absence of inhibitor and cultured for 16 hours. Activin A content in the medium was determined by enzyme-linked immunosorbent assay. Bars show the mean $\pm$ SD of 8 different cultures. *** = $P < 0.0001$ versus untreated cartilage (none), by unpaired t -test. **C**, Cartilage was dissected directly into ice-cold TRI Reagent or was dissected and cultured for 4 hours in DMEM in the presence or absence of inhibitor prior to homogenization of cartilage explants, RNA extraction, and reverse transcriptase-polymerase chain reaction for determination of inhibin β A and GAPDH levels.

show that the activin A produced by cartilage was active and accounted for essentially all Smad2 phosphorylation caused by the conditioned medium.

Factors regulating the production of activin A by articular cartilage. Having shown that activin A is synthesized by injured cartilage, is biologically active, and accounts for the TGF β -like activity produced by tissue in culture, we investigated factors regulating its synthesis. We have previously shown that wounding or dissecting articular cartilage causes the release of FGF-2 from a pericellular pool where it is sequestered by perlecan (23,24). The release causes prolonged chondrocyte activation, as judged by stimulation of ERK (23). Dissection also rapidly activates inflammatory signaling pathways. There is strong activation of JNK and p38 MAPK and degradation of I κ B (25). The signaling upon injury is sufficient to induce the expression of inflammatory response genes. The mechanism of activation of inflammatory signaling is unknown. FGF-2 release explains the prolonged ERK activation, but FGF-2 does not activate JNK or NF- κ B in cartilage. It is likely that activin A production was dependent on the chondrocyte activation caused by dissection.

FGF-2 added to porcine explants adapted to culture induced activin A mRNA (Figure 4A). We therefore first assessed the role of FGF-2 in activin A production caused by dissection and culture by using the compound PD173074, an inhibitor of the FGFR tyrosine kinase. This agent is selective for FGFR but also exerts some activity against the platelet-derived growth factor receptor (23,26). Figure 4B shows the results of an experiment conducted with porcine cartilage. Medium was removed from freshly dissected tissue at the indicated times and replaced with fresh serum-free DMEM. Activin A content in medium samples harvested at different time points was determined. Production of activin A peaked between 8 and 16 hours and declined thereafter. When cartilage was dissected into medium containing PD173074, activin A synthesis was inhibited ~60% (Figure 4B). A similar experiment was carried out with human femoral head cartilage, and activin A production was inhibited to a similar degree (Figure 4C).

Activation of signaling caused by dissection of cartilage is very rapid, and we have found that to achieve maximal inhibition of signaling with pharmacologic agents, it is better to not only dissect the cartilage into a solution containing the inhibitor, but also to first inject the inhibitor into the joint so that it distributes through the cartilage before the tissue is injured. To test the effect of the FGFR inhibitor on the activation of chon-

Table 1. Summary of the effects of signaling pathway inhibitors on the production of activin A caused by dissection and culture*

Inhibitor (concentration tested)/target	% inhibition, mean (range)
BMS345541 (10 μ M)/IKK	71 (63–78)
MG-132 (10 μ M)/proteasome	49 (28–70)
SB202190 (20 μ M)/p38 MAPK	17 (2–33)
PD173074 (250 nM)/FGFR	82 (79–84)
U0126 (10 μ M)/MEK-1	51 (34–66)
LY294002 (10 μ M)/PI 3-kinase	12
Gö6983/Gö6976 (100 nM)/PKC	0
PP2 (10 μ M)/Src family kinases	60 (52–66)
PP1 (10 μ M)/Src family kinases	51

* Intact porcine metacarpophalangeal joints were injected with 3 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) plus 10 μ g/ml cycloheximide, the given inhibitor, or the vehicle (serum-free DMEM plus 0.1% DMSO) or were left untreated and equilibrated at 37°C for 2 hours. Joints were then opened and cartilage was dissected into 1 gm/ml of DMEM in the presence or absence of inhibitor and cultured for 16 hours. Activin A content in medium was determined by enzyme-linked immunosorbent assay. Each experiment was performed with quadruplicate cultures for each condition. Mean activin A production (ng/ml) was measured in the presence and absence of each inhibitor, and the percentage inhibition was calculated. The range shows minimum and maximum percentage inhibition where >1 experiment was performed. FGFR = fibroblast growth factor receptor; PI 3-kinase = phosphatidylinositol 3-kinase; PKC = protein kinase C.

drocytes caused when cartilage is dissected, we injected it into porcine MCP joints. After 2 hours the joints were opened, and the articular cartilage was dissected into DMEM containing the inhibitor. The explants were either lysed immediately or after 30 minutes and were Western blotted for determination of phospho-ERK and total ERK protein content (Figure 5A).

The explants left for 30 minutes after dissection showed, as expected, strong phosphorylation of ERK when compared with those lysed immediately. This activation was unaffected by the presence of vehicle, but was nearly completely prevented by the presence of PD173074 (Figure 5A). A similarly effective inhibition of ERK activation was seen when U0126, an inhibitor of MEK-1, was used (Figure 5A). Figure 5B shows that when PD173074 was injected before dissection, activin A production was inhibited over 80%. Taken together, the results of these pharmacologic experiments strongly suggested that activin A production was highly dependent on FGF-2.

It was likely that other signaling pathways activated by injury also contributed to the change in expression of the cytokine. One candidate is the pathway leading to the activation of NF- κ B. Performing the dissection in the presence of the IKK inhibitor BMS345541 reduced the production of activin A by about 70% (Figure 5B). Another way of inhibiting

activation of NF- κ B is to use proteasome inhibitors, which prevent the degradation of phosphorylated I κ B. MG-132, a widely used inhibitor, also reduced activin A production (Table 1). Combining the IKK inhibitor with the FGFR inhibitor did not have a significantly greater effect than either agent alone (Figure 5B).

To achieve maximal inhibition, we used CHX in the experiment, which reduced activin A production by more than 90% (Figure 5B). Since NF- κ B is antiapoptotic, we were concerned that the reduction in activin A production caused by inhibitors of NF- κ B activation over a period of 20 hours was due to cell death. However, inhibition of inhibin β A mRNA was seen at an early time point (4 hours) after dissection with each inhibitor alone and in combination (Figure 5C). The p38 MAPK inhibitor SB202190 was also tested but had no significant effect on activin A production under these conditions (Table 1). We were unable to block JNK signaling pharmacologically in cartilage, so its contribution could not be determined. These results suggest that the burst of activin A production after dissection and culture, which peaks between 8 and 16 hours, is dependent on both FGF-2 and NF- κ B.

Stimulation of FGFR activates several signaling pathways. In addition to the ERK MAPK pathway (which is probably Ras-dependent), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-kinase), and Src family kinases are all likely to be activated. The effects of inhibitors of these pathways were tested (Table 1). LY294002, a PI 3-kinase inhibitor, and PKC inhibitors had no effect on activin A production. However, both the MEK-1 inhibitor U0126 and the Src family kinase inhibitors PP1 and PP2 caused inhibition (Figure 5B and Table 1). It is therefore likely that the ERK pathway and Src family kinases are important for the effect of FGF-2.

DISCUSSION

The results of the present study showed that a function of activin A in cartilage may be to oppose cartilage-catabolizing stimuli such as IL-1. This means it is potentially an autocrine anticatabolic cytokine and might limit cartilage degradation in inflammation. The mechanism of action is unclear, since activin A did not regulate mRNA for the known IL-1-inducible aggrecanases ADAMTS-4 and ADAMTS-5. It also did not alter the amounts of TIMP-3 mRNA or protein in the medium. Activin A modestly increased TIMP-1 production, but this was not seen when IL-1 was present (TIMP-1 is only a weak inhibitor of aggrecanases).

Investigation of the mechanism of action of activin A was hampered by our inability to detect the aggrecanases in cartilage cultures by Western blotting.

It was surprising that activin A accounted for virtually all of the active material that activated Smad2 in the culture medium from human OA and porcine cartilage. However, we did not acidify the medium in order to release TGF β from its latency peptide, to which it may be bound. A study published in 1991 showed that calf articular cartilage produced TGF β and sequestered it in the extracellular matrix (27), and several other studies have demonstrated the expression of TGF β mRNA and activity by cultured chondrocytes (28–31). In light of the findings of the present study, the relative importance of TGF β and activin A as potentially chondroprotective proteins endogenous to articular cartilage needs to be investigated.

Activin A was clearly induced by wounding the cartilage and could provide a homeostatic mechanism to limit damage to the extracellular matrix. Production of the cytokine was dependent partly on FGF-2 and partly on inflammatory signaling, since it was markedly reduced by the FGFR inhibitor, the IKK inhibitor, and the proteasome inhibitor. In the context of inflammatory signaling, FGF-2 therefore appears to enhance the induction of the gene. Since FGF-2 is a normal pericellular constituent and activates chondrocytes upon physiologic loading (24,32), the question arises as to whether FGF-2 is a sufficient stimulus, and whether activin A is produced in the absence of inflammatory stress and exerts a chondroprotective action normally in the tissue.

Activin A mRNA was detectable in the cartilage *ex vivo*, and when we used FGF-2 to treat explants that had been adapted to culture, we saw an increase in mRNA. This suggests that FGF-2 is a sufficient stimulus for activin A expression. However, we were not able to detect a significant increase in secreted activin A from these explants. This was puzzling, but the explants were spontaneously secreting some activin A all the time, and the amount that is continuously removed by the cells is unknown. In previous studies using isolated chondrocytes (1) or fibroblasts (5), FGF-2 was shown to be a sufficient stimulus for activin A production.

The *in vitro* experiments undertaken in the present study should be interpreted with caution, but it is likely that activin A is produced at a low level normally, and not solely after wounding or injury. However, further experiments are needed to determine whether activin A functions in the tissue under normal, as well as stressful, conditions. It is difficult in these experiments to separate the effects of dissection from

the effects of tissue culture. OA cartilage explants secreted more of the cytokine than did normal tissue; to what extent this reflects increased production *in vivo*, perhaps as an attempt to reduce aggrecan lysis, or represents a change in responsiveness of the tissue to the insult of dissection and culture is not known.

We have only examined the expression and response of one component of the activin/inhibin system to cartilage injury. Inhibin α -chains and follistatin may be produced, and their expression might also be altered by injury. Interestingly, follistatin mRNA expression is increased in OA cartilage (33), and if this is reflected at the protein level, there should be a reduction in activin activity.

It is possible to draw some tentative conclusions regarding the intracellular signaling pathways controlling activin A production in cartilage. Activin A production was dependent on NF- κ B, as evidenced by the reduced production caused by both the IKK and proteasome inhibitors. With regard to other inflammatory pathways, blocking p38 MAPK had no effect, and we were unable to block JNK specifically in cartilage with the available agents. FGF-2 strongly activates ERK in cartilage, and the inhibitor of activation of MEK-1, which blocks the activation of ERK, had an effect comparable with that of the inhibitor of FGFR itself. FGF-2 would be expected to activate PKC and PI 3-kinase, but these did not appear to be necessary for activin A induction. The Src family kinase inhibitors reduced activin A production; therefore, FGF-2 controls activin A expression via ERK and possibly Src.

It should be stressed that although wounding the cartilage rapidly activates inflammatory signaling and causes instantaneous release of FGF-2, the mechanism of these phenomena is unknown. FGF-2 alone does not activate inflammatory signaling (NF- κ B and JNK), and we have not been able to identify any soluble material released from the injured tissue as a stimulus of the inflammatory activation.

The possibility that articular cartilage acts via endogenous anticatabolic mechanisms has implications for the pathogenesis and treatment of OA. The disease is generally thought to result from matrix destruction due to production of proteinases caused by a cryptic stimulus. However, if there are endogenous anticatabolic mechanisms, and activin A may represent one, then failure of these could lead to an imbalance between catabolism and anabolism, resulting in excessive aggrecan degradation. By the same logic, therapy could be directed at boosting endogenous protective mechanisms.

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AUTHOR CONTRIBUTIONS

Dr. Watt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Alexander, Watt, Sawaji, Hermansson, Saklatvala.

Acquisition of data. Alexander, Watt, Sawaji.

Analysis and interpretation of data. Alexander, Watt, Sawaji, Hermansson, Saklatvala.

Manuscript preparation. Alexander, Watt, Saklatvala.

Statistical analysis. Alexander, Watt.

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