CYCLICAL HYPEROSMOLAR LOADING OF CHONDROCYTES ENHANCES EXTRACELLULAR MATRIX PRODUCTION AND OCCURS AS A CONSEQUENCEOF P38 MAPK/ERK SIGNALLING AND INCREASED MRNA HALF LIFE

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy

by

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Abstract

It was hypothesised that the application of cyclical hyperosmolar loading on chondrocytes enhanced extracellular matrix (ECM) production and was due to p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase signalling and increased SOX9 mRNA half life. In addition the regulation of COX-2 mRNA was effected by hyperosmotic loading.

SOX9 is a transcription factor required for cartilage formation and is essential for cartilage ECM formation. In man SOX9 gene expression is regulated by osmotic loading. COX-2 is a bifunctional enzyme with both oxygenase and peroxidase activities, responsible for the formation of prostanoids. Aberrant expression of COX-2 protein in articular tissues is a feature of arthritis. SOX9 and COX-2 mRNA regulation was characterised through static and cyclical application of hyperosmotic conditions in human articular cartilage (HAC) derived from OA joints and in addition normal and osteoarthritic of equine chondrocytes in monolayer culture. The roles of ERK1/2 and p38 MAPK signalling pathways were investigated using pharmacological inhibition to identify mechanisms of SOX9 regulation. SOX9 and COX-2 half lives ($t_{1/2}$) were determined in HAC (SOX9 and COX-2) and equine articular cartilage (SOX9). ECM production was measured by *de novo* glycosaminoglycan (GAG) synthesis of equine articular during static hyperosmolar loading and the expression of the ECM genes aggrecan and COL2A1was identified.

Decay curves generated in freshly isolated HAC for SOX9 and COX-2 identified that hyperosmolarity increased the mRNA stability of both genes and ERK signalling was not required for the stabilisation of SOX9. HAC exposed to static and dynamic hyperosmotic loading showed a significant increase in SOX9 and COX-2 mRNA. There was a significant increase in SOX9 and COX-2 mRNA following treatment with MEK1/2 inhibitor during normosmotic static loading. Equine chondrocytes in contrast demonstrated that static hyperosmotic conditions significantly reduced SOX9 mRNA but increased COX-2 mRNA in normal P2 and OA P0 but not normal P0 chondrocytes. Cyclical loading of equine derived normal P2 and OA P0 but not normal P0 cells led to an increase in SOX9 gene expression and this was prevented by both p38 MAPK and MEK1/2 inhibition. Furthermore in equine articular chondrocytes there was no effect on COX-2 mRNA of cyclic hyperosmolar loading, although p38 MAPK signalling reduced COX-2 expression. Hyperosmotic loading in HAC increased the activation of p44/42 MAPK and p38 MAPK. In HAC the presence of either the MEK1/2 inhibitor U0126 or the p38 MAPK inhibitor SB202190 in conjunction with cyclical hyperosmotic loading reduced the induction of SOX9 mRNA. Only the presence of the p38 MAPK inhibitor SB202190 effected a reduction in COX-2 under these conditions. In equine articular explants static hyperosmolar loading increases GAG synthesis and this was reduced by ERK inhibition.

The response to osmotic loading of SOX9 and COX-2 mRNA is dependent on the nature of the osmotic stimulation, the chondrocyte phenotype and the species. MEK-The p38 MAPK and ERK1/2 pathways were involved in the induction of SOX9 under cyclical hyperosmotic loading in most chondrocytes. Additionally COX-2 is regulated by hyperosmotic conditions post transcriptionally.

These findings suggest that the response of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes. The altering sensing of the osmotic environment and inappropriate responses of the resident cell population may be important in the progression of OA.

Abbreviations

ADAM-TS	A disintegrin and metalloproteinase with thrombospondin
ASK	Apoptosis signal regulated kinase
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BGT	Betaine-GABA cotransporter
cAMP	Cyclic-AMP
CD	Campomelic dysplasia
COl2A1	Collagen 2A1
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
CRE	Cyclic-AMP responsive element
CREB	Cyclic-AMP responsive element binding protein
CS	Chondroitin sulphate
DMEM	Dulbecco's eagles medium
DNA	Deoxyribonucleic acid
ECM	Extra cellular matrix
ERK	Extracellular regulated kinase
ES	Embryonic stem cell
FCD	Fixed charge density
FCS	Foetal calf serum
FGF	Fibroblast growth factor

GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanidine diphosphate
GTP	Guanidine triphosphate
HA	Hyaluronic acid
HAC	Human articular chondrocytes
HMG	High mobility group
HXT	Basic helix-loop-helix transcription factor gene
IGD	Interglobular domain
IGF	Insulin like growth factor
IL	Interleukin
JNK	Jun N-terminal Kinase
KS	Keratin sulphate
LoxP	Locus of X-ing over
MAPK	Mitogen activated protein kinase
MAPKAPK	MAPK activated protein kinase
MEF	Myocyte enhancer factor
MEK	Mitogen extracellular kinase
MKP	MAPK phosphatase
M-MLV	Moloney Murine Leukemia Virus
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell

NaCl	Sodium chloride
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Non-steroidal anti-inflammatory
OA	Osteoarthritis
P0	Freshly isolated cells
P2	Passage 2
PG	Proteogylcan
PGG	Prostaglandin G
PKA	Protein Kinase A
PTHrP	Parathyroid hormone related protein
Ras	Small GTPase
RhoA	Ras homolog gene family member A
ROCK	Rho-associated protein kinase
RNA	Ribonucleic acid
SAPK	Stress-activated protein kinase
SMIT	Myo-inositol transporter
SOX	Sry-related HMG box
Src	Sarcoma
SRY	Sex-determining region Y
$t_{1/2}$	Half-life
TonEBP	Tonicity-responsive enhancer binding protein
TNF	Tumour necrosis factor
Wnt	Wingless and Int signalling pathway

Introduction

1. General remarks

The Arthritis Research Campaign web site states that more than 6 million people in the UK suffer from osteoarthritis (OA) of the knee and 650,000 have osteoarthritis of the hip. More than 1 million adults consult their GP each year with OA(http://www.arc.org.uk/). In the future OA is projected to rank second for women and fourth for men in the developed countries in terms of years lived with a disability (Lohmander, 2000). Osteoarthritis is an extremely common cause of morbidity in both man and animals. OA involves the biomechanical failure f articular cartilage, together with changes in the underlying (subchondral) bone and inflammation of the joints and leads to a variety of symptoms including pain, stiffness and reduced mobility. One of the ways to provide new insights into the development and treatment of osteoarthritis is to obtain an understanding of how cartilage responds to physical stress in the joint at the cellular and molecular level.

This study investigates the specific signalling processes p38 MAPK and MEK- which occur following the application of one such stress: hyperosmolarity to cartilage cells (chondrocytes) are involved. The aim is to link these processes to tissue maintenance.

1.1 The structure and function of cartilage

Cartilage is a specialised connective tissue, which consists, like other connective tissues, of cells (the chondrocyte) and extracellular components; the extracellular matrix (ECM) (Poole et al., 2001). Unlike other adult connective tissues it does not contain blood vessels and nerves and so receives nutrients via synovial fluid and subchondral bone. Depending on the composition of the matrix, cartilage is classified into elastic, fibro-cartilage and hyaline cartilage. The gliding surfaces of synovial joints are covered with hyaline cartilage, also known as ‘articular cartilage’. Hyaline cartilage provides a low-friction gliding surface, which compared to bone has increases compressive strength and resistance to wear under normal physiological conditions (Buckwalter and Mankin, 1997). The main function of articular cartilage is load-bearing. In low friction articulation it acts as a shock absorber and minimizes peak pressure on subchondral bone. Cartilage is customarily

subjected to high stresses and during normal activity pressures may arise to 100-200 atmospheres (10-20MPa) within msec (Hodge et al., 1986). Compressive forces are transient and rise from 1-2 atmospheres when unloaded (Grushko et al., 1989, Afoke et al., 1987) to 100-200 atmospheres on standing, and cycle between 40-50 atmospheres when walking (Afoke et al., 1987).

During embryonic development cartilage arises from mesenchymal condensations. Mesenchymal cells aggregate to form a blastema, the cells of which begin to secrete cartilage matrix and are then called chondroblasts. Further development pushes the cells apart due to ECM production. The ECM consist of, ground substance (hyaluronan, chondroitin sulphates and keratin sulphate) and tropocollagen, which polymerises extracellularly into fine collagen fibres. The cells encased in this tough and specialized matrix are called chondrocytes. The mesenchymal tissue surrounding the blastema gives rise to a membrane called the perichondrium. After growth has ceased there is no detectable cell division of chondrocytes in healthy adult articular cartilage (Muir, 1995).

In diarthrodial joints the hyaline cartilage faces the joint cavity on one side and is linked to the subchondral bone on the other by a narrow layer of calcified cartilage tissue. A capsule encloses the entire joint and retains the synovial fluid (Figure 1.1).

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Figure 1.1 Hierarchical structures of diarthrodial joints and articular cartilage (Mow and Hayes, Basic Orthopaedic Biomechanics 1997). Clockwise from top left (following the arrows) illustrates the composite structure of diarthriodal joints. The next level indicates in more detail the actual bearing surface of the joint. The next level shows the existence of the structural features of articular cartilage including the chondrocytes and the organization of type II collagen fibrils.

1.2 Mechanotransduction

Mechanotransduction refers to the many mechanisms by which cells convert mechanical stimulus into chemical activity (Katsumi et al., 2004). Chondrocytes sense and convert the mechanical signals they receive into biochemical signals, which subsequently direct and mediate both anabolic and catabolic processes. These processes include the synthesis of matrix proteins (type II collagen and proteoglycans), proteases, protease inhibitors, transcription factors, cytokines and growth factors (Fitzgerald et al., 2004). A four-step mechanism has been proposed for bone which can be extended to cartilage (Duncan and Turner, 1995);

1. Mechanocoupling, applied force is converted to a detectable force or physical phenomena.

2. The various forces (primary or secondary) are converted into electrical, chemical or biochemical responses.

3. Transduction, i.e., intracellular conversion of a signal into other signals then occurs.

4. In the fourth step a terminal cellular response is seen, i.e., up or down regulation of gene expression or cellular proliferation, autocrine or paracrine factors may be released

Furthermore a number of mechanisms involved in the first two steps have been proposed (Stoltz et al., 2000).

1.3 Components of cartilage

1.3.1 Chondrocyte

Chondrocytes fulfil two major roles in mammals. During development, most bones form through endochondral ossification in which bone is first laid down as cartilage precursors (Karsenty and Wagner, 2002). However, in the adult, chondrocytes are the sole cell type of articular cartilage and play crucial roles in joint function (Aigner et al., 2002).

Articular cartilage has the lowest cellular density of any tissue in the human body. In humans, chondrocytes contribute to only about 1% of the tissue volume and are

situated in small cavities called lacunae within the cartilage and have an average size of 13μm. The spherical cells are found in a 'chondron', a structural unit comprising one or two chondrocytes and its pericellular microenvironment (Benninghoff, 1925). Even in chondrons there is no cell-cell contact (Elfervig et al., 2001). A single cilia extends into the surrounding ECM (Buckwalter and Mankin, 1998, Scherft and Daems, 1967). Recently it has been elucidated that the cilium act like switches, that when toggled by cyclical pulses of lacunocanalicular fluid or cartilage compression send signals such as $Ca^{2+.}$ This surges into the cell to trigger a cascade of events that include appropriate gene activations to maintain and strengthen bone and cartilage (Whitfield, 2008)**.** The cells sense the structure and composition of the ECM and carry out their primary function which is to maintain it. Furthermore is the chondrocytes themselves that synthesize all the ECM components (Buckwalter and Mankin, 1998).

Irrespective of the size of a given animal, there is an inverse relationship between cell density and cartilage thickness (Stockwell, 1971). As cartilage is avascular, its nutrition depends on diffusion from outside and this may limit the total number of cells that can be sustained in a given volume (Stockwell, 1979). Moreover chondrocytes can exist under very low oxygen tensions and metabolise glucose primarily by glycolysis to produce lactate. This anaerobic pathway is maintained even under aerobic conditions (Marcus, 1973).

Articular chondrocytes have great longevity and normally live as long as their owners. The metabolic state of the arrested cell division breaks down, however, whenever the integrity of the collagen network is compromised, as happens in the vicinity of lesions in OA. Here cell division appears to be reactivated, although any division is slow (Muir, 1995).

1.3.2 Collagen

Collagen accounts for two thirds of the dry weight of adult articular cartilage with the large aggregating proteoglycan aggrecan accounting for a large part of the remainder. The materials strength depends upon the extensive cross-linking of the collagen as well as the zonal changes in fibrillar architecture with tissue depth. Collagen concentration is highest at the surface and collagen is under constant tension. Currently, there are at least 28 members of the collagen super family, which

function as structural components of the peri- and ECM in vertebrate tissue (Eyre et al., 2004). Articular cartilage contains at least eight collagens types; II, VI, IX, X, XI, XVI, XX and XXVII Types II, IX and XI form the characteristic basic architecture whilst the remainder are found in small fractions (Eyre, 2002). Recently collagen XXVII has been discovered and is thought to play a role in the later stages of the cartilage modelling phase of endochondral bone formation. It is also a candidate for a scaffold of mineralization in cartilage and as the supporting environment for invading blood vessels (Pace et al., 2003, Hjorten et al., 2007). In articular cartilage type II collagen constitutes 90-95% of collagen in the ECM. In association with type XI it forms a meshwork wherein type IX member of the collagen subgroup FACIT (Fibril Associated collagens with Interrupted Triple Helices) is covalently linked to the surfaces of the type II fibrils and further enables a cross-linked framework to aggrecan (Eyre, 1995). Thus collagen II, IX and XI consist as heterotypic copolymers. The non-fibrillating type VI forms elastic fibres and can be found pericellularly in middle zone and throughout the ECM in small amounts of up to 1% of overall collagen (Wu and Eyre, 1989). Furthermore it has been shown that Collagen-VI interacts directly with the cell surface (Poole et al., 1988).

Collagen has a high level of structural organization (Figure 1.3) and is represented as extended extracellular proteins composed of three polypeptide chains (α-chains), each possessing a characteristic tripeptide sequence (gly-x-y) that forms a left-handed helix. The three α-chains in each molecule are twisted tightly into a right-handed helix to form a rope-like structure that is stabilised by hydrogen bonds.

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Figure 1.3 Schematic representation of the collagen fibril structure (Mow, 1991)

Glycine placed at every third residue of the tripeptide sequence, is small enough to occupy interior of the helix, while frequent other amino acids are proline and hydroxyproline. The collagen precursors, or procollagen are synthesized with large C- and N- terminal extensions which aid in chain assembly. These extension propeptides are cleaved by procollagen peptidases after secretion but before fibril formation. Furthermore collagen fibrils are stabilized by cross-links that involve

lysine residues with fibrillar collagen, the biologically functional form, resulting from a series of post translational modifications (Muir, 1995).

Mature collagen fibres provide the capacity to withstand tensile and shear forces. Type II collagen (a fibrillar collagen), the dominant type in mature collagen, is specific to cartilage, and is a marker of chondrocyte differentiation. Moreover the triple helix is composed of three identical alpha chains synthesized from the COL2A1 gene. Type II exists in two splice variants (IIA and IIB), in IIB, the dominant form found in mature cartilage, exon 2 is spliced out (encodes a 69 amino acid cys-rich domain in the N-terminal propeptide). In IIA, a transient embryonic form found in prechondrogenic mesenchyme, perichondrium and vertebrae, this domain is retained (Sandell, 1994).

1.3.3. Proteoglycans

Proteoglycans (PG) are protein polysaccharide molecules that form 10-20% wet weight and provide a compressive strength to articular cartilage. There are two major classes of PG found in articular cartilage, large aggregating PG monomers or aggrecan and small proteoglycans including decorin, biglycan and fibromodulin (Buckwalter, 1997). The cartilage PG aggregate is a unique structure of macromolecules that, together with type II collagen and a number of minor accessory molecules, gives cartilage its specific biomechanical properties. What's more, aggrecan is immobilized in the collagen network and the importance of aggrecan in articular cartilage function is emphasized by altered metabolism and abnormal expression in animal models with arthritis (Pfander et al., 2004).

Aggrecan has a 220- to 250-kDa multiple domain protein core which is substituted with both chondroitin sulphate (CS) and keratin sulphate (KS) chains in addition to N- and O-linked oligosaccharides (Kiani et al., 2002). There are two types of CS, type 4 and type 6. Whilst type 6 remains constant throughout life, type 4 decreases with age (Bhosale and Richardson, 2008). The core protein possesses two globular regions near the amino-terminus, known as G1 and G2, separated by an interglobular domain (IGD). A third globular region, G3, is found at the carboxy terminal end of the core protein, whilst an extended region containing KS and CS attachment sites is found between G2 and G3 domains. The cartilage PG aggregate is made from aggrecan monomers bound into large aggregates with hyaluronan were the

interaction is stabilized by link protein. This is a 45- to 50-kDa glycoprotein, which is bivalent and so binds to both G1 and hyaluronan. Hyaluronan (HA) is a polysaccharide having repeating disaccharide structure and in cartilage, HA functions in the supramolecular assembly of PG and link protein into aggregates. Furthermore there are receptors on the surface of chondrocytes for HA and these function to provide a gel to which chondrocytes attached (Hardingham, 1981).

The glycosaminoglycan chains impart many of the physical properties to the molecule. CS is composed of repeating disaccharide units of glucuronic acid and galactosamine, with a sulphate group per disaccharide. KS consist of repeating disaccharide units of glucosamine and galactose, also averaging a sulphate group per disaccharide. The sulphate and carboxy groups on the CS and KS chains become charged in solution and in-situ. The total fixed charge density (FCD) in cartilage ranges from 0.05 to 0.3 mEq/g wet weight of tissue (Mow et al., 1999b) and it is this FCD that is responsible for the high Donnan equilibrium ion distribution in the interstitium. It is the Donnan osmotic pressure measured in cartilage (Lai et al., 1991), which contributes to the overall compressive stiffness of cartilage. The fixed negative charges of the PG serve to maintain a high degree of hydration in articular cartilage by generating a substantial osmotic pressure within the tissue (Hopewell and Urban, 2003, Urban, 1994). This explains why cartilage has a tendency to swell, but this is resisted by the collagen network, which is therefore under constant tension, even when unloaded. High transient loads are accommodated by changes in osmotic and hydrostatic pressure when fluid is forced from loaded to unloaded areas, while aggrecan remains immobilized within the collagen network provided it is intact and bound to the hyaluronan.

1.3.4 Other molecules in cartilage

Cartilage contains a large number of extracellular matrix proteins in addition to collagen and PG. These have a wide range of roles e.g., in facilitating matrix assembly, in maintaining mechanical properties of the tissue, in sequestering growth factors and proteinases to specific parts of the matrix, and in interacting with the cells important in regulating the cellular activities. Examples include thrombospondins such as cartilage oligomeric matrix protein (COMP), matrilins, cartilage intermediate layer proteins and small leucine-rich repeat proteoglycans.

1.4 Collagen-proteoglycan interactions

In articular cartilage there are molecular interactions through collagen-collagen covalent (cross-links) and non-covalent interactions, and proteoglycan-proteoglycan and collagen-proteoglycan noncovalent interactions. The size of the PG and its ability to form large aggregates play important roles in keeping the molecules in the tissue and in providing an elastic network that contributes to the material properties of the cartilage (Kimura et al., 1979). Moreover it is the collagen interactions which provide cohesion for the collagen network and a means to immobilize the special arrangement of the collagen network. Furthermore it is the pore size in articular cartilage which is very small, ranging from 25 to 74 Å (Armstrong and Mow, 1982) and this provides an effective barrier against the transport of large molecules through the tissue. Thus the shape and size of the branching PG aggregates serve to immobilize the molecules within the matrix, as long as the integrity of the surrounding collagen network is maintained (Hardingham et al., 1984).

There are electrostatic interactions between the positive charge groups along the collagen and the negative charge groups along the PG molecules which may contribute to the overall mechanical properties of the tissue (Hardingham et al., 1983).

1.5 Organisation of ECM

The thickness of the cartilage varies in the human. In the human medial femoral head it measures 2 to 3 mm thick and on the patella it can be up to 5mm thick. The organisation of the ECM and its distribution into zones differs between immature and mature cartilage. In immature cartilage the articular cartilage is thicker and unstratified. Chondrocytes are distributed in a random fashion and as the tissue matures the matrix becomes arranged in defined zones. An increase in the mechanical competence of the cartilage occurs with these changes. The zones of articular cartilage are superficial (tangential), the middle (transitional), the deep (radial) and calcified cartilage. Where different zones are isolated and cultured, differences in terms of morphology, metabolism, phenotypic stability and responsiveness to signalling molecules, such as the cytokine IL-1 are evident

(Homandberg et al., 1992a). The superficial zone includes the articular surface, is approximately 200μm thick and has collagen fibres tangential to the articular surface, with transition to more randomly orientated fibres in deeper regions. This parallel arrangement of fibrils is responsible for providing the great tensile and shear strength. Additionally chondrocytes in this zone (which are flattened ellipsoid) synthesize high concentrations of collagen and low concentrations of PG. The middle zone is approximately 1mm thick and has randomly orientated collagen fibres. Here collagen fibres are orientated perpendicular to the joint surface in the deep zone, which is approximately 600μm thick. This zone contains the highest concentration of PG; however, the cell density is the lowest. It is at this point that a smoothly undulating tidemark separates the deep zone from the calcified cartilage. This is characterised by rounded chondrocytes arranged in columns, a high PG content and a radial collagen network (Figure 1.2).

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Figure 1.2 Schematic of the zones within articular cartilage (www.kneejointsurgery.com)

Articular cartilage is composed of a 'fluid phase' consisting of water and electrolytes (Na^+, CL^- ,Ca^{2+} etc.) and a 'solid phase' containing collagen, PG, glycoproteins and cells, together with other proteins and lipids (Heinegard and Oldberg, 1989). Whilst 60-87% of articular cartilage is water, 30% of this water is in the intrafibrillar space of the collagen. What's more the amount of water present depends upon the 'fixed charge density' (FCD), organisation of the collagen network and the strength and stiffness of the network (Buckwalter and Rosenberg, 1988). Water allows load dependant deformation of the cartilage as well as providing nutrition and medium for lubrication, creating a low friction gliding surface. In osteoarthritis water content can increase to approximately 90% due to increased permeability and disruption of the matrix (Aigner and McKenna, 2002).

1.5.1 Osmotic stress

The response of articular cartilage to loading is a complex phenomenon due to the numerous factors related to the mechanical strain of the tissue. At the beginning of

the load application, a hydrostatic pressure gradient develops in the matrix and the proportion of the interstitial fluid is driven out. The fluid escaping carries positive counterions and induces streaming potentials and currents (Frank and Grodzinsky, 1987). External compression of the cartilage deforms chondrocytes (Broom and Myers, 1980). Decreased water content of cartilage leads to physicochemical changes in the matrix by both increasing the osmotic pressure, and FCD and by reducing interstitial pH (Parkkinen et al., 1993). Any of these phenomena have biological consequences. Therefore models in which individual physical phenomena can be studied separately are important in revealing the cellular mechanisms of joint loading.

The mechanical environment of the chondrocytes is an important factor that influences the health and function of the diarthrodial joint. Chondrocytes in articular cartilage utilize mechanical signals in conjunction with other environmental and genetic factors to regulate their metabolic activity. This capability provides a means by which articular cartilage can change its structure and composition to meet the physical demands of the body. Under loading, water is expressed from articular cartilage, the matrix deforms and the hydrostatic pressure of tissue increases; if the load is maintained for any length of time, the PG concentration increases, due to water loss and effects the counter ion concentration in the matrix and around the chondrocyte. Thus physiologically, load bearing cartilages experience changes in extracellular ion composition and hence osmotic pressure under cyclic and static loading (Urban and Bayliss, 1989). In addition the physicochemical environment has a strong influence on the viscoelastic and physical properties of the chondrocyte (Guilak et al., 2002).

Cartilage has a highly negative FCD due to the ability of aggrecan to concentrate negative charge. In addition aggrecan also forms macromolecular aggregates with HA (Hardingham and Fosang, 1992). Articular cartilage thus contains high cation concentrations and tissue osmolarity relative to other body fluids, 380 - 450mOsm/kg water depending on the type and zone, with cation concentrations and hence interstitial osmolarity, following PG gradients (Urban et al., 1993, Urban and Hall, 1994). Thus it is very different to plasma which is about 280mOsm/l. Along with zonal variations across cartilage, the osmotic environment of the chondrocyte also changes under loading and in pathological states (Bush and Hall, 2001b, Urban,

1994). Periods of static loading or prolonged cyclical loading without recovery disturb the osmotic balance of cartilage(McArthur and Gardner, 1992). Furthermore joint loading of articular cartilage leads to matrix deformation and so fluid is expressed from the tissue, increasing the concentration of PGs and hence cations and osmotic pressure in the matrix, thus exposing chondrocytes to a hypertonic environment. Once the load is removed fluid is slowly reimbibed and normal cartilage hydration and osmolarity re-established (Urban, 1994). Thus the combination of fluid-flow and deformation-induced changes in FCD expose the chondrocytes to osmotic changes in their milieu. These osmotic variations represent one important physical stimulus in the regulation of chondrocyte activities (Mow et al., 1999a) and even isolated chondrocytes behave as perfect osmometers (Bush and Hall, 2001b, Borghetti et al., 1995).

Under pathological conditions osmotic gradients are also altered, with an increase in hydration being an early indicator of osteoarthritis (OA) (McArthur and Gardner, 1992). When the collagen network is damaged PG are able to imbibe water and hence decrease interstitial osmolarity (Maroudas, 1976). Later stage OA is characterised by PG loss from the tissue, this further exacerbates osmotic perturbations (Maroudas and Venn, 1977). What's more when loaded, OA chondrocytes are subjected to a greater daily variation in osmolarity compared to normal chondrocytes, due to an increase in the rate and extent of fluid loss from swollen cartilage tissue (Hopewell and Urban, 2003).

1.5.2 Charged nature of cartilage and the Donnan Osmotic Pressure

FCD is produced by the charged sulphate and carboxy groups attached to the hyaluronan chains that comprise the major glycosaminoglycans (GAG) of the proteoglycan aggregate in cartilage (Lai et al., 1991). These charges have profound effects on the tissue hydration and control of fluid and ion transport through the interstitium (Maroudas and Venn, 1977). As well as determining the distribution of osmotically active ions, the concentration of fixed negative charges in the matrix also determines the distribution of H^+ and thus the pH. The H^+ concentration of

cartilage is always higher in the tissue than in the external solution, and so the pH is lower (Gray et al., 1988) and hence the extracellular pH of articular cartilage may be as low as pH 6.9-7.0.

Ion distributions in cartilage matrix are governed by the Gibbs-Donnan equilibrium conditions (Grodzinsky, 1983). The Donnan osmotic pressure is used to describe cartilage swelling. It may be considered as an elemental volume of articular cartilage containing typical amounts of collagen, proteoglycan, water, ions and cells. On this micro-scale the elemental volume acts as a microscopic osmotic chamber. The semi-permeable membrane of the osmotic chamber is analogous to the collagen network that surrounds and traps the PG within the tissue. If the semi-permeable membrane of the micro-osmotic chamber is placed against an external electrolyte solution (for example NaCl solution at any concentration) water and ions will flow into and out of the micro-osmotic chamber in order to maintain the electroneutrality and to achieve electrochemical equilibrium with the charged PG contained within the micro-osmotic chamber (Donnan, 1924,(Gu et al., 1998). Donnan derived a mathematical expression for the equilibrium ion concentration (e.g., $Na^{+)}$ within such a semi-permeable chamber of charged molecules (Donnan, 1924).

1.5.3 Regulation of matrix synthesis rates by osmotic environment

The metabolic activity of chondrocytes in articular cartilage is influenced by alterations in the osmotic environment of the tissue, which occur secondary to mechanical compression. When chondrocytes are isolated by digesting away the collagens and PG of the matrix using enzymes and then suspended in standard tissue culture media, the chondrocytes are exposed to an environment different from that in the tissue. The Donnan distribution does not apply and Na^+ and Cl^- have a similar concentration of 140 mM and the extracellular osmolarity falls to that of the culture medium. The K^+ and Ca^{2+} concentrations in the media are lower than in tissue, and the anion concentration is higher. Therefore the pH rises to that of the medium, pH 7.4, and lactate gradients are dissipated. The osmolarity of culture media is 250-280mOsm instead of 350-480 mOsm in the tissue

The mechanisms by which chondrocytes ‘sense’ and respond to their physico-chemical environment is poorly understood (Urban et al., 1993).

Loading patterns that cause fluid loss, result in a fall in matrix synthesis in cartilage explants (Sah et al., 1989), whereas those that result in a rise in hydrostatic pressure (Hall et al., 1991), or fluid flows/ stream potentials, tend to stimulate matrix synthesis. Although some of these effects in the short term are significant in terms of matrix turnover, their roles in long term control of ECM remodelling are unknown.

Caution should be exercised when extrapolating *in-vitro* experiments into the *in-vivo* situation, especially as the relative turnover of matrix molecules, which for sulphated PGs in adult animals can be hundreds of days (Bush and Hall, 2001a).

Previously the effects of osmolarity, on chondrocyte ECM synthesis have been undertaken in a number of studies with differing outcomes. Urban and Hall (Urban et al., 1993) indicated that maximum measured synthesis rate occurred when chondrocytes were closest to their *in-vivo* osmolarity and synthesis rates decreased in proportion to the increase or decrease in osmolarity. These effects were evident in both explants culture and freshly isolated cell experiments indicating that isolated chondrocytes may be used as model for investigating the effects of osmotic environment on articular cartilage. This was similar to findings from experiments undertaken in bovine cartilage explants (Urban and Bayliss, 1989). Work carried out in isolated chondrocytes demonstrated that hyperosmotic loading conditions regulates aggrecan gene expression (Palmer et al., 2001). Culture of cartilage explants from human femoral heads in osmotically active solutions resulted in a reduction in the sulphate uptake by chondrocytes (Schneiderman et al., 1986). In contrast Hopewell and Urban (Hopewell and Urban, 2003) demonstrated that after 24 hour culture in hyper-osmotic conditions there was an increase in s^{35} incorporation, indicating an increase in GAG synthesis. Furthermore a more recent study demonstrated that the application of dynamic hypo-osmotic conditions caused an increase in cartilage ECM genes (Chao et al., 2006). There is a potential pitfall on the conditional equivalence of chemical loading versus mechanical loading on articular cartilage experiments. Osmotic loading has been used, in studies as an equivalent to mechanical loading. The exact conditions under which osmotic pressure loading of cartilage can be considered to be equivalent to a mechanical loading have been derived. The mechanical loading conditions that satisfy this equivalency criterion are an isotropic loading delivered via a porous-permeable rigid

platen, uniformly applied around the specimen, and is not practically achievable. Furthermore, the interstitial fluid pressure caused by the two loading conditions is not the same (Lai et al., 1998). Care must therefore be taken when interpreting changes due to osmotic stress as they do not necessarily relate to an equivocal change due to mechanical loading. However the interest in osmotic loading is not always as an equivalent to mechanical load. There are other mechanisms involved in the cells ability to identify mechanotransduction, for instance by integrins (Millward-Sadler and Salter, 2004). Thus one question that needs to be addressed in this field is the difference between mechanotransduction and osmotic loading.

Dynamic osmotic loading may modulate chondrocyte signalling and gene expression differently than static osmotic loading. Using a flow machine that minimized fluid shear stress effects, Chao et al.,(Chao et al., 2006) demonstrated that dynamic loading increased chondrocyte aggrecan gene expression. Work undertaken on bovine explants using dynamic and static compression in a polysulphone loading chamber revealed that most matrix genes were up regulated by 24 hours of dynamic compression, but down regulated by 24 hours of static compression, suggesting that cyclic matrix deformation is a key stimulator of matrix protein expression. In addition, in the static compression of cartilage, in contrast to dynamic compression, causes a dose dependant decrease in biosynthetic activity (Gray et al., 1988, Sah et al., 1989, Schneiderman et al., 1986). As periods of cyclical loading together with data from compressive loading experiments show that dynamic compression of cartilage induces increases in ECM synthesis by chondrocytes (Sah et al., 1989), one of the aims of this project is to assess the nature of the osmotic load applied. Unpublished data in our laboratory using cyclical osmotic loading on chondrocytes supports a model where increased frequencies of compression in cartilage leads to enhanced ECM production by the residing chondrocytes.

1.6.1 Osteoarthritis

Osteoarthritis (OA), the most common form of arthritis, is a chronic disease that affects diarthrodial joints. The disease is characterized by progressive destruction of articular cartilage, but it also affects the entire joint, including the synovial membrane, joint capsule, ligaments, peri-articular muscles and tendons and subchondral bone (Altman et al., 1986). Primary OA is a chronic degenerative

disorder related to but not caused by aging. It is characterised by its late onset and no obvious cause. Secondary OA has an earlier onset and an identifiable cause such as injury or a developmental abnormality (Goldring and Goldring, 2007). Clinical manifestations include pain, stiffness and impairment of joint motion. There are, as yet no recognisable disease modifying treatments for OA. More recently it has been demonstrated that OA is not exclusively a disorder of articular cartilage (Brandt et al., 2006) and it has been suggested that the collateral ligaments could be a principle sits of wear and tear in early disease. Additionally, there is a suggested role for subchondral bone adaptation in traumatic overload arthrosis in the racehorse (Barr et al., 2009) and this is in agreement with proposals that one of the mechanisms of initiation of joint failure may be steep stiffness gradient in the underlying subchondral bone (Radin and Rose, 1986).

1.6.2 Risk Factors

In OA a variety of potential forces; hereditary, developmental, metabolic and mechanical may initiate processes leading to a loss of cartilage matrix. There are two fundamental mechanisms that segregate the risk factors for the development of OA. These are related to the effects of 'abnormal' loading on normal cartilage or of 'normal' loading on abnormal cartilage. Aging has been suggested as the main factor contributing to the state of abnormal cartilage. While changes in the composition and structure of the cartilage matrix are inevitable, the development of OA with ageing, while common, is not universal (Carrington, 2005). Genetic factors can cause disruption of chondrocyte differentiation and function and influence the composition and structure of the cartilage matrix thus contributing to abnormal biomechanics despite age. Genetic abnormalities can result in earlier onset of OA (Valdes et al., 2006). Various epidemiological studies have implicated hereditary predisposition as a risk factor (Anderson and Felson, 1988, Felson et al., 1998). There are also rare subtypes of arthritis that that have a basis in single gene mutations and are associated with early age onset (Jimenez et al., 1997). Environmental factors can interact with the variable genetic background. Joint malalignment, overloading and injury are recognized risk factors that predispose to OA to varying degrees (Hunter and Felson, 2006). Systemic factors that increase the vulnerability of the joint to OA include increasing age, female sex, and possibly nutritional deficiencies.

1.6.3 Pathophysiology

OA is characterized by progressive destruction of articular cartilage, remodelling of bone and intermittent inflammation. Changes in subchondral bone, synovium and ligaments are also seen at an early stage. The cellular pattern during the OA process is heterogeneous. This variety is reflected in the widespread changes observed in osteoarthritic chondrocyte phenotype and behaviour (Figure 1.4). However the reaction patterns can actually be summarised into five categories (Sandell and Aigner, 2001):

1) proliferation and cell death

2) changes in synthetic activity

3) changes in degradation

4) phenotypic modulation of the articular chondrocytes

5) formation of osteophytes

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Figure 1.4 Cell biology of osteoarthritis Osteoarthritic chondrocytes are exposed to severely abnormal extracellular stimuli, including autocrine and paracrine factors, synovial factors and altered matrix constituents, that induce a plethora of abnormal cellular responses made apparent by the changes in anabolism, catabolism, and phenotype that have been demonstrated in the cells. Also, chondrocyte numbers are modified by proliferation or apoptosis. In addition, cells might become presenescent, leading to an overall loss of chondrocyte function. In this schematic, an osteoarthritic chondrocyte is embedded in a cartilaginous extracellular matrix of type II collagen, aggrecan, and fibronectin, for simplicity. Other collagens, proteoglycans and noncollagenous proteins are also present at varying levels (Aigner et al., 2007).

Osteoarthritic chondrocytes show very low proliferative activity which is, in contrast to normal chondrocytes, which show no activity. A potential explanation for this may be the better access of the chondrocytes to proliferative factors due to loosening of the collagen network (Meachim and Collins, 1962) or damage to the collagen matrix itself (Lee et al., 1993). The (Rozenblatt-Rosen et al., 2002) Apoptosis is also a feature of OA though there has been a large variation in the amount of cell death reported ranging from un published data of 0.1% (Sandell and Aigner, 2001) to 51% (Kouri et al., 2000), both in knee OA cartilage samples.

The complexity of the osteoarthritis process is underlined by this increase in the synthesis of cartilage matrix components that can be detected concurrently with an increase in the degradation of cartilage ECM (Matyas et al., 1999). The degeneration in OA can also be characterized in two phases which occur simultaneously: a biosynthetic phase, during which the chondrocytes attempt to repair the ECM; and a degradative phase, in which the activity of the enzymes produced by the chondrocyte digests the matrix, matrix synthesis is inhibited and erosion of cartilage is evident (Howell et al., 1976). One of the first events in articular cartilage degeneration is the disruption of the molecular structure and composition of the ECM (Buckwalter and Mankin, 1997). There is an early and potentially reversible degradation of PG which is then followed by an irreversible breakdown of collagen. Furthermore there is a loss of proteoglycan and cleavage of type II collagen resulting in an increase in water content and loss of tensile strength in the cartilage matrix as the lesion progresses. The tissue damage stimulates a chondrocyte synthetic and proliferative response that may maintain or even restore the cartilage. In progressive joint degeneration the chondrocytic anabolic response declines and the imbalance between synthesis and degradation leads to the thinning of articular cartilage.

The degradation and synthesis of ECM in OA are driven by mediators released by chondrocytes and synoviocytes. These include cytokines (a number of substances that are secreted by certain cells of the immune system and which carry signals locally between cells) such as IL-1 (a degradative cell signalling molecule released during inflammation), nitric oxide (Studer et al., 1999) and pro-inflammatory cytokines with a role in the regulation of immune cells, for example TNF-α (a cytokine with a role in the regulation of immune cells) (Amin, 1999). The inflammatory cytokines IL-1, TNF-α, IL-17 and IL-18 (the latter two are cytokines

that has a role in inducing and mediating proinflammatory responses in the joint) act to increase synthesis of matrix metalloproteinases (MMP) and other proteinases, decrease MMP enzyme inhibitors, and decrease extracellular matrix synthesis. The anabolic growth factors IGF-1, 2, 3, fibroblast growth factors (FGF) 2, 4 and 8 and the bone morphogenetic proteins (BMP) as well as TGFβ1-3 generally act to stimulate ECM synthesis. Platelet derived growth factor BB may also have a tissue protective role in inflammatory joint disease (Roth, 2002). It is believed that the production of catabolic and anabolic cytokines activates the chondrocyte, but no single cytokine can stimulate the metabolic reactions observed in OA (Goldring, 1999). The increased release of these mediators may induce changes in chondrocyte loading, caused by joint overload or injury (Lohmander, 2000).

Proteases derived from cartilage and synovium play an important part in ECM degradation, including matrix metalloproteases(MMP-1, MMP-3, MMP-8, MMP-13) and aggrecanases (ADAMTS 4 and 5) (Lark et al., 1997, Porter et al., 2005) as well as tissue inhibitors of metalloproteinases (TIMPs) (Dean et al., 1989). ADAMTS 4 and 5 have been implicated in the early cleavage of the proteoglycans aggrecan, verisan and brevican and show altered expression in OA (Porter et al., 2005). The MMPs and aggrecanases cleave aggrecan at distinct sites in the core protein (Tortorella et al., 2000). Most MMP activity is increased in OA, either by mechanisms of increased synthesis, increased activation of proenzymes by other MMPs or decreased inhibitor activity. In nearly all OA cells, MMP-3 (stromelysin), MMP-8 (collagenase-2) and MMP-13 (collagenase-3) are elevated (Sandell and Aigner, 2001). The levels of TIMPs are reduced in OA (Naito et al., 1999). MMP-13 is responsible for most of the collagen degradation (Billinghurst et al., 1997). In addition, MMP-3 can cleave the non-helical telopetide of type II and type IX collagen, leading to the breakdown of a collagen crosslink (Wu et al., 1991). Disruption of the collagen network will eventually lead to the destabilization of the joint. Together, the proteases have the ability to degrade the major macromolecular constituents of the cartilage matrix; collagens, aggrecan and matrix proteins, leading to a complete loss of cartilage function.

Phenotypic modulation may be one of the reasons for anabolic failure of chondrocytes in OA cartilage. Changes in chondrocyte phenotype occur in chondrocyte differentiation in foetal growth plate cartilage. Several factors such as

IL-1 and retinoic acid induce so called ‘dedifferentiation’ or modulation of the phenotype to a fibroblastic state. The chondrocytes stop expressing cartilage specific anabolic genes; aggrecan and collagen type II and express collagen types I,III and V (Benya et al., 1978).

The presence of osteophytes more than any other pathological feature distinguishes OA from other arthritides (Altman et al., 1986) Bone remodeling and osteophyte formation rarely take place in active rheumatoid arthritis (RA) although a small number may be evident on remission (Cabral et al., 1989). Osteophytes represent a new cartilage and bone development in OA joints. They arise from tissue association with the chondro-synovial junction or from progenitor cells residing in the perichondrium (Matyas et al., 1997) and may play a role in stabilizing joints affected by OA (Pottenger et al., 1990).

1.6.4 Tissue Engineering

Tissue engineering, applies the principles of biology and engineering to the development of functional substitutes for damaged tissue (Langer and Vacanti, 1993). Moreover tissue engineering is emerging as a treatment option for cartilage repair and provides a potential method for the production of 3-dimensional implants (Langer and Vacanti, 1993). Effective engineering protocols have already been developed in which chondrocytes, usually from young animals, are seeded onto biodegradable scaffolds and cultured in a bioreactor (Freed et al., 1998). Due to its avascular nature, articular cartilage exhibits a very limited capacity to regenerate and to repair (Hunziker, 1999). Although much of the tissue-engineered cartilage in existence has been successful in mimicking the morphological and biochemical appearance of hyaline cartilage, it is generally mechanically inferior to the natural tissue.

The restoration of the three-dimensional collagen structure and the integration of newly synthesized matrix with the resident tissue is a major challenge in tissue engineering. Procedures currently adopted include tissue debridement, microfracture of the subchondral bone, and the transplantation of autologous (from the patient) or allogenic (from another donor) osteochondral grafts (Hunziker, 2002). The feasibility of using mesenchymal stem cells (MSCs) from bone marrow and other tissue sites is under research as bone marrow derived MSCs have the capacity to

differentiate into cartilage (Barry and Murphy, 2004). One strategy for improving cartilage tissue engineering is the transduction of the chondrogenic transcription factor Sox9, either alone or together with L-Sox5 and Sox6, into MSCs ex vivo or into joint tissue in vivo to induce cartilage formation (Ikeda et al., 2004, Tew et al., 2005).

1.7 The transcription factor SOX9

1.7.1 Introduction

SOX9 is a transcription factor; a protein that binds to specific DNA sequences and thereby controls the transcription of DNA to mRNA (Latchman, 1997). Transcription factors use a variety of mechanisms for the regulation of gene expression (Gill, 2001). Thus they provide important biological roles in functions including basal transcription regulation, differential enhancement of transcription, development, response to intracellular signals and the environment as well as cell cycle control.

The identification of the mammalian testis-determining factor, SRY, first led to the description of a class of genes encoding transcription factors, the Sox gene family. Sox genes are involved in governing cell fate decisions in a number of diverse developmental processes. Sox proteins are a sub-family of 20 members, identified in human and the mouse, in a large family of transcription factors that contain 1 or more high-mobility-group (HMG) DNA-binding domains (Denny et al., 1992).The DNA-binding domain is encoded by a variant of HMG box (the acronym of Sox is derived from the term SRY-type HMG box) (Figure 1.5). The HMG domain itself consists of a 79 amino acid motif with 3 α-helices arranged in a twisted L-shape. The HMG domain of SOX proteins is 50% identical or more to the founding member of the group, mouse SRY, a testis-determining factor (Sinclair et al., 1990, Gubbay et al., 1990). Sox HMG domain recognizes DNA sequences with the consensus C (A/T)TTG(A/T)(A/T). Target gene specificity is achieved by subtle preferences for flanking nucleotides and this is dictated by signature amino acids in their HMG box (Mertin et al., 1999).

Sox proteins are classed into 7 subgroups A to G. Sox9 belongs to subgroup E, whose members have a well conserved HMG domain and a transactivation domain.

Sox proteins have been shown to bind to DNA and activate transcription in vitro (Dubin and Ostrer, 1994). There is a tissue specific expression pattern of some Sox genes (Dunn et al., 1995). Furthermore the expression of a particular Sox gene is not necessarily restricted to a particular cell type or lineage. The Sox9 protein has critical functions in many developmental processes, including sex-determination, skeletal formation, pre-B and T cell development and neural induction (Pevny and Lovell-Badge, 1997). Mutations of Sox genes in mouse and human produce severe developmental defects and disease; mutation of Sry (which maps to the Y chromosome and is involved in testis differentiation) results in sex reversal in XY humans (Yuan et al., 1995).

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Figure 1.5 Schematic representation of Sox9. Sox9 is a typical transcription factor with a DNA-binding domain and a transcriptional activator domain. The DNA binding domain consists of a HMG high-mobility-group box which binds to a specific sequence in the minor groove of DNA (de Crombrugghe et al., 2000).

In contrast to other types of transcription factors which contact DNA in the major grove, Sox (and other HMG proteins) target the minor grove of DNA. The binding of the Sox proteins forms a sharp DNA bend so that the concave binding surface of the HMG domain perfectly fits with the DNA groove (Ferrari et al., 1992, Pineda et al., 1992). Due to this bending property SOX proteins in addition to acting as classical transcription factors, are believed to act as architectural proteins that organize chromatin structure to promote assembly of macromolecular regulatory complexes involving transcription factors bound to DNA at proximal sites (Laudet et al., 1993).

1.7.2 Sox9 the master chondrogenic transcription factor

SOX9 was identified as the first chondrogenic transcription factor (Bi et al., 1999). Indeed it is now recognized as a master chondrogenic transcription factor. To qualify as a master chondrogenic transcription factor, Lefebvre et al., 2001 stated that the gene must be expressed during chondrogenesis and in all sites; it must activate chondrocyte specific genes and thus bind to chondrocyte marker genes. Finally

mutation of the gene must produce dramatic consequences on cartilage formation. Mutant mice experiments using both loss and gain of function analyses indicates Sox9 is essential for the commitment of osteochondroprogenitors, chondrogenic mesenchymal condensations and chondrocyte proliferation, differentiation, maturation and hypertrophic conversion, suggesting that Sox9-L-Sox5, Sox6 form the regulatory axis of chondrogenesis (Smits et al., 2001, Lefebvre et al., 2001, Lefebvre and de Crombrugghe, 1998)

L-Sox5 and Sox6 are co-expressed with Sox9 in all precartilaginous condensations and also in mouse embryo cartilage (Lefebvre and de Crombrugghe, 1998**).** They bind the Col2A1 chondrocyte specific enhancer *in vitro* and induce its expression in non-chondrocytic cells (Bell et al., 1997**).** Along with Sox9 they induce the endogenous expression of chondrocyte differentiation marker genes *in vivo.*L-Sox5 and Sox6, consistent with their identical pattern of expression, have redundant function *in vivo* as single-gene null mutant mice are apparently normal. Deletion of both L-Sox5 and Sox6 leads to embryo lethality due to chondrodysplasia (Smits et al., 2001)**,** demonstrating their essential role in chondrocyte differentiation.

Sox9 commits undifferentiated mesenchymal cells to osteochondroprogenitors. Sox9 is also needed for chondrogenic mesenchymal condensation, whilst both overt chondrocyte differentiation, and normal chondrocyte proliferation, are in part mediated by Sox5 and Sox6, the expression of which requires Sox9. Meanwhile Sox9 has also been shown in mice embryos to inhibit the transition of proliferating chondrocytes to hypertrophy (Akiyama et al., 2002).

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Figure 1.6 Diagram of the action of Sox5, Sox6, and Sox9 in the different steps of chondrogenic differentiation pathway during endochondral ossification (Akiyama et al., 2002).

1.7.3 Role of Sox9 in chondrogenic differentiation

Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway (Akiyama et al., 2002)**.** A Cre/loxP recombination system of bacteriophage

P1 was used to generate mouse embryos in which either Sox9 was absent from undifferentiated mesenchymal limb buds or the Sox9 gene was inactivated after chondrogenic mesenchymal condensations. A summary of their findings is summarized in figure 1.6. The inactivation of Sox9 in limb buds prior to mesenchymal condensations resulted in a complete lack of cartilage and bone, and in this model expression of Sox5 and Sox6 was no longer detected. When Sox9 was deleted after mesenchymal condensations a severe, generalized chondrodysplasia was evident, similar to that seen in Sox5:Sox6 double null mutant mice.

The earliest molecule required for specifying a cell fate during skeletogenesis is Sox9 (Yang and Karsenty, 2002). The role of Sox9 in chondrocyte differentiation is summarised by figure 1.7.

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Figure 1.7 Role of Sox9 in chondrocyte differentiation. *Sox9* homozygous mutant cells are unable to express chondrocyte-specific markers such as *Col2A1*, *Col9A2*, *Col11A2* and *Aggrecan*. The cells have the aspect of mesenchymal cells. The block in differentiation occurs at the stage of mesenchymal condensation (de Crombrugghe et al., 2000).

Initially there were two lines of evidence that suggested SRY-related gene Sox9 was important in chondrogenesis in mammalian embryos. Firstly Wright et al. (Wright et al., 1995) showed that Sox9 mRNA was expressed in chondrocyte condensations in mice. Bi et al. (Bi et al., 1999) also showed that Sox9 was expressed in all chondroprogenitor cells and in differentiating chondrocytes but not in hypertrophic cells. Secondly, mutations of the SOX9 gene are associated with the skeletal malformation disorder campomelic dysplasia (CD) (Wagner et al., 1994, Foster et al., 1994). Most children affected with CD die in the perinatal period. CD represents an autosomal dominant condition caused by haploinsufficiency of the gene i.e. 50% of Sox9 being insufficient to fulfil the physiological function of Sox9. Heterozygous mutations in and around Sox9 causes clinical features in patients including disproportionally short stature, bowing of the limbs, low ears, a depressed nasal bridge. Radiological findings suggest an important role of Sox9 in developing bone and cartilage (Akiyama, 2008). Further convincing evidence that Sox9 was required for the formation of mesenchymal condensations was seen from mouse genetic

studies (Bi et al., 1999). In these studies chimaeric mouse embryos, $Sox9^{-/-}$ embryonic stem cells (ES) but not wild type ES cells were excluded from mesenchymal condensations, indicating that Sox9 was essential for their formation. Teratomas derived from $Sox9^{-/-}$ ES but not wild type ES failed to develop cartilage in mouse chimaeras. Also $Sox9^{+/-}$ mice were seen to be phenotypically similar to human patients with CD. Ectopic expression of Sox9 also activated the Col2A1 gene in transgenic mice.

Evidence has been established that there is an interaction between Sox9 and β-catenin/ Wnt signalling pathway in the control of chondrocyte differentiation (Akiyama et al., 2004).

1.7.4 Targets for Sox9

Once Sox9 was identified as a transcription factor it was necessary to identify target genes in order to clarify its developmental role. It has been demonstrated that SOX9 protein binds SOX/SRY consensus sequence present in the regulatory region of human COL2A1 (Ng et al., 1997). Work carried out by Kypriotou et al. (Kypriotou et al., 2003) in the rabbit, demonstrated that Sox9 exerted a bifunctional effect on the transcription activity of human COL2A1 gene, which was dependent upon its expression level and the differentiation state. The differential action implicated two distinct regions of the COL2A1gene are involved in its regulation. The activation of COL2A1 in freshly isolated cells and dedifferentiated chondrocytes involved the first intron specific enhancer, whereas inhibition in dedifferentiated chondrocytes was mediated by a short promoter region. The work concluded that although Sox9 played a crucial role in chondrocyte differentiation, it cannot restore the phenotype of osteoarthritic chondrocytes alone.

In contrast, studies undertaken by Aigner and Dudhia (Aigner and Dudhia, 2003), in adult articular chondrocytes suggested that Sox9 was not a key regulator of COL2A1 promoter activity. Here it was shown that normal adult articular chondrocytes in vivo contained high Sox9 mRNA levels, which decreased in osteoarthritic cartilage. No positive correlation between Sox9 and COL2A1 was observed, the expression of COL2A1 was actually significantly increased in the diseased chondrocytes. In equine articular cartilage in early post-traumatic osteoarthritis (OA) Sox9 gene

expression is up-regulated and is expressed at equivalent levels to normal tissue in late OA. Furthermore the differences may be species related or there may be contrasts in the disease phenotype between the studies (Clegg PD, 2005).

In addition to its major role in chondrogenesis, it has also been suggested that Sox9 was important for other developmental processes, with expression observed in CNS, notochord, lungs, heart and the urogenital system (Elluru and Whitsett, 2004). The functional significance of these sites was previously evident by observations that SOX9 mutations in CD patients commonly affect a variety of non skeletal organs (Lee et al., 1972). Bell et al. (Bell et al., 1997) later discovered that SOX9 protein specifically binds to sequences in the first intron of human COL2A1, directly regulating the type-II collagen gene. Moreover in chondrocytes, Sox9 binds as a homodimer (Bell et al., 1997) to a pair of the consensus sequences of Col2A1. This binding is mediated by a dimerisation domain located closer to the N-terminus than the HMG domain (Jenkins et al., 2005, Bernard et al., 2003, Genzer and Bridgewater, 2007). Following initial work in transgenic mice and transient transfection experiments, it was shown that SOX9 binds directly to a 48 base pair Col2A1 enhancer at a site essential for chondrocyte specific expression (Lefebvre et al., 1997). Mutations in the enhancer that inhibited Sox9 abolished the enhancer activity.Sox9 effect is also enhanced by two other cooperative members of the same family, L-Sox5 and Sox6 (Lefebvre and de Crombrugghe, 1998) . Zhao et al. (Zhao et al., 1997) found a correlation between high levels of Sox9 expression and high levels of Col2a1 expression in mouse chondrocytic cells. However, no Sox9 expression was evident in hypertrophic chondrocytes and only low levels of Col2a1 RNA were detected in the upper hypertrophic zone. Together with the previous results from the author showing that the chondrocyte-specific enhancer element of the Col2a1 gene is a direct target for Sox9, these findings suggest that Sox9 plays a major role in the expression of Col2a1 and indicates that high levels of Sox9 are needed for the full expression of the chondrocyte phenotype.

Sox9 is also required for the expression of a series of chondrocyte-specific marker genes apart from Col2a1. In work by Bi et al (Bi et al., 1999), with mouse embryo chimeras, $Sox9^{-/-}$ mutant cells were not able to express chondrocyte-specific marker genes for collagen types II, IX, XI and aggrecan. Thus the genes Col9a1 (Zhang et al., 2003), Col11a2 (Bridgewater et al., 1998), and aggrecan (Bi et al., 1999, Sekiya

et al., 2000)**,** but also Col27a1 (Jenkins et al., 2005), and cartilage link protein (Kou and Ikegawa, 2004) require Sox9 for their expression.

Experiments undertaken in TC6, a clonal chondrocyte cell line derived from articular cartilage, examined the effects of Sox9 on the aggrecan gene promoter and regulation of Sox9 gene expression in TC6 cells. It was found that Sox9 enhances the promoter activity of the aggrecan gene**.** Bi et.al. (Bi et al., 1999) also reported that $SOX9^{-/-}$ cells do not express aggrecan in mouse chimaeras, suggesting that Sox9 activates aggrecan gene expression *in vivo* as well. Further evidence for a role for Sox9 in aggrecan expression was found when the Sox9 transduction of cultured human articular chondrocytes caused an increase in aggrecan gene expression. However, Sox9 transduction did not increase the expression of chondroitin sulphate glycosyltransferase and sulfotransferase genes (two of the enzymes responsible for GAG chain synthesis); therefore the chondroitin sulphate synthetic capacity increase may occur by indirect regulation of enzyme activity through control of enzyme protein translation or enzyme organisation (Tew et al., 2008b)**.**

1.7.5 Regulation of Sox9 expression

Some of the mechanisms responsible for the regulation of Sox9 expression in chondrocytes have been elucidated (Gordon et al., 2009). A number of factors increase Sox9 expression, including fibroblast growth factor (FGF) (Murakami et al., 2000b)**,** insulin-like growth factor (IGF-1) (Shakibaei et al., 2006)**,** human cartilage glycoprotein (Jacques et al., 2007)**,** and Src inhibitor (Bursell et al., 2007)**,** Sonic hedgehog (Tavella et al., 2004), CCAAT-binding factor (Colter et al., 2005), CREB (Piera-Velazquez et al., 2007) and hypoxia-inducible factor 1α (Robins et al., 2005) activate the Sox9 proximal promoter. Interleukin-1 (IL-1) and tumour necrosis factor α (TNFα) down regulate the expression of Sox9 in chondrocytes and the activity of a Col2a1 chondrocyte specific enhancer. Both are these effects are mediated by the NFκB pathway (Murakami et al., 2000a)**.**

Huang et al. (Huang et al., 2000), showed that posttranslational modification of Sox9 protein affects the activity of Sox9. Using a yeast two-hybrid method it was shown that Sox9 is a target of cAMP signalling and that phosphorylation of Sox9 by protein kinase A (PKA), a downstream intracellular signalling molecule of parathyroid hormone related peptide (PTHrP)/PTHrP receptor enhances its transcriptional and

DNA-binding activity. For example it increases Sox9 binding to COL2A1 enhancer element and stimulates Sox9 transcriptional activity. Sox9 is phosphorylated at one of two PKA phosphorylation sites, mainly localized to the prehypertrophic zone of the growth plate in vivo, suggested that Sox9 may be a target for PTHrP signalling. Sox9 translocation into the nucleus is controlled by Sox9-calmodulin interaction through the nuclear localization signal of the HMG domain. Mutations within the calmodulin-binding region decrease the ability of Sox9 to activate the transcription of cartilage genes (Argentaro et al., 2003) and causes CD.

The RhoA/ROCK pathway (Rho effector kinase) is a well recognised regulator of cytoskeletal organization in chondrogenesis. Studies undertaken in mouse revealed that inhibition of ROCK signalling with the pharmacological inhibitor Y27632 resulted in an increase in the expression of Sox9 mRNA and protein, whereas over expression of RhoA in the chondrogenic line ATDC5 had the opposite effect. The suppression of Sox9 seen was achieved through the repression of Sox9 promoter activity. Further experiments using compounds that affected the cytoskeletal dynamics revealed that RhoA/ROCK signalling suppresses chondrogenesis through the control of Sox9 expression and actin organization (Woods et al., 2005). RhoA is an important regulator of cytoskeletal structure and focal adhesion maturation (Burridge and Wennerberg, 2004). Its activity increases the formation of actin stress fibres through downstream effector kinases; ROCK-1 and 2. These act by both increasing tension in the cell through direct phosphorylation of myosin II regulatory light chain (Kimura et al., 1996) and controlling events downstream that inhibit the actin depolymerising protein cofilin (Maekawa et al., 1999). Work undertaken by Tew and Hardingham, (Tew and Hardingham, 2006) using cyclohexamide (a protein synthesis inhibitor, known to superinduce gene expression) to stimulate SOX9 in monolayer, showed that HAC from passaged cells only responded by increasing SOX9 gene expression (by 4-fold) when actin stress fibre formation was abrogated. Furthermore, studies in passaged HAC revealed that a hyperosmotic induction of SOX9 mRNA was only observed when the formation of actin stress fibres by the ROCK inhibitor Y27632 was prevented. However on re-differentiation of the chondrocytes in alginate culture for 72 hours a 5 hour exposure to hyperosmotic conditions increased SOX9 mRNA without a requirement for Y27632. Freshly

isolated HAC also did not require Y27632 treatment in order to respond to hyperosmotic conditions (Tew and Hardingham, 2006).

1.7.6 Sox9 in articular cartilage repair

Changes in the expression of ECM genes and the down regulation of Sox9 are characteristic of cartilage in OA (Aigner and Dudhia, 2003). Sox9 decreases in the cartilage of individuals with osteoarthritis (Tew et al., 2007). Furthermore the expression of Sox9 declines rapidly in chondrocytes that are isolated and cultured in monolayer (Stokes et al., 2001).

Salminen et al. (Salminen et al., 2001), characterized the involvement of Sox9 in articular cartilage repair and in the maintenance of the articular chondrocyte phenotype. An experiment was carried out in transgenic Del1 mice, which develop early onset OA, and in their non-transgenic littermates. Results indicated that chondrocytes in mature articular cartilage were capable of inducing the production of Sox9 and type IIA procollagen, which is typical of early chondrogenesis. The group concluded that stimulation of the expression of Sox9, and possibly L-Sox5 and Sox6 in OA joints could have a favourable effect on type II collagen production, which is one of the limiting factors in articular cartilage repair. Evidence has been provided of the ability of Sox9 to compensate for the loss of extra cellular matrix (ECM) components in human OA cartilage. The synthesis and content of proteoglycans and type II collagen, in 3 dimensional cultures of human normal and OA articular cartilage, following direct application of a recombinant adeno-associated virus (rAAV) SOX9 vector, in vitro and in-situ was monitored. After gene transfer the amounts of proteoglycan and type II collagen increased over time in normal and OA articular chondrocytes in vitro. In situ, over expression of Sox9 in normal and OA articular cartilage stimulated proteoglycan and type II collagen, two key ECM components of cartilage in a dose dependant manner (Cucchiarini et al., 2007).

Transduction with Sox9 in passaged human articular chondrocytes with adenoviral, retroviral and lentiviral vectors has been investigated as a method to reinitiate cartilage matrix gene expression (Li et al., 2004). Results indicated that adenoviral and retroviral vectors efficiently induced Sox9 expression. Efficient transduction with a retroviral vector expressing Sox9 resulted in the up-regulation of chondrocyte matrix protein genes and showed the potential for the recovery of key features of

chondrocyte phenotype. Previously it has been shown that passaged human articular chondrocytes, after retroviral transduction of Sox9, regain their chondrogenic response to three-dimensional cell aggregate culture and to growth factors and greatly increased their production of GAG-rich cartilage matrix (Tew et al., 2005). Given the importance of Sox9 in the development and maintenance of the chondrocyte phenotype, its reduction in OA is likely to contribute to the cartilage pathology. These findings suggest that approaches which control SOX9 expression have a potential clinical value.

Cell therapy and tissue engineering have the potential to become important treatments in human articular repair but suffer a major limitation, as chondrocytes *in vitro* lose the differentiated phenotype. The use of autologous chondrocytes as a cell source in cartilage repair procedures has been in place for a number of years (Peterson et al., 2002). Establishing the ideal chondrocyte phenotype is paramount. If Sox9 expression and chondrogenic commitment of expanded cells was preserved cell based therapy could be successful. Malpeli et al. (Malpeli et al., 2004) developed a serum-free medium that supported cell proliferation and preserved the differentiation potential; indeed expression of Sox9 was maintained. The system may have potential implications for future cartilage regeneration strategies.

1.8 The MAPK signalling cascade

The mitogen-activated protein kinase (MAPK) signaling pathways play important roles in the regulation of gene expression in eukaryotic cells. One of the major mechanisms for effecting changes in gene expression is through MAPKs altering the activity of transcription factors and hence the transcription of their target genes. Numerous mammalian transcription factors have been identified as targets of the different MAPK cascades (Johnson and Lapadat, 2002).

Protein kinases are enzymes that covalently attach phosphate to the side chain of serine, threonine or tyrosine of specific proteins. Phosphorylation in this way can modify the target protein and regulate enzymatic activity, cellular location and interactions with other proteins/molecules (Johnson and Lapadat, 2002). The MAPKs are a class of protein-serine kinases that are involved in the transduction of

messages from the membrane to the nucleus. A key characteristic of MAPKs is the formation of a core functional unit of three sequentially activated kinases. Each kinase is activated by dual phosphorylation within a group of amino acids known as the activation loop by the kinase immediately upstream of it (Canagarajah et al., 1997). Thus, a MAP kinase kinase kinase (MAPKKK, MAP-3-kinase) will phosphorylate and activate a MAP kinase kinase (MAPKK, MAP-2-kinase), which will then phosphorylate and activate a MAP kinase (MAPK) (Hoeflich and Woodgett, 2001). The final MAPK in the cascade will then activate a target transcription factor such as AP-1 by phosphorylation.

The activity of MAPKs is reversed when they are dephosphorylated by the MAPK phosphatases (MKPs). These dual specificity phosphatases (DSPs), dephosphorylate MAPKs at both threonine and tyrosine residues. Each MKP has a specific tissue and subcellular localization pattern and specific target recognition sequence (Camps et al., 2000). The expression and function of MKPs is regulated by growth factors and inflammatory cytokines and the MAPKs themselves creating an effective negative feedback loop for MAPK signalling (Lasa et al., 2002).

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Figure 1.7 Organisation of mammalian MAPK cascades. MAPK cascades feature a core triple kinase module consisting of MAPKKKs, MAPKKs and MAPKs. There are a number of MAPKKK families and individual MAPKKKs are often components of more than one MAPK cascade. MAPKKs selectively target a particular MAPK. A potential exception is MKK4 which has been reported to activate p38 (dashed line) in addition to its major target JNK. MAPKs can phosphorylate transcriptional targets directly or this can occur via the indicated downstream protein kinases (Yang et al., 2003).

There are 4 major groups of MAPKs in mammalian cells, each named after the final MAPK in the cascade, the extracellular-signal related kinase (ERK), c- jun kinase (JNK), p38 and extracellular signal regulated kinase-5 MAPK cascades (Figure 1.7). These groups contain a number of gene products and additional isoforms are generated by alternative splicing of the pre-mRNAs. For example 10 JNK isoforms are expressed in cells and these are derived from three JNK genes (Whitmarsh et al., 1997). The best-described MAPK pathway is the ERK or p42/44 MAPK pathway.

This pathway is activated mainly by growth and differentiation factors through specific tyrosine kinase receptors but can also be activated by cytokines and G-protein coupled receptors (Cobb et al., 1991, Zanke et al., 1996, Widmann et al., 1999). The pattern of MAPK cascade is not restricted to growth factor signaling and it is now known that signaling pathways initiated by phorbol esters, ionophors, heat shock, and ligands for seven transmembrane receptors use distinct MAPK cascades with little or no cross-reactivity between them (Yang et al., 2003). Activated receptors recruit the small G-protein Ras^{GTP} as a second messenger. Members of the low molecular weight G-protein families such as Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf and Ran act as molecular switches in cell signalling. They are switched off when bound to guanidine diphosphate (GDP), and are switched on when bound to guanidine triphosphate (GTP). G-proteins act as relays for various cell signals, the most common of which is from the tyrosine kinase receptors. These receptors activate G-proteins causing a conformational/allosteric change, the displacement of GDP and the binding of GTP. The activated G-protein can then bind to a second enzyme effector such as phospholipase C which catalyses a further reaction (Lodish *et al*., 1995). There are around 20 different G-proteins and they all have different responses. For example, Gαs stimulates adenylyl cyclase and Gαq activates phospholipase C. Most G-proteins possess intrinsic GTPase activity which hydrolyses the GTP back to GDP and thus deactivates itself (Takai et al., 2001).

In the case of ERK signalling, $Ras\text{-}^{GTP}$ acts to recruit Raf1, a MAP-3-kinase, to the cell membrane where it is activated by membrane bound tyrosine kinases including c-Src. Activated Raf1 can then phosphorylate the MAP-2-kinases, MEK1 and MEK2. The final MAPK in this family is ERK, and this is normally sequestered in the cytoplasm by MEK1. ERK1 and ERK2 are isoforms of the classical MAPK pathway and are referred to as ERK1/2. MEK1/2 phosphorylates ERK1/2 releasing it from the cytoplasm so that it can translocate to the nucleus and phosphorylate transcription factors (Widmann et al., 1999). To date, 8 ERK MAP kinases have been identified, but ERK 1/2 are the most ubiquitous and well-characterised family members (Court et al., 2004). ERK has many transcription factors substrates including the transcription factors Ets, Elk and Myc (Widmann et al., 1999). It has also been shown that ERK1/2 mediates the up-regulation of the transcription factor

Sox9 by fibroblast growth factor in mouse primary chondrocytes (Murakami et al., 2000a).

The JNK pathway (also known as the stress activated protein kinase (SAPK) pathway) is activated by inflammatory cytokines and stress factors (Kyriakis et al., 1994). There are around 13 MAP-3 kinases that activate the JNK pathway (Johnson and Lapadat, 2002), these include the MEK kinases 1-4, the TGF-β activated kinase-1 (TAK1) and the apoptosis signal-regulated kinase-1 (ASK1) (Hoeflich and Woodgett, 2001). Two MAP-2 kinases have been identified upstream of JNK, these are SAPK/ERK kinase-1 (SEK1) (also known as MAPK kinase-4 (MKK4) or JNK Kinase-1 (JNKK1)) and SEK2 (also known as MKK7 or JNKK2) (Hoeflich and Woodgett, 2001). There are 3 major members of the JNK family, termed JNK1, 2 and 3 (SAPK-α,β and γ). The major targets of JNKs are the Jun family components of the AP-1 complex (Hoeflich and Woodgett, 2001).

The p38 MAPK pathway (also known as the p54 MAPK pathway) is activated by inflammatory cytokines and stress factors (Raingeaud et al., 1995). The major MAPK-3 kinases that activate p38 are TAK1 and ASK1 (Ichijo et al., 1997, Wang et al., 2001). The MAP-2 kinases involved in p38 MAPKs are MKK3 and MKK6 (Raingeaud et al., 1996). There are 4 members of the p38 MAPK family termed p38-α,β,δ and γ (New and Han, 1998). The major targets of p38 MAPKs are the transcription factor ATF-2 (Raingeaud et al., 1995) the kinase, MAPK activated kinase-2 (MAPKAPK-2) (Rouse et al., 1994) and its substrate, heat shock protein 27 (Hsp27) (Freshney et al., 1994). One transcription factor that may be a target of the p38 MAPK pathway is SOX9 were it was shown that its regulation is controlled at the transcriptional level by complex long range enhancer elements (Bagheri-Fam et al., 2006). Studies under taken in transgenic mice provided *in vivo* evidence for the role of p38 in endochondral ossification and suggested that Sox9 was a likely downstream target of the p38 MAPK pathway (Zhang et al., 2006). Furthermore work undertaken in HAC has determined that there is evidence of the early involvement of SOX9 in chondrocyte redifferentiation in which a novel post-transcriptional regulatory mechanism activated by p38 MAPK, stabilized SOX9 mRNA (Tew and Hardingham, 2006).

The ERK 5 pathway is stimulated by both stress stimuli and growth factors (Kyriakis and Avruch, 2001). The MAPKKK that activates ERK 5 is MEKK2 and MEKK3. The MAPK-2 kinase involved is MEK 5 (Chao et al., 1999). The ERK5 mitogen-activated protein kinase (MAPK) differs from other MAPKs in possessing a potent transcriptional activation domain (Sohn et al., 2005), an alternative mode of activation utilized by the ERK5 MAPK. ERK5 possesses a unique transcriptional coactivator domain, which mediates protein-protein interactions with the myocyte enhancer factor 2 (MEF2) transcription factors and provides a potent coactivator function toward MEF2-driven transcription (Karasseva et al., 2003). Major targets of ERK5 include c-myc and SAP-1 (Yang et al., 2003).

Nearly all aspects of cell life are controlled by the reversible phosphorylation of proteins. About one-third of mammalian proteins contain covalently bound phosphate, and there are likely to be 1000 protein kinases encoded by the human genome. Furthermore the `average' protein kinase phosphorylates about 30 proteins. A major challenge is therefore to identify the physiological substrates of each protein kinase. The availability of inhibitors has helped to clarify the roles of MAPK pathways in the cell and may ultimately offer therapeutic benefit. Several small, cell-permeable inhibitors of protein kinases have been developed that exhibit a relatively high degree of specificity for a particular protein kinase, and which may be useful for identifying the physiological substrates and cellular functions of these enzymes (Cohen, 1999). Pharmacological inhibitors have been identified that impact on the MAPKs ERK1, ERK2, two of the four p38 isoforms, three Jun-N-terminal kinase/stress activated kinases (JNK/SAPKs) and ERK5. Most significantly, the identification of p38 MAPK as a target for pyridinyl imidazole anti-inflammatory drugs reaffirmed the idea that intracellular enzymes with multiple functions are potentially valuable therapeutic agents for specific applications (Lee et al., 1994). The pyridinyl imidazoles like SB202190 are specific inhibitors of p38α and p38β and have been widely used in investigation of the biological functions of p38. They act as specific inhibitors through competition with ATP for the same binding site on the p38 kinase (English and Cobb, 2002). The development of MEK1 and MEK2 inhibitors has progressed but a lack of three-dimensional structures for many members of the MEK family has impeded efforts. This is particularly relevant with the inhibitor U0126 as this does not appear to compete with ATP and so is likely to

have a distinct binding site on MEK (Favata et al., 1998). In a comparison of multiple kinase inhibitors, the MEK1/2 inhibitors appeared to be the most specific kinase inhibitors because they inhibited the fewest non-target kinases in a panel of 24 kinases (Davies et al., 2000).

1.9 COX-2

Cyclooxygenase (COX) is a bifunctional enzyme with both oxygenase and peroxidase activities, and is responsible for the formation of prostanoids (Needleman et al., 1986). Prostanoids are members of a large group of hormonally active, oxygenated C18, C20, and C22 fatty acids collectively known as eicosanoids that are derived from n-3 and n-6 polyunsaturated fatty acids (Smith et al., 2000). There are three main groups of prostanoids; prostaglandins, prostacyclins and thromboxanes; each involved in the inflammatory process. Two isoforms of COX have been identified: a housekeeping enzyme, COX-1 is constitutively expressed and produces low physiological levels of prostanoids, whereas the expression of the inducible isoforms, COX2, is inducible by a wide variety of stimuli including proinflammatory cytokines or bacterial products (Vane, 1998) and physical and chemical stresses (Sun et al., 2008). COX-3 is a splice variant of COX-1, which retains intron one and has a frameshift mutation; this is also known as COX-1b (Chandrasekharan et al., 2002). There is a high degree of homology between COX-1 and COX-2: 61% of amino-acids are identical and 84% are similar (Hawkey, 2001). COX-1 is involved in the maintenance of the normal gastric mucosa and is also involved in kidney and platelet function. COX-2 is primarily present at sites of inflammation.

COX converts arachidonic acid to prostaglandin H_2, the precursor of the series-2 prostanoids (Figure 1.8). The enzyme contains two active sites; a heme with peroxidase activity, responsible for the reduction of PGG_2 to PGH_2, and cyclooxygenase site, where arachidonic acid is converted into hydroperoxy endopeptidase prostaglandin G_2 (PGG_2). The reaction proceeds through H atom abstraction from arachidonic acid by a tyrosine radical generated by the peroxidase active site. Two molecules of oxygen then react with the arachidonic acid radical, yielding PGG_2 (Smith et al., 2000).

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Figure 1.8 Biosynthetic pathway for the formation of prostanoids derived from arachidonic acid (Smith et al., 2000)

The action of COX-2 isoenzyme is characterized first by the need for latency (at least half an hour) before induction of the protein; and secondly by the short duration of the gene expression, which is due to a long 3' untranslated region of COX-2 mRNA, that contains several different polyadenylation signals and multiple 'AUUUA' instability sequences that mediate the rapid degradation of the transcript (Kujubu et al., 1991, Ristimaki et al., 1994).

COX-2 is highly expressed in rheumatoid (RA) and osteoarthritic cartilage (Amin et al., 1997, Pelletier et al., 2001). COX-2 selective inhibitors have been developed, for the use in diseases include OA since the late 1990s, due to their anti-inflammatory and analgesic effects. Theoretically drugs which were developed as COX-2 selective inhibitors have less side effects (ulcers, prolonged bleeding time, nephrotoxicity) than general non-steroidal anti-inflammatory (NSAID) treatment such as aspirin and ibuprofen, as the latter inhibit both COX-1 and COX-2 (Hawkey, 2001).

In experiments previously undertaken in synovia from patients with rheumatoid arthritis (RA) and OA, as well as joints of rats with streptococcal cell wall and adjuvant arthritis it was concluded that COX expression was upregulated in inflammatory joint diseases. Furthermore the level of expression was genetically controlled and was a biochemical correlate of disease severity. Additionally, the expression was down-regulated by antiinflammatory glucocorticoids (Sano et al., 1992). Moreover unstimulated chondrocytes did not contain detectable COX-2 mRNA.

Interestingly OA-affected cartilage in ex vivo conditions shows an up-regulation of COX-2 (Amin et al., 1997). Furthermore a study using IL-1 activated human articular chondrocytes either at passage 0 or passage 1 in monolayer, determined that there was an increase in the expression of COX-2 mRNA and protein which was a protein tyrosine kinase-dependant response (Geng et al., 1995).

It has also been previously shown that hyperosmolarity stimulates COX-2 expression in cultured medullary epithelial cells (Yang et al., 1999) and colonic epithelium (Arbabi et al., 2001). Furthermore a study has shown that p38 MAPK regulates COX-2 gene expression in cardiocytes. Therefore we were interested in the

role that hyperosmotic conditions played in controlling COX-2 in normal and OA chondrocytes.

The regulation of SOX9 in chondrocytes is beginning to be revealed. One way in which SOX9 mRNA levels are controlled is through p38 MAPK-dependant regulation of its mRNA stability (Tew and Hardingham, 2006). Medium osmolarity, a known regulator of p38 MAPK ,controls SOX9 by the same mechanism (Tew et al., 2009). This represents an important means of chondrocyte mechanotransduction. Consequently there is a need to further investigate other potential mechanisms of control of SOX9 in order to allow us to understand more clearly how chondrocytes are able to respond to, and regulate, their extracellular environment. This will allow new insights into developing treatments of diseases such as OA. The central hypothesis of this study was to determine whether cyclical hyperosmolar loading of chondrocytes enhances ECM production and occurs as a consequence of p38 MAPK/ERK signaling and increased SOX9 mRNA half life.

The three main aims of the project were;

1. To determine activation of the ERK1/2 and p38 MAPK pathways by hyperosmotic conditions in human and equine chondrocytes and explore whether regulation of SOX9 under these conditions can be controlled by the pharmacological inhibition of these pathways.
2. To characterize the induction of SOX9 mRNA by cyclical application of hyperosmotic conditions in monolayer cultures of human and equine chondrocytes.
3. To explore the regulation of SOX9 mRNA half life and ECM production by hyperosmolarity in equine cartilage explants cultures.

In addition the project will investigate the role of COX-2 in under hyperosmotic conditions in normal and OA chondrocytes of both human and equine monolayers.

Manuscript 1

A ROLE FOR THE ERK1/2 PATHWAY IN REGULATING SOX9 and COX-2 mRNA DURING CYCLICAL HYPEROSMOTIC LOADING OF HUMAN ARTICULAR CHONDROCYTES

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ABSTRACT

Objective: To assess the role of the MEK-ERK and p38MAPK signalling pathways in hyperosmotically-induced, regulation of SOX9 and COX-2 in human articular chondrocytes.

Method: Freshly isolated and passage 2 human articular chondrocytes (HAC) from total knee arthroplasty were subjected to different osmotic loading patterns in monolayer culture. The involvement of p38 MAPK and MEK-ERK signalling was determined by using pharmacological inhibitors. SOX9 and COX-2 half lives ($t_{1/2}$)for freshly isolated cells were determined by measuring decay following administration of 1μM actinomycin D. Samples were analyzed for SOX9, Cox-2, aggrecan and COL2A1 using qT-PCR gene expression analysis and for p44/42 MAPK, P38 MAPK, COX-2 SOX9 protein using western blotting.

Results: Decay curves generated for SOX9 and COX-2 showed that hyperosmolarity increased the mRNA stability of both genes. HAC exposed to static and dynamic hyperosmotic loading showed a significant increase in SOX9 and COX-2 mRNA. Under static control conditions in freshly isolated HAC there was a significant increase in SOX9 and COX-2 mRNA following treatment with the MEK1/2 inhibitor, U0126. Hyperosmotic loading increased the activation of p44/42 MAPK and p38 MAPK. The presence of either the MEK1/2 inhibitor U0126 (10μM) or the p38 MAPK inhibitor SB202190 (20μM) in conjunction with cyclical hyperosmotic loading reduced the induction of SOX9 mRNA. Only the presence of the p38 MAPK inhibitor SB202190 effected a reduction in COX-2 under these conditions

Conclusions: Static and cyclical hyperosmotic loading increased SOX9 mRNA. MEK-ERK signalling was not required for the stabilisation of SOX9 in static hyperosmotic conditions. However, both p38 MAPK and MEK-ERK signalling were involved in the induction of SOX9 under cyclical hyperosmotic loading. We have identified that COX-2 is regulated by hyperosmotic conditions post transcriptionally whilst under normosmolar conditions MEK-ERK signalling results in suppression of COX-2 expression.

Introduction

The surface of long bones within diarthrodial joints is lined with articular cartilage, an avascular connective tissue that provides a nearly frictionless bearing surface for transmitting and distributing mechanical loads between the bones of the skeleton. The unique load bearing properties of articular cartilage are dependent upon its structural composition and organisation, particularly the interactions between collagens and proteoglycans of the extracellular matrix (ECM) (Poole et al., 2001). These matrix macromolecules are regulated by chondrocytes embedded within the cartilage. Chondrocytes utilize mechanical signals (Guilak et al., 1999), such as mechanical loading and local osmotic environment (Schulz and Bader, 2007), in conjunction with environmental and genetic factors to regulate their metabolic activity(Guilak, 2000). Under loading, water is expressed from articular cartilage causing the matrix to deform and the proteoglycan concentration to increase due to water loss which, along with elevated counter ion concentration exposes the chondrocytes to a hyperosmotic environment. Thus physiologically, load bearing cartilages experience changes in extracellular ion composition and hence osmotic pressure under cyclic and static loading (Urban and Bayliss, 1989).

The effects of osmolarity on chondrocyte ECM synthesis have been undertaken in a number of studies with differing outcomes. These studies have shown that proteoglycan synthesis can be reduced in the presences of hyper-, or hypo-osmotic conditions (Hopewell and Urban, 2003, Schneiderman et al., 1986, Palmer et al., 2001, Urban and Hall, 1994, Urban et al., 1993). A reduction in COL2A1 gene expression has also been identified as a consequence of hyperosmotic conditions(Urban and Bayliss, 1989). Although a further study demonstrated that the application of dynamic hypo-osmotic conditions caused an increase in cartilage ECM genes (Chao et al., 2006). The mitogen activated protein kinases (MAPK); extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) have been implicated in the adaptive responses of chondrocytes to hyperosmotic conditions (Hopewell and Urban, 2003). The MAPK families are a cell signalling transduction pathway which connect extracellular signals to intracellular responses such as gene expression in eukaryotic cells (Yang et al., 2003). Both p38 MAPK and JNK signalling pathways have previously been implicated in osmotic stress signalling in studies in yeast and many mammalian cells

(Sheikh-Hamad and Gustin, 2004, Brewster et al., 1993, Galcheva-Gargova et al., 1994, Rouse et al., 1994, Capasso et al., 2001).

It has been previously identified that SOX9 (Tew et al., 2009) and COX-2 (Le et al., 2006) expression are regulated by hyperosmotic conditions. The chondrogenic transcription factor SOX9 controls the expression of many cartilage ECM genes including collagen type II (Bell et al., 1997) and aggrecan (Bi et al., 1999, Sekiya et al., 2000). Campomelic dysplasia, a severe syndrome caused by inadequate cartilage formation during development, is due to a haploinsufficiency of SOX9 and underlines its importance to the chondrocyte phenotype (Foster et al., 1994, Wagner et al., 1994). One of the mechanisms responsible for the control of SOX9 expression in chondrocytes is the p38 MAPK-dependant regulation of its mRNA stability (Tew and Hardingham, 2006). Further studies have concluded that human articular chondrocytes (HAC) exposed to hyperosmotic culture resulted in an increase in the stability of SOX9 mRNA, a process which was also sensitive to p38 MAPK inhibition (Tew et al., 2009).

COX-2 is a bifunctional enzyme with both oxygenase and peroxidase activities, responsible for the formation of prostanoids (Needleman et al., 1986). A superinduction of COX-2 in human osteoarthritic affected cartilage and aberrant expression of COX-2 protein in articular tissues is a feature of arthritis (Kang et al., 1996, Amin et al., 1997, Sano et al., 1992). However normal mature unstimulated HAC did not contain detectable COX-2 mRNA (Geng et al., 1995). It has been previously shown that hyperosmolarity stimulates COX-2 expression in cultured medullary epithelial cells (Yang et al., 1999), colonic epithelium (Arbabi et al., 2001) and kupffer cells (Zhang et al., 1995). In juvenile bovine cartilage there is an osmolarity dependant potentiation of COX-2 (Le et al., 2006) Furthermore involvement of the intracellular signalling proteins p38 MAPK and ERK in inducing COX-2 gene expression has been reported (Chen et al., 2001). Moreover, COX-2 mRNA stability at the post-transcriptional level is necessary for maximal COX-2 expression (Newton et al., 1997, Inoue et al., 1995).

The response of articular cartilage to loading is a complex phenomenon due to numerous factors related to the mechanical strain of the tissue. Therefore models in which individual physical phenomena can be studied separately are important in

revealing the cellular mechanisms of joint loading. With this in mind our present study was performed to explore the effects of osmolarity on SOX9 and COX-2 expression, in particular by examining whether the activation of the ERK or p38 MAPK signalling pathways were required. We also wished to examine the nature of the osmotic load applied to the cells, as data from compressive loading experiments clearly indicate that dynamic compression of cartilage produces increases in ECM synthesis by chondrocytes (Sah et al., 1989).

MATERIALS AND METHODS

Chondrocyte isolation and cell culture

HAC were obtained following total knee arthroplasty with informed consent and ethical approval. Isolation of chondrocytes has been described previously (Tew et al., 2008a). The chondrocytes were grown as monolayers in Dulbecco's modified eagles medium (DMEM) (Invitrogen, Paisley, UK), supplemented with 10% foetal calf serum (FCS), 100units/ml penicillin, 100μg/ml streptomycin (all from Invitrogen, Paisley, UK) and 500ng/ml amphotericin B (BioWhittaker, Lonza, USA). Experiments were undertaken using either freshly isolated chondrocytes plated at 100,000 cells/cm^2 within 48 hours, or with cells at the end of passage 2 (with a 1:2 split ratio). Experiments were replicated using cells from different donors with an age range of 48 – 85 years (mean 69 years). In order to elucidate the effects of the osmolarity of the media on the cells they were grown for 5 hours in serum-free and antibiotic-free DMEM containing either 207mM NaCl or 527mM NaCl in order to yield 380mOsm.kg^{-1}.H_2O(mOsm) or 550mOsm solutions. Following production of the defined media a freezing point depression osmometer (Loser, Berlin, Germany) was used to confirm the osmolarity was within an acceptable range of +/- 2% variation. The osmotic loading magnitude was chosen from existing literature. The use of these osmolarities was based upon previous experiments in bovine articular cartilage where 380mOsm (control) is close to that experienced by healthy chondrocytes *in-situ* and 550mOsm represented a hyperosmotic condition similar to that experienced by chondrocytes under load (Hopewell and Urban, 2003, Urban et al., 1993). Previously it has been demonstrated that an increase in SOX9 mRNA in 550mOsm conditions was observed when the formation of actin stress fibres by the

cells was prevented in the presence of the ROCK1/2 inhibitor Y27632 (Tew et al., 2009). However we have previously demonstrated that the up-regulation of SOX9 in response to osmolarity in freshly isolated HAC is not dependant on actin stress fibres (Tew et al., 2009). Therefore passaged cultures only were supplemented with 10μM of the ROCK1/2 inhibitor Y27632 (Calbiochem, Nottingham, UK). In addition where necessary cultures were supplemented at doses which have been shown to maximally inhibit the MEK-ERK; 10μM (data not shown) or p38 MAPK; 20μM (Tew et al., 2009) signalling pathways (both Sigma-Aldrich, Dorset, UK), for 2 hours prior to the commencement of experiments.

Gene expression analysis

Total RNA was prepared from monolayer cultures in 12 well culture plates using 0.5ml Tri Reagent (Ambion, Warrington, UK) per well. The Guanidium-thiocyanate-phenol-chloroform extraction technique was used as previously described (Chomczynski and Sacchi, 1987). M-MLV reverse transcriptase and random hexamer oligonucleotides were used to synthesize cDNA from RNA (both from Promega, Southampton, UK) in a 25μl reaction. Aliquots (1μl) were amplified by PCR in 20μl reaction volumes on an ABI 7700 Sequence Detector using either a SYBR Green PCR mastermix or a Taqman mastermix were appropriate (Applied Biosytems, Warrington, UK). The fitness of GAPDH as a valid normalisation factor under different osmolarities has been previously established by us (Tew et al 2009) (Appendix1). Relative expression levels were normalized to GAPDH and calculated using the $2^{-\triangle Ct}$ method (Livak and Schmittgen, 2001). Primers and probe for SOX9 were designed by Applied Biosystems Assays-by-Design and had the following sequences: Forward 5'-3' CGCCGAGCTCAGCAAGA; Reverse 5'-3' CGCTTCTCGCTCTCGTTCA; and Probe 5'-3' AAGCTCTGGAGACTTC. GAPDH primers and probes have been described previously (Martin et al., 2001). For determination of aggrecan, collagen II (COL2A1) and cyclo-oxygenase (COX-2), SYBR Green detection was used. The primer sequences for aggrecan, COL2A1 and COX-2 have been previously reported (Martin et al., 2001, Johnson et al., 2002) and were synthesised by Eurogentec (Seraing, Belgium).

For decay experiments freshly isolated HAC was grown in monolayers and treated under experimental conditions for 2 hours before the addition of 1μM of the

transcription inhibitor actinomycin D (Sigma- Aldrich, Dorset, UK) mRNA decay was then measured following total RNA extraction at a number of time points 0-3 hours later. For SOX9 mRNA decay experiments, the copy number in each sample was calculated using a calibration curve created from known dilutions of the pcDNA3SOX9_UT_FLAG vector (Lefebvre et al., 1997). Copy numbers were then normalized to input RNA concentrations, which were measured using a Nanodrop ND-100 spectrophotometer (Labtech, East Sussex, UK). For COX-2 mRNA decay curves were generated using GAPDH as a normalization factor. For both genes data was plotted on semi-log charts and exponential regression lines generated in Microsoft Excel. The slope (m) of the regression lines were used to calculate the mRNA half life ($t_{1/2}$) using the equation $t_{1/2} = ln(2)/m$.

Western blot analysis of cell extracts

For western blot analysis, culture media was removed from the 12 well plate and cell layers washed with cold PBS. SDS sample-extraction buffer (62.5mM Tris-HCL, pH 6.8, 2% w/v SDS, 10% glycerol, and 0.01% w/v bromophenol blue)(100µl) was used to extract the cells with the aid of a cell scraper. Samples were reduced by adding dithiothreitol to a final concentration of 50mM, heated to 80^{o}C for 10 minutes and then run on Novex 4-12% SDS-PAGE gels (Invitrogen, Paisley, UK). Protein transfer to nitrocellulose was performed using the Invitrogen X Cell Sure Lock apparatus according to standard protocol. Membranes were probed with the following antibodies: anti p38 MAPK phospho Th180/Tyr182 #9219, anti phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204) #9101, anti p44/42 MAPK (ERK1/2) (Thr202/Tyr204) #4344, anti COX-2 #4842 (all used at 1:1000 dilution and obtained from Cell Signalling Technologies, Danvers, USA), anti SOX9 (used at 1:2000 from Chemicon, Hampshire, UK) and anti GAPDH-horseradish peroxidase (HRP) conjugate (used at 1:10,000 from Sigma, Dorset, UK). Primary antibodies were detected using a HRP-conjugated goat anti-rabbit secondary antibody (Sigma, Dorset, UK) at 1:2000 and Western Lightning™ and Western Lightning Plus Chemiluminescence reagents (Perkin Elmer, Beaconsfield, USA).

Statistical analysis

Statistically significant differences between gene expression values of control and treated cultures were analysed using mixed effects linear regression to allow for donors with significant biological variation. Significant changes in $t_{1/2}$ data was performed using paired student t-test. The analyses were undertaken using S-Plus, SPSS and Excel software.

RESULTS

Hyperosmotic dependant increase in SOX9 and COX-2 mRNA

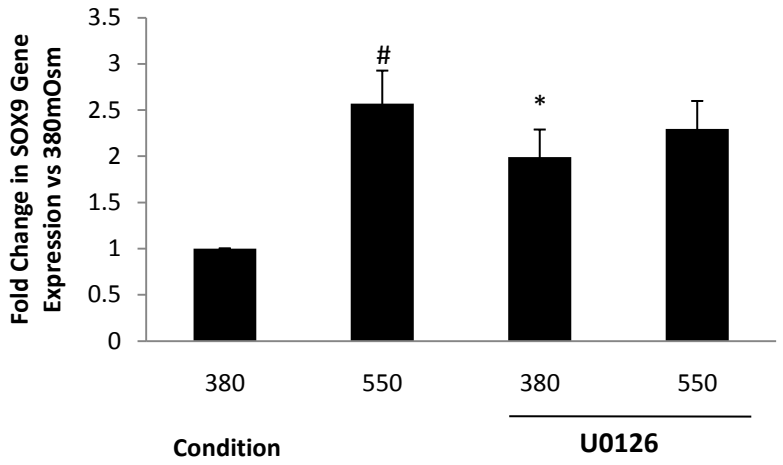
Hyperosmotic conditions for 5 hours significantly increased SOX9 mRNA in freshly isolated human articular chondrocytes (2.5 fold, p=0.0027) (Figure 1a). In addition a significant 50-fold increase (p=0.0001) in COX-2 mRNA was also evident in these conditions (Figure 1b). Interestingly with western blot analysis there was no apparent alteration in either SOX9 or COX-2 protein levels in hyperosmotic conditions (data not shown).

Examining the role of ERK in hyperosmotic loading

Hyperosmolar loading activates ERK signalling

Western blot analysis using a phosphorylated p44/42 MAPK specific antibody demonstrated an increase in p44/42 activation following 5 hours incubation at 550mOsm medium in freshly isolated HAC (Figure 1c). This activation of p44/42 in hyperosmotic conditions was abolished by addition of U0126, a selective inhibitor for the upstream kinase MEK1 and 2, a specific inhibitor of ERK1/2 [43].

1a



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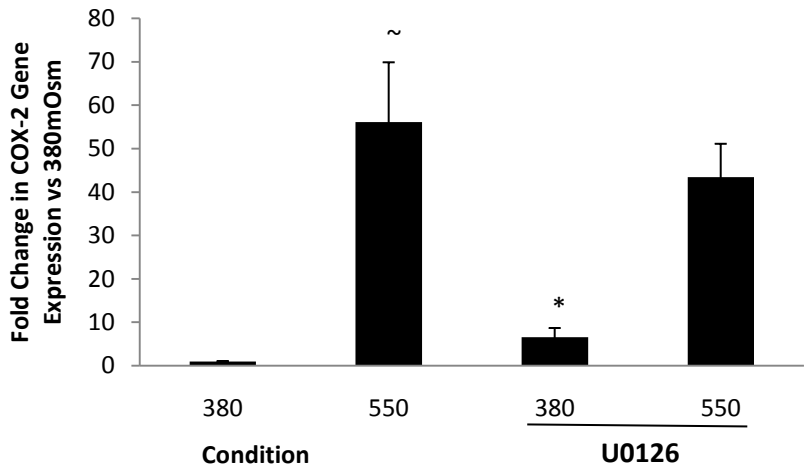


Figure 1: Effect of hyperosmotic conditions on SOX9 and COX-2 mRNA. Real time PCR analysis of **(a)** SOX9 mRNA levels and **(b)** COX-2 mRNA levels in freshly isolated HAC cultured at 380 or 550mOsm in the presence or absence of MEK1/2 inhibitor U0126 (10μM) for 5 hours. Data are presented as the fold change in expression compared to cells under 380mOsm conditions without the inhibitor. Histograms represent means ± SEM (n=6 freshly isolated HAC). Data was evaluated using mixed effect linear regression and * # ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as *P<0.05, # P<0.01, ~P<0.001.

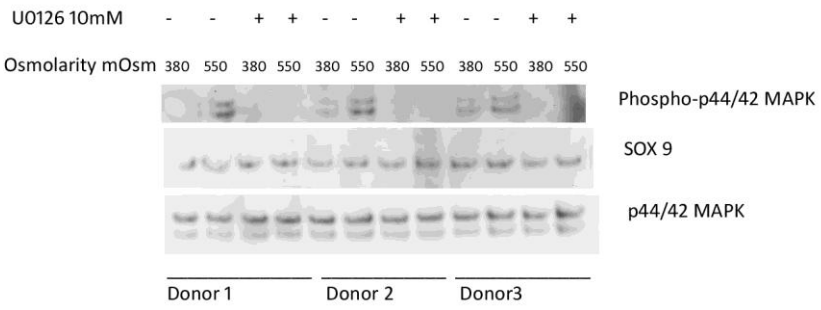


Figure 1c: Effect of hyperosmotic conditions on SOX9 and phosphorylated p44/42 protein levels in freshly isolated HAC. Western blot analysis, using antibodies to phosphorylated p44/42 MAPK and SOX9, of cell extracts from freshly isolated HAC which had been cultured in 380 and 550 mOsm media with and without the MEK inhibitor U0126 (10μM) for 5 hours.

ERK signalling reduces SOX9 and COX-2 in normosmolar conditions

Experiments undertaken in freshly isolated chondrocytes in the presence of the MEK1/2 inhibitor; U0126, significantly increased SOX9 and COX-2 gene expression in 380mOsm (control) cultures (2 fold, $p=0.002$ and 7 –fold, $p<0.012$ respectively) (Figure 1a and b respectively) but no effect of the inhibitor was seen on this gene expression at 550mOsm.

ERK signalling has no effect on SOX9 mRNA stability

Interestingly, it has previously been demonstrated that hyperosmotic conditions increase the SOX9 mRNA $t_{1/2}$ which is partially controlled by p38 MAPK signalling (Tew et al., 2009). Therefore we investigated the effect of hyperosmolarity and MEK-ERK inhibition on the decay of SOX9 mRNA in freshly isolated HAC. Decay curves generated using mean values for all donors showed that culture in 550mOsm increased the $t_{1/2}$ of SOX9 mRNA (Figure 2a). To further quantify this we calculated the $t_{½}$ for each donor individually and performed Student t-test analysis on the values (Table 1). At control conditions, the $t_{½}$ of SOX9 mRNA was 2.9 ±2.3 hours, but this was increased to 8.1±3.6 hours when the culture medium was 550mOsm. Statistical analysis showed a significant effect of 550 mOsm on the $t_{½}$ of SOX9 mRNA in

freshly isolated HAC ($p<0.01$). There was no evidence that the MEK1/2 inhibitor, U0126 had any effects on SOX9 mRNA stability under either condition.

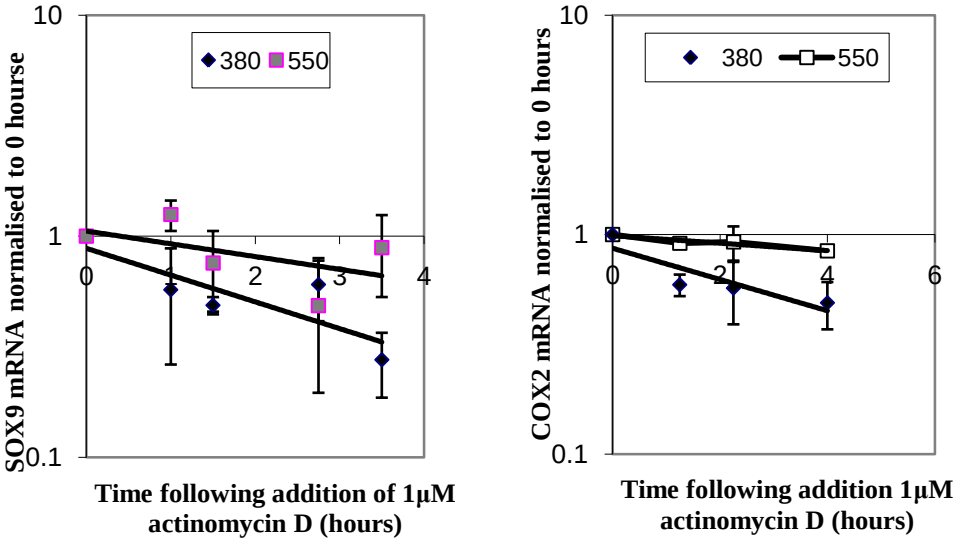


Figure 2: Hyperosmolarity acts post transcriptionally in freshly isolated HAC (a) SOX9 and (b) COX-2decay in freshly isolated HAC cultured at different osmolarities. HAC were cultured at 380 or 550mOsm for 2 hours prior to the addition of actinomycin D. RNA was then extracted at time periods over 3.5 hours for reverse transcription and analysed by real-time PCR. Histograms represent means ± SEM of the fold changes in mRNA levels compared to time point 0 (n=5 for SOX9 and n=3 for COX-2).

Medium Osmolarity (mOsm)	Inhibitor Present	Number of Donors	SOX9 mRNA t ½ (hours) #
380	**None**	**5**	**2.9 (SD2.3)**
550	**None**	**5**	**8.1 (SD3.6)**
380	**U0126**	**3**	**3.3 (SD1.4)**
550	**U0126**	**4**	**8.9 (SD7.9)**

Table 1: Half life of SOX9 mRNA in freshly isolated HAC cultured in osmotically defined media, with and without the MEK ½ inhibitor, U0126 at 10mM.# Results shown are mean values, with standard deviation in brackets. Significant effect of 550 mOsm on SOX9 mRNA t ½ compared to 380mOsm ($p<0.01$) tested by Student t-test.

In addition an RNA decay curve was also generated for COX-2 using mean values for donors, as we wished to determine if COX-2 was post-transcriptionally regulated in HAC. We found that hyperosmolarity also increased the $t_{1/2}$ of COX-2 mRNA, from 3.27±0.9 hours to 16±5.1hours ($p<0.05$) (Figure 2b).

Effect of hyperosmolarity on cartilage ECM genes

We next looked at the gene expression of COL2A1 and aggrecan in freshly isolated HAC and found that application of hyperosmotic conditions for a 5 hour period resulted in a significant decrease in aggrecan and COL2A1 mRNA ($p<0.0012$, $p< 0.0008$ respectively). The MEK1/2 inhibitor U0126 (10μM) had no significant effect on this decrease although, interestingly, its presence in control cultures (380mOsm) elicited a greater than 3-fold increase in COL2A1 (data not shown).

Effect of static versus cyclic osmotic loading on SOX9 and COX-2 mRNA levels

We wished to quantify the effects of hyperosmolarity and the MEK1/2 inhibitor U0126 on passaged HAC, which are a useful model system given the restricted number of freshly isolated chondrocytes that could be studied in a given experiment. HAC which were expanded in monolayer to passage 2 (P2), were used to investigate the effects of different hyperosmotic loading regimes. To identify whether passaged HAC acted in a similar manner to primary cultures of these cells, we initially assessed whether static hyperosmotic loading of these cells induced a similar up regulation of SOX9 and COX2 in P2 HAC. We identified an induction of SOX9 and COX-2 mRNA caused by culture under 550mOsm conditions (3 fold, $p <0.003$ and 7-fold $p<0.037$ respectively) in comparison to that seen at 380mOsm (control).

We were interested in investigating how the nature of the osmotic load applied to cells effected SOX9 expression. *In vivo*, chondrocytes are subjected to periods of cyclical loading which will generate fluid flow and osmotic fluctuations within the tissue. First we investigated the effect of exposure to hyperosmolarity (550mOsm), for different time periods before returning to control (380mOsm) conditions. During a 5 hour experiment, HAC were exposed to hyperosmotic stress for a time period ranging from between 10 minutes to 4 hours prior to returning to control conditions for the remainder of the 5 hour period. In both freshly isolated HAC and passaged HAC there were small but significant increases in SOX9 mRNA when chondrocytes were exposed to 550mOsm for 2, 3, 4 and 5 hours (Figure 3).

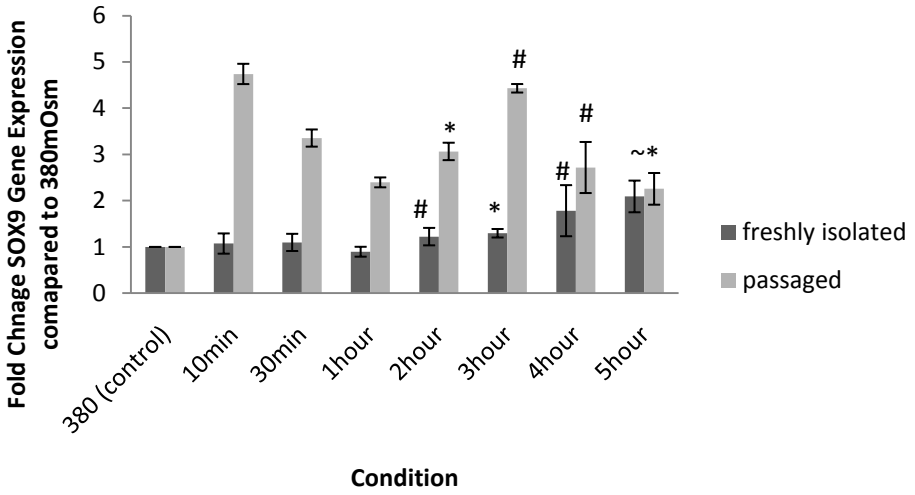
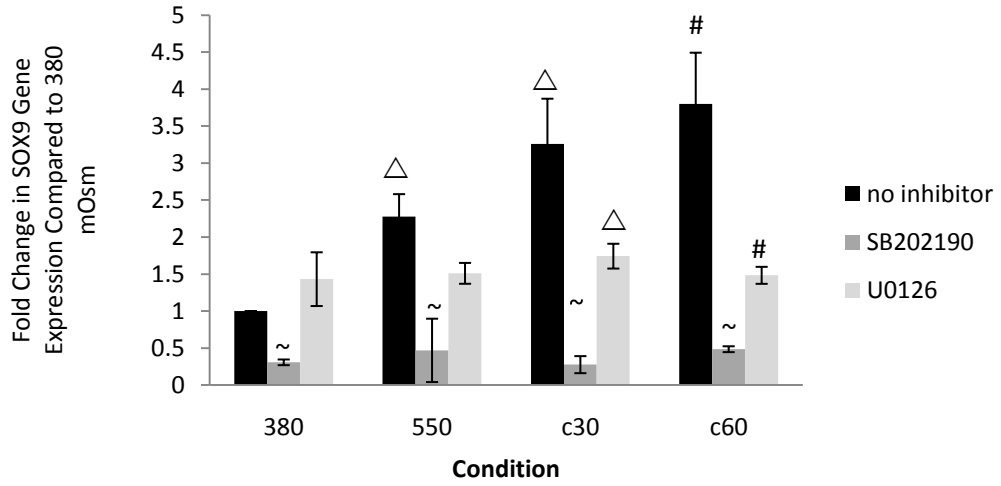


Figure 3: Effect of different hyperosmotic stress loading patterns on SOX9 mRNA in HAC Real time PCR analysis of SOX9 mRNA levels in freshly isolated and passaged HAC, cultured for different amounts of time initially at 550mOsm, prior to culture in 380mOsm media. For example 10 minutes denotes the initial time the HAC were exposed to 550 mOsm prior to culture at 380mOsm for a total of 5 hours. Passaged HAC had the addition of the ROCK1/2 inhibitor Y27632 (10μM). Data point 5 hour represents HAC exposed to 550mOsm for the whole time period of 5 hours. Data are presented as the fold change in expression compared to cells under 380mOsm conditions. Histograms represent means ± SEM for 3 donors. * # ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as *P<0.05, # P<0.01, ~P<0.001.

There was no statistically significant up-regulation in either cell type when the cells were cultured in hyperosmolar conditions for 10, 30 or 60 minutes. Next we examined the effect of a cyclical application of hyperosmotic conditions on monolayer cultures of P2 HAC. Media was adjusted with periods of 380 or 550mOsm alternating every 30 (c30) or 60 (c60) minutes over a 5 hour period. Chondrocytes cultured in serum-free media at 380mOsm was used as the control as we had previously determined that there was no change in SOX9 when media was changed for the same osmolarity, every 30 or 60 minutes over a 5 hour period (data not shown). At each frequency the final incubation period was under 550mOsm. Cyclical application of hyperosmotic conditions up-regulated SOX9 and COX-2 mRNA more than static application. For SOX9 (Figure 4a) and COX-2 (Figure 4b) this was significant at both 30 and 60 minute frequencies (SOX9; 3.25 fold; p=0.03, 3.75 fold p=0.006 and COX-2; 118fold, p<0.0003 and 78 fold, p<0.003 respectively). There was no significant difference between the frequencies of

cyclingfor either gene. Despite the changes in mRNA levels, when we examined SOX9 and COX-2 protein we again found no difference in protein levels.

4a



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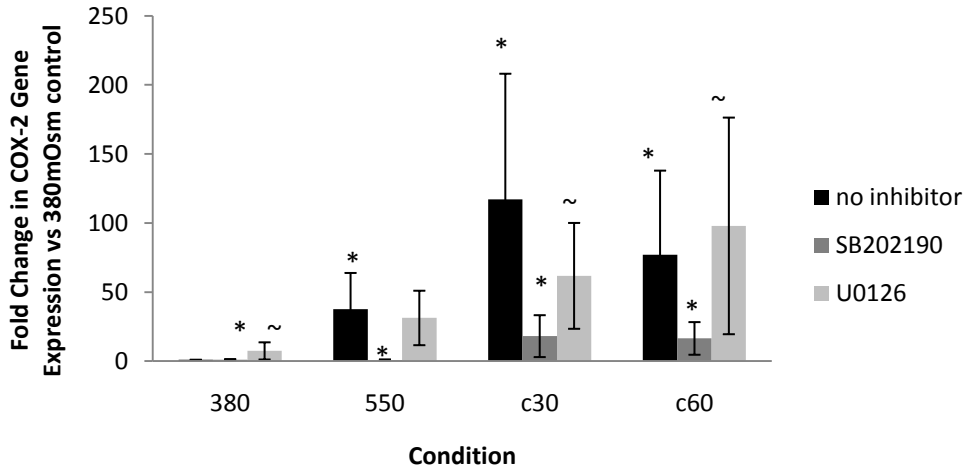

Figure 4: Effect of cyclical hyperosmotic loading on passaged HAC Real-time PCR analysis of (a) SOX9 mRNA levels and (b) COX-2 mRNA levels in monolayer culture of passage 2 HAC, incubated with cyclical application of 380mOsm and 550mOsm every 30 or 60 minutes for the 5 hour period with the addition of the ROCK1/2 inhibitor Y27632 (10μM) and either p38 MAPK inhibitor SB202190 (20μM) or the MEK1/2 inhibitor U0126 (10μM). In unloaded cultures media was changed at the same frequencies to account for possible shear induced effects on the cells during the loading period. At each frequency, the final incubation period was under 550mOsm conditions. Histograms represent means ± SEM of fold change in expression compared to cells at 380mOsm (n=3). * # ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as *P<0.05, # P<0.01, ~P<0.001, △represents a trend (P<0.1).

Interestingly exposure of passaged HAC to cyclical hyperosmotic loading did not cause a change in the expression of either aggrecan or COL2A1 (data not shown).

Role of MAPK signalling on SOX9 and COX-2 expression following cyclical hyperosmotic loading

We examined the activity of the ERK1/2 pathway in passaged HAC which had been exposed to cyclical hyperosmotic stimulation. We found that similarly to the freshly isolated chondrocytes, culture in 550mOsm conditions for 5 hours induced p44/42 MAPK (ERK 1/2) phosphorylation. However, both c30 and c60 cultures had greater levels of p44/42 MAPK phosphorylation than that observed under static hyperosmotic conditions (Figure 5). We therefore examined the effect of the MEK1/2 inhibitor U0126 on the response of the chondrocytes to cyclical osmotic loading. Real-time PCR analysis showed that inhibition prevented the further increase in SOX9 mRNA caused by cyclical osmotic loading at both time points (c30; $p=0.02$, c60; $p=0.005$) (Figure 4a). Similarly to SOX9 mRNA, addition of the MEK1/2 inhibitor U0126 increased COX-2 mRNA levels in control conditions ($p<0.001$). However, there was also evidence of a variable effect in the presence of the MEK1/2 inhibitor U0126 on COX-2 mRNA in cyclical hyperosmotic loading, with a down regulation under c30 conditions ($p<0.0001$) but an up regulation in c60 cultures ($p<0.001$) (Figure 4b).

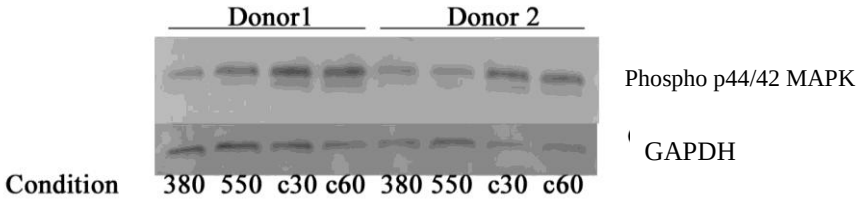

Figure 5: ERK activation following cyclical hyperosmolar loading Western blot analysis, using phosphorylated p44/42 ERK and GAPDH antibodies, of cell lysates from passage 2 HAC cultured in 380, 550mOsm media or following cyclical hyperosmotic loading every 30 (c30) or 60 (c60) minutes for 5 hours. 2 donors representative of the findings are shown here.

Further western blot analysis using a phosphorylated p38 MAPK specific antibody confirmed the previous findings of Tew et al. (Tew et al., 2009) by identifying an increase in activation of p38 MAPK at 550mOsm compared to 380mOsm (control) under static hyperosmolar conditions. Cyclical hyperosmolar loading every 60 minutes led to an increase in p38 MAPK activation compared to control, although this was at a much lower level than seen with 5 hours of static hyperosmolar loading. There was no identified regulation of p38 MAPK by hyperosmolar loading every 30 minutes (Figure 6a). Western blot analysis was undertaken, using SOX9 specific antibodies. p38 MAPK inhibition with SB202190, but not MEK1/2 inhibition with U0126 caused a reduction in SOX9 protein expression in all conditions (Figure 6b).

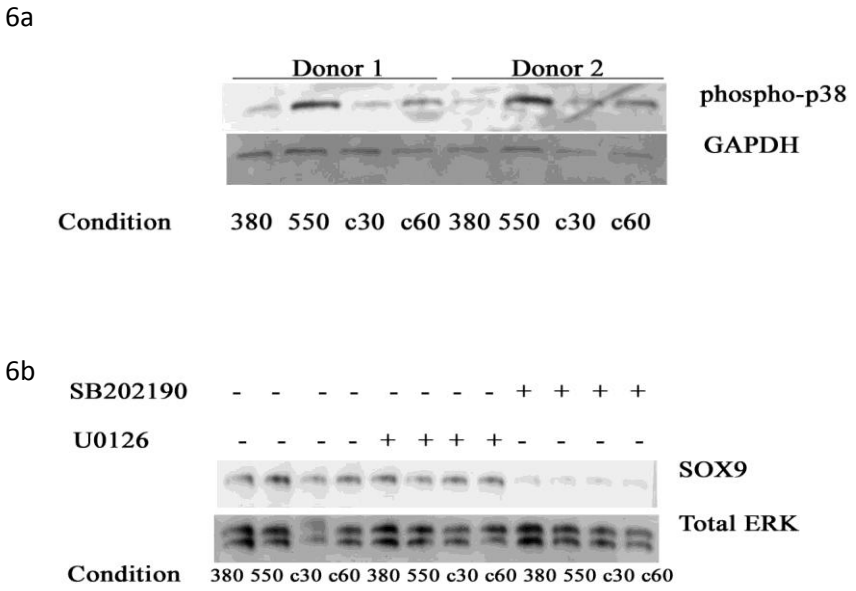

Figure 6: p38 MAPK activation is required for hyperosmotic induction of SOX9 mRNA in passaged HAC (a) Western blot analysis, using phosphorylated p38 MAPK and GAPDH specific antibodies, of cell extracts from passage 2 HAC cultured in 380, 550mOsm media or following on-off hyperosmotic loading every 30 (c30) or 60 (c60) minutes for a 5 hour period. 2 donors are shown here. (b) Western blot analysis, using SOX9 and total p44/42 ERK antibodies of cell extracts from passage 2 HAC cultured in 380, 550mOsm media or following on-off hyperosmotic loading every 30 (c30) or 60 (c60) minutes for a 5 hour period, in the presence of the ROCK1/2 inhibitor Y27632 (10μM) with or without the p38 MAPK inhibitor SB202190 or the MEK1/2 inhibitor U0126. The p38 inhibitor SB202190 but not the MEK1/2 inhibitor U0126 reduced SOX9 protein expression. The figure represents data from 1 of the two independent experiments that produced similar result.

Following the p38 MAPK inhibition by SB202190 of cultured P2 HAC, real-time PCR analysis showed that this inhibition reduced SOX9 during cyclical loading ($p<0.0001$) (Figure 4a). In addition p38 MAPK inhibition with SB202190 also prevented COX-2 induction by hyperosmolarity under both static and dynamic loading when compared to controls ($p<0.03$) (Figure 4b).

The ROCK1/2 inhibitor Y27632 was present in all passage cell cultures as it had previously been demonstrated that an increase in SOX9 mRNA in 550mOsm conditions was observed more consistently when the formation of actin stress fibres by the cells was prevented (Tew et al., 2009). We wished to identify whether the presence of the ROCK1/2 inhibitor Y27632 was indeed required for the hyperosmotic induction of SOX9 in passaged chondrocytes. Monolayer culture of passaged HAC, for 3 donors was incubated at 380mOsm and 550mOsm for 5 hours in the presence or absence of the ROCK1/2 inhibitor Y27632 (10μM). It was identified that the inhibition of actin stress fibres has no significant effect on hyperosmolar induction of SOX9 in P2 HAC (data not shown).

DISCUSSION

The expression of SOX9 is essential for the ability of the chondrocyte to produce cartilage matrix (Stokes et al., 2001, Tew et al., 2005). Given the importance of SOX9 in the development and maintenance of the chondrocyte phenotype, its reduction in OA (Haag et al., 2008, Tew et al., 2005, Aigner et al., 2003, Tchetina et al., 2005) may contribute to the cartilage pathology. Cell therapy and tissue engineering have the potential to become important treatments in human articular repair but suffer a major limitation, as chondrocytes *in vitro* lose the differentiated phenotype (Aulthouse et al., 1989, Lin et al., 2008). Finding ways of regulating chondrocyte phenotype is an important goal of cell based therapies and so our findings demonstrating means of controlling SOX9 mRNA expression have a potential clinical value. The regulation of SOX9 in chondrocytes is beginning to be identified. One mechanism for the regulation of SOX9 gene expression is through p38 MAPK-dependant signalling leading to post-transcriptional stabilisation of its

mRNA. Furthermore medium osmolarity, a known regulator of p38 MAPK, controls SOX9 by this mechanism (Tew et al., 2009). This represents one method of chondrocyte mechanotransduction. Consequently there is a need to further identify other potential mechanisms of control of SOX9 in order to understand more clearly how chondrocytes are able to respond to, and regulate, their extracellular environment. It is known that SOX9 gene expression is influenced by many different signalling pathways (Murakami et al., 2000a, Tavella et al., 2004, Piera-Velazquez et al., 2007). In this study, in addition to confirming the role of p38 MAPK during osmotic stress, we examined whether MEK-ERK signalling pathway was involved in regulating SOX9 in our system.

We were interested in characterising how loading patterns of hyperosmolarity could affect SOX9 expression levels. Osmotic changes will occur *in vivo* as a consequence of mechanical loading. These changes will naturally occur dynamically as water is expelled and drawn into the tissue as its levels of load change. It has formerly been shown that subjecting chondrocytes to different patterns of osmotic loading affects their biosynthetic activity (Urban and Hall, 1994, Borghetti et al., 1995, Urban and Bayliss, 1989, Urban et al., 1993) and there is a differential effect of variable mechanical loading regimes on the chondrocyte biosynthetic response. Static compression has been found to decrease proteoglycan and protein synthesis (Gray et al., 1988, Sah et al., 1989, Schneiderman et al., 1986, Korver et al., 1992), while dynamic compression at certain frequencies and amplitudes stimulated synthesis of these matrix constituents (Korver et al., 1992, Parkkinen et al., 1993, Sah et al., 1989). Our experiments show that a constant application of hyperosmolarity is not necessary for SOX9 mRNA induction. Significant increases in SOX9 gene expression were observed after just a 2 hour exposure to hyperosmotic media following which the chondrocytes were returned to control conditions for the remaining 3 hours. Furthermore, when the HAC are exposed to alternating applications of hyperosmotic and control conditions over 5 hours, the induction of SOX9 mRNA is significantly higher than that seen following static hyperosmotic exposure alone. Whilst these conditions are not meant to precisely simulate the osmotic conditions experienced by a chondrocyte *in vivo*, they do demonstrate that chondrocytes are able to perceive periods of hyperosmotic stimulation over differing time scales and that repeated applications of these stimuli can enhance their

production of factors such as SOX9. A previous study, undertaken using immature bovine chondrocytes, showed some evidence of increased aggrecan mRNA expression following exposure of the cells to dynamic 0.0017Hz fluctuations in osmolarity (Palmer et al., 2001). We examined aggrecan and COL2A1 mRNA levels. Although there was no apparent change in the gene expression of these matrix genes in cyclical loading of passaged HAC, in our freshly isolated HAC cultures hyperosmolarity caused a reduction in their expression agreeing with the majority of studies that have noted a decrease in ECM production under static hyperosmotic loading (Palmer et al., 2001, Hung et al., 2003, Urban and Bayliss, 1989, Schneiderman et al., 1986, Urban et al., 1993). We recently showed that post-transcriptional control of COL2A1 occurs under hyperosmotic conditions and that COL2A1 mRNA is destabilised (Tew et al., 2009). This may explain the reduced levels of collagen type II produced by chondrocytes under hyperosmotic conditions. Equally, this may be a result of osmotic stress inhibiting translational machinery which has been demonstrated in other cell types (Gray et al., 1988, Tew and Hardingham, 2006). Indeed, this could account for our failure to detect an increase in SOX9 protein levels following 5 hours of hyperosmotic stimulation, a process that would appear to be slow in relation to the change in the mRNA levels. Interestingly SOX9 protein levels are increased by p38 MAPK signalling but not MEK-ERK signalling. We have formerly demonstrated that hyperosmotic culture led to an increase in the half life of SOX9 mRNA, a process that is dependent on p38 MAPK signalling (Tew et al., 2009), but we have established here, that MEK-ERK signalling is not involved. We show here that p38 MAPK inhibition leads to a rapid decrease in levels of SOX9 protein within HAC, although when p38 MAPK is stimulated, by hyperosmolar loading, no accumulation of SOX9 protein was seen within the 5 hour duration of experiments performed. However, previously we had found that hyperosmotic stimulation for 24 hours led to an increase in SOX9 protein levels (Tew et al., 2009). It is unclear why there is a difference in this process, although it is possible that the narrow range of regulation of SOX9 mRNA produces no discernible effect using the detection techniques used here, which may be relatively insensitive. There is a much bigger effect on SOX9 destabilisation, and hence reduction of cellular levels when p38 MAPK signalling is abrogated. ERK1/2 has no effect on SOX9 protein production in our system.

Both static and cyclic hyperosmotic stimulation led to a rapid increase in ERK1/2 phosphorylation. Hyperosmotic stress has been previously shown to activate ERK in tissue culture in a variety of cells (Terada et al., 1994, Kwon et al., 1995, Itoh et al., 1994). However, blocking this pathway using the MEK1/2 inhibitor U0126 demonstrated that the activation of the MEK-ERK pathway is not required for hyperosmotic induction of SOX9 mRNA. We were interested to note though that control cultures which had been treated with MEK1/2 inhibitor U0126 had significantly increased SOX9 mRNA levels. This was not associated with mRNA stabilisation and indicates that constant low level ERK activity has a repressive role on SOX9 mRNA levels in HAC. Contrasting roles for ERK signalling in SOX9 regulation have been previously demonstrated. Chick limb mesenchyme showed an increase in SOX9 mRNA when treated with the MEK1/2 inhibitor U0126 (Bobick and Kulyk, 2004) whilst in young murine primary chondrocytes increased Sox9 expression, caused by FGF2 stimulation was inhibited by U0126 (Murakami et al., 2000a). This could suggest that ERK signalling represses basal SOX9 expression but may also be a signalling component controlling SOX9 induction under some circumstances. Alternatively these results could simply demonstrate a significant contrast between the reactions of young murine costal chondrocytes and the osteoarthritic HAC which were used in this study.

It has previously been demonstrated that 550mOsm conditions only induced SOX9 mRNA in passaged cells when the formation of actin stress fibres by the cells was prevented (Tew and Hardingham, 2006) therefore in all our passaged cell experiments we co-cultured with the ROCK inhibitor Y27632. However, experiments performed at the end of this study did identify that the presence of actin stress fibres in these current experiments did not inhibit the identified hyperosmolar response. It is unclear why there is a difference between our data and previous studies (Tew et al., 2009, Tew and Hardingham, 2006).

Many cells exist in an environment where osmolarity can fluctuate and have a variety of responses, many of which appear to be controlled by a signalling network of protein kinases and transcription factors. In mammalian cells hypertonicity activates many MAP kinases including ERK1/2 and p38 MAPK. In yeast cells although ERK activity is not essential for the transcriptional regulation of BGT1 and SMIT, two genes that encode for osmolyte transporters (Kwon et al., 1995),

inhibition of MEK1 down regulated TonE-mediated reporter gene expression (Nadkarni et al., 1999) and it has been proposed that the activation of ERK pathway in hyperosmotically stressed cells serves as a cell survival signal (Michea et al., 2000). Interestingly studies undertaken in rat nucleus pulposus cells, which produce an extracellular matrix similar to that of chondrocytes, have found that exposure to a hyperosmotic environment caused an increase in the transcription factor TonEBP with a subsequent activation of its target genes including aggrecan (Tsai et al., 2006). This transactivation was sensitive to inhibition of ERK and p38 signalling (Tsai et al., 2006). Intriguingly, others have demonstrated that MEK-ERK signalling is activated in articular chondrocytes at normosmotic conditions exposed to fluid-flow leading to a down-regulation of aggrecan (Hung et al., 2000). In the present study, the adaptive response of SOX9 to dynamic hyperosmotic loading is reduced by MEK-ERK inhibition. Meanwhile, inhibition of p38 MAPK leads to a general down regulation of SOX9 mRNA in all cultures and inhibits hyperosmotic induction of SOX9 and stabilisation of SOX9 mRNA (Tew et al., 2009). The dynamic hyperosmotic loading of HAC is associated with the activation of ERK1/2 above that of both control and static hyperosmotic conditions whilst activation of p38 MAPK is greatest under static hyperosmotic loading. These results indicate that MEK-ERK signalling plays a role in the elevated response of SOX9 mRNA under dynamic hyperosmotic loading, whereas the findings with p38 MAPK inhibitor are consistent with previous studies where SOX9 mRNA levels are controlled through p38 MAPK-dependant regulation of its mRNA stability (Tew and Hardingham, 2006). The same group recently determined that medium osmolarity, a known regulator of p38 MAPK controls SOX9 by the same mechanism(Tew et al., 2009). Results from this study suggest that the activation of MEK-ERK signalling is important for the increase in SOX9 evident in cyclical hyperosmotic loading.

We have demonstrated that the regulation of COX-2 under hyperosmotic loading is remarkably similar to SOX9. COX-2 has been shown to be regulated by hyperosmotic conditions in a number of cells including lipopolysaccaharide-activated macrophages, (Zhang et al., 1995) renal medullary interstitial cells (Hao et al., 2000) and human umbilical vein endothelial cells (Arbabi et al., 2000). In bovine articular chondrocytes, hyperosmotic-dependent potentiation of COX-2 occurred only in IL-1 stimulated bovine cartilage explants culture (Le et al., 2006). In this

study we demonstrate for the first time that COX-2 mRNA is up-regulated in hyperosmotic conditions alone. We were interested that COX-2 was regulated post transcriptionally by hyperosmotic conditions. Post transcriptional control of COX-2 mRNA levels is well established and there is good evidence for a role for p38 MAPK in stabilising COX2 mRNA in response to a number of external stimuli including IL-1α, dexamethasone and IL-17 (Miyazawa et al., 1998, Lasa et al., 2000, Faour et al., 2001). However, in contrast Thomas et al (Thomas et al., 2002) found that p38 MAPK had no effect on COX-2 half-life in chondrocyte cell lines. It will be interesting to investigate whether increased stability of COX-2 mRNA evident under hyperosmotic conditions is acting through p38 MAPK mediated post transcriptional regulation similar to that described previously in SOX9 (Tew and Hardingham, 2006).

Under control conditions the expression of COX-2 mRNA was increased following MEK1/2 inhibition. It was also shown that the increase in COX-2 gene expression under dynamic hyperosmotic loading was abrogated by p38 MAPK inhibition but MEK1/2 inhibition had a variable effect on the hyperosmotic induction of COX-2. Nieminen et al (2005) (Nieminen et al., 2005) suggested that the activation of ERK1/2 and p38 MAPK pathways are two of the signalling cascades that mediate the up regulation of COX-2 expression in HAC exposed to IL-1. However others have found that in chondrocytes p38 MAPK signalling alone is involved (Thomas et al., 2002). Inhibitors of p38 MAPK and ERK1/2 have been associated with a suppression of the hypertonicity stimulated COX-2 expression in cultured medullary epithelial cells (Yang et al., 2000). Other reports in human monocytes and RAW264 macrophages (Jones et al., 1999, Subbaramaiah et al., 2000, Caivano and Cohen, 2000, Dean et al., 1999, Shalom-Barak et al., 1998) have indicated that p38 MAPK and ERK1/2 signalling pathways are involved in the cellular events leading to the up-regulation of COX-2 gene transcription. Our findings support a major role for p38 MAPK in the hyperosmotic induction of COX-2; however our results indicate that the role of MEK-ERK signalling is more complex. As mentioned previously, under normosmotic conditions MEK-ERK signalling results in a reduction in COX-2. This may indicate that constant low-level ERK activity has a repressive role on COX-2 mRNA levels in HAC. The variable effect of the signalling cascade on the up-regulation of COX-2 under cyclical osmolar loading requires further investigation

as these findings are in contrast to our studies in freshly isolated HAC grown as monolayers where MEK-ERK signalling had no affect on the increase in COX-2 mRNA evident under static hyperosmotic conditions. It would be interesting to further investigate the role of hyperosmotic stress in the regulation of COX-2 in normal HAC.

In summary we have shown that, in freshly isolated HAC, hyperosmolarity increases SOX9 mRNA and that induction is highest when osmolarity is cyclically applied. MEK-ERK signalling has a role in controlling SOX9 gene expression under normal osmotic conditions but is not involved in SOX9 regulation by static hyperosmotic stimulation. However, MEK-ERK signalling does control the enhanced SOX9 mRNA response to cyclical osmotic variation. We have demonstrated that COX2 is regulated in these cells in a similar way to SOX9 with a role for post-transcriptional regulation following exposure to increased osmolarity.

APPENDIX TO MANUSCRIPT 1

Introduction

A useful model system given the restricted number of freshly isolated chondrocytes that could be obtained from HAC derived from knees from total knee replacement surgery was passaged chondrocytes. HAC expanded in monolayer to passage 2 (P2) were therefore used in some of the experiments in this thesis. The effects of hyperosmolarity and the MEK1/2 inhibitor U0126 on passaged HAC,were demonstrated by undertaking identical studies, over a 5 hours, for static hyperosmotic loading in freshly isolated and passaged HAC. This was in order to assess whether static hyperosmotic loading of these cells induced a similar up regulation of SOX9 in P2 HAC and in addition to determine the effect of the ME1/2 inhibitor U0126.

Materials and Methods

Materials and methods were as described in the previous manuscript.

Results

Hyperosmotic (550mOsm) conditions significantly increased SOX9 in passaged chondrocytes (3 fold, $p<0.003$) in comparison to controls as described previously (Manuscript 1). In passaged cells there was a reduction in SOX9 mRNA gene expression when the MEK1/2 inhibitor U0126 was present (50% reduction, $p<0.008$).

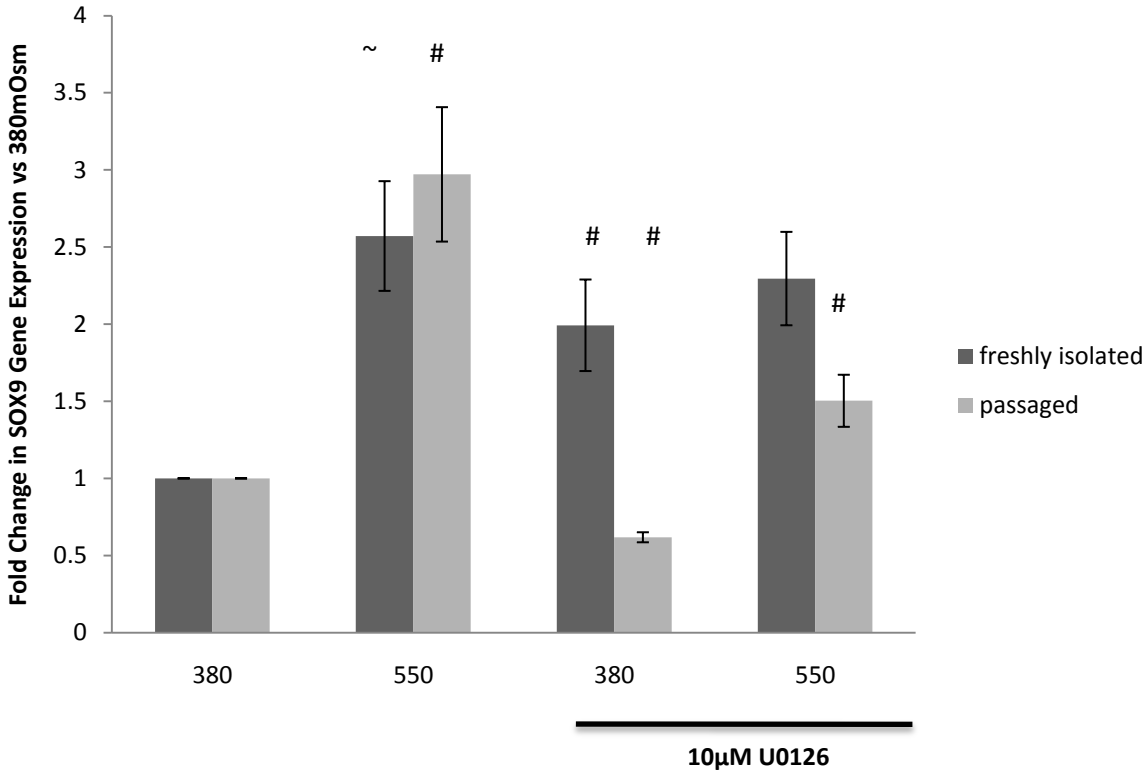

Figure 1: Effect of static hyperosmotic loading on SOX9 mRNA expression in freshly isolated (n=6) and passaged HAC (n=3). Histograms represent means ± SEM of fold change in expression compared to cells at 380mOsm. # ~ indicates significant difference relative to 380mOsm control where statistical significance is defined for this study as # P<0.01, ~P<0.001. Hyperosmotic conditions significantly increased SOX9 mRNA levels in freshly isolated cells and passaged cells (P<0.001 and P<0.003 respectively). In freshly isolated chondrocytes overall there was a trend for the MEK1/2 inhibitor U0126 (10μM) to increase SOX9 gene expression (P<0.06), however there was a significant statistical interaction, with the MEK1/2 inhibitor causing a significant increase in SOX9 in 380mOsm alone (P<0.002). In passaged cells there was a significant reduction in both osmotic conditions (P<0.008).

Discussion

In primary cells it would appear that the effect of ERK1/2 signalling on the expression of SOX9 mRNA is dependent upon the osmolarity of the media to which the chondrocytes are exposed. Although this alteration in fold change of SOX9 is small, changes in SOX9 mRNA gene expression in chondrocytes are restricted and the importance of small changes is evident from mouse Sox9 knock-out/knock-in studies (Akiyama et al., 2004). In contrast in passaged cells, which have lost their chondrocyte phenotype and become more fibroblastic in appearance, it seems that

the presence of ERK1/2 signalling has a positive effect on SOX9 induction. These differences would suggest that the mode of regulation is dependent upon the differentiation state of the cell.

Acknowledgements

This study was supported by a Wellcome Trust veterinary research entry level grant. MJP was a recipient of a Wellcome Veterinary Research Entry Level Fellowship. SRT was supported by the Arthritis Research Council. We thank the University of Liverpool Veterinary School, Leahurst, Wirral, for providing equine cartilage samples.

Manuscript 2

Regulation of SOX9 and COX-2 in normal and OA equine articular chondrocytes by hyperosmotic loading

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Running title: **A ROLE FOR ERK1/2 IN REGULATING SOX9 and COX-2 mRNA DURING CYCLICAL HYPEROSMOTIC LOADING**

Keywords: Equine articular cartilage, SOX9, COX-2, ERK, p38 MAPK, hyperosmotic loading

Abstract

Introduction SOX9 is a transcription factor required for cartilage formation and is essential for cartilage extracellular matrix (ECM) formation. Osteoarthritis (OA) is characterized by a loss of ECM. In human SOX9 gene expression is regulated by osmotic loading. Here we characterize SOX9 and COX-2 mRNA regulation through static and cyclical application of hyperosmotic conditions in normal and osteoarthritic monolayer cultures of equine chondrocytes. The roles of ERK1/2 and p38 MAPK pathways were investigated using pharmacological inhibition to identify mechanisms of SOX9 regulation.

Methods Equine chondrocytes harvested from normal or OA joints were subjected to different osmotic loading patterns in monolayer culture as either primary (P0) or passaged (P2) cells. The involvement of p38 MAPK and MEK-ERK signalling was determined by using pharmacological inhibitors; SB212190 (p38 MAPK inhibitor, 20 μM) and U0126 (MEK1/2 inhibitor, 10 μM). SOX9 mRNA stability for freshly isolated cells was determined by measuring decay following administration of 1μM actinomycin D. Levels of transcripts encoding SOX9, COX-2, Col2A1 and aggrecan were measured using qRT-PCR. *De novo* glycosaminoglycan (GAG) synthesis of explants was determined with ^{35}S sulphate during static hyperosmolar loading. Statistical analyses were undertaken using S-Plus, SPSS and Excel software.

Results Static hyperosmotic conditions significantly reduced SOX9 mRNA but increased COX-2 mRNA in normal P2 and OA P0 but not normal P0 chondrocytes. Cyclical loading of normal P2 and OA P0 but not normal P0 cells led to an increase in SOX9 gene expression and this was prevented by both p38 MAPK and MEK1/2 inhibition. In these cells there was no effect on COX-2 mRNA of cyclic hyperosmolar loading although p38 MAPK signalling reduced COX-2 expression. Static hyperosmolar loading in explants increases GAG synthesis and this was reduced by ERK inhibition.

Conclusions The response to osmotic loading of SOX9 and COX-2 mRNA is dependent on the nature of the osmotic stimulation and the chondrocyte phenotype. The p38 MAPK and ERK1/2 pathways are involved in the adaptive response in SOX9 regulation, to cyclical osmotic loading in normal P2 and OA P0 chondrocytes.

Thus the response of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes suggesting that altering sensing of the osmotic environment and inappropriate responses of the resident cell population may be important in disease progression.

Introduction

The surfaces of long bones within diarthrodial joints is lined with articular cartilage, an avascular connective tissue that provides a nearly frictionless bearing surface for transmitting and distributing mechanical loads between the bones of the skeleton (Mow et al., 1992). The unique load bearing properties of articular cartilage are dependent upon its structural composition and organization, particularly the interactions between collagens and proteoglycans of the extracellular matrix (ECM) (Poole et al., 2001). These matrix macromolecules are turned over by chondrocytes embedded within the cartilage. Chondrocytes utilise mechanical signals (Guilak et al., 1999), such as mechanical loading and local osmotic environment (Schulz and Bader, 2007), in conjunction with environmental and genetic factors to regulate their metabolic activity (Guilak, 2000). During loading, water is expressed from articular cartilage causing the matrix to deform and the proteoglycan concentration to increase due to water loss which, along with elevated mobile cation concentration exposes the chondrocytes to a hypertonic environment. Thus physiologically, load bearing cartilages experience changes in extracellular ion composition and hence osmotic pressure under cyclic and static loading (Urban and Bayliss, 1989).

Progressive degeneration of articular cartilage leads to joint pain and dysfunction that is clinically identified as osteoarthritis. Under normal circumstances, there is equilibrium between matrix deposition and degradation; however this equilibrium is disrupted in OA leading to the excessive degradation of matrix and progressive loss of important matrix components such as collagen and aggrecan (Martel-Pelletier et al., 1994, Poole et al., 1993). In early OA, disruption of the collagen network results in an increase in water content of the tissue (Guilak et al., 1994, Maroudas, 1976) and a corresponding decrease in pericellular osmolarity (Maroudas et al., 1985). PG loss in later stage OA further exacerbates osmotic perturbations resulting in a decrease in interstitial osmolarity (Venn and Maroudas, 1977). Under loading OA chondrocytes are subject to greater variations in osmolarity than normal

chondrocytes due to an increase in the rate and extent of fluid loss in this swollen cartilage (Hopewell and Urban, 2003, Bush and Hall, 2005).

The effects of osmolarity on chondrocyte ECM synthesis have been undertaken in a number of studies with differing outcomes. These studies have shown that proteoglycan synthesis can be reduced in the presences of hyper-, or hypo-osmotic conditions (Hopewell and Urban, 2003, Schneiderman et al., 1986, Palmer et al., 2001, Urban and Hall, 1994, Urban et al., 1993). A reduction in COL2A1 gene expression has also been identified as a consequence of hyperosmotic conditions (Tew et al., 2009, Urban and Bayliss, 1989, Urban et al., 1993). A further study demonstrated that the application of dynamic hypo-osmotic conditions caused an increase in cartilage ECM genes (Chao et al., 2006). The mitogen activated protein kinases (MAPK); extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) have been implicated in the adaptive responses of chondrocytes to hyperosmotic conditions (Hopewell and Urban, 2003). The MAPK families are a cell signalling transduction pathway which connect extracellular signals to intracellular responses such as gene expression in eukaryotic cells (Yang et al., 2003). Both p38 MAPK and Jun N-terminal Kinase (JNK) signalling pathways have previously been implicated in osmotic stress signalling in studies in yeast and many mammalian cells (Sheikh-Hamad and Gustin, 2004, Brewster et al., 1993, Galcheva-Gargova et al., 1994, Rouse et al., 1994, Capasso et al., 2001).

SOX9 is an essential transcription factor controlling the expression of many cartilage ECM genes including collagen type II (Bell et al., 1997) and aggrecan (Bi et al., 1999, Sekiya et al., 2000). Campomelic dysplasia, a severe syndrome caused by inadequate cartilage formation during development, is due to a haploinsufficiency of SOX9 and underlines its importance to the chondrocyte phenotype (Foster et al., 1994, Wagner et al., 1994). One of the mechanisms responsible for the control of SOX9 expression in chondrocytes is the p38 MAPK-dependant regulation of its mRNA stability (Tew et al., 2009, Tew and Hardingham, 2006). Further studies have concluded that human articular chondrocytes (HAC) exposed to hyperosmotic culture resulted in an increase in the stability of SOX9 mRNA, a process which was also sensitive to p38 MAPK inhibition (Tew et al., 2009).

Another gene COX-2 has also been identified as being regulated by osmolarity. Hyperosmolarity stimulates COX-2 expression in activated chondrocytes (Le et al., 2006) in addition to cultured medullary epithelial cells (Yang et al., 1999), colonic epithelium (Arbabi et al., 2001) and kupffer cells (Zhang et al., 1995). COX-2 is a bifunctional enzyme with both oxygenase and peroxidase activities, responsible for the formation of prostanoids (Needleman et al., 1986). IL-1 stimulated HAC have been shown to express COX-2 mRNA and protein (Geng et al., 1995). Additionally there is a superinduction of COX-2 in human OA cartilage and aberrant expression of COX-2 protein in articular tissues is a feature of arthritis (Kang et al., 1996, Amin et al., 1997, Sano et al., 1992). Furthermore studies in animal models demonstrated that COX-2 expression was detected in inflamed, but not normal, paw tissue from rats with adjuvant-induced arthritis (Anderson et al., 1996, Sano et al., 1992). The involvement of the intracellular signalling proteins p38 MAPK and ERK in inducing COX-2 gene expression has also been reported (Chen et al., 2001). Moreover, COX-2 mRNA stability at the post-transcriptional level is necessary for maximal COX-2 expression (Newton et al., 1997, Inoue et al., 1995).

Few studies have been undertaken comparing the effects of hyperosmotic loading on normal versus OA chondrocytes, which are known to undergo changes in phenotype. Hence, the objective of this study was to compare the response to hyperosmotic loading of cultured normal and OA primary chondrocytes. In addition we also wished to identify whether the altered phenotype demonstrated in passaged chondrocytes effects SOX9 and COX-2 gene expression during hyperosmotic loading. The response of articular cartilage to loading is a complex phenomenon due to numerous factors related to the mechanical strain of the tissue. Therefore models in which individual physical phenomena can be studied separately are important in revealing the cellular mechanisms of joint loading. With this in mind our present study was performed to explore the effects of osmolarity on SOX9 and COX-2 expression and the biosynthetic response, by examining whether the activation of the p38 MAPK or ERK signalling pathways were required. As data from compressive loading experiments clearly indicate that dynamic compression of cartilage produces increases in ECM synthesis by chondrocytes (Sah et al., 1989) we also examined the nature of the osmotic load applied to cells. We found that the nature of the response

to osmotic loading of SOX9 and COX-2 mRNA is dependent on the character of the osmotic stimulation and on the chondrocyte phenotype.

Materials and methods

Chondrocyte isolation, expansion and culture

Equine articular cartilage was obtained from the surfaces of metacarpophalangeal joints of skeletally mature horses with grossly normal or arthritic joints. OA joints were derived from clinically diagnosed cases following euthanasia, which on visual inspection exhibited typical patterns of cartilage fibrillation and erosion. Sample collection was subject to institutional ethical review. Isolation of chondrocytes has been described previously (Tew et al., 2008a). Chondrocytes were grown as monolayers in Dulbecco's modified eagles medium (DMEM) (Invitrogen, Paisley, UK), supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin, 100μg/ml streptomycin (all from Invitrogen, Paisley, UK) and 500ng/ml amphotericin B (BioWhittaker, Lonza, USA). Experiments were undertaken using either freshly isolated chondrocytes plated at 100,000 cells/cm^2 within 48 hours, or with cells at the end of passage 2 (with a 1:2 split ratio). Experiments were replicated using cells from different donors. In order to elucidate the effects of medium osmolarity on the cells they were grown for 5 hours in serum-free and antibiotic-free DMEM containing either 207mM NaCl or 527mM NaCl in order to yield 380mOsm or 550mOsm solutions. Following production of the defined media a freezing point depression osmometer (Loser, Berlin, Germany) was used in order to check the osmolarity was within an acceptable range of +/- 2% variation. The use of these osmolarities was based upon previous experiments in bovine articular cartilage where 380mOsm, used as a control, is close to that experienced by healthy chondrocytes *in-situ,* and 550mOsm represented a hyperosmotic condition similar to that experienced by chondrocytes under load (Hopewell and Urban, 2003, Urban et al., 1993). Previously it has been demonstrated that an increase in SOX9 mRNA in 550mOsm conditions was observed when the formation of actin stress fibres by the cells was prevented in the presence of the ROCK1/2 inhibitor Y27632 (Tew et al., 2009). Therefore passaged cultures were supplemented with 10μM of the ROCK1/2 inhibitor Y27632 (Calbiochem, Nottingham, UK). Where necessary, cultures were supplemented with the MEK1/2 inhibitor U0126 or the p38 MAPK inhibitor

SB202190 at doses which have been shown to maximally inhibit the MEK-ERK; 10μM (data not shown) or p38 MAPK; 20μM (Tew et al., 2009) signalling pathways (both Sigma- Aldrich, Dorset, UK), for 2 hours prior to the commencement of experiments.

Gene Expression analysis

Total RNA was prepared from monolayer cultures in 12 well culture plates using 0.5ml Tri Reagent (Ambion, Warrington, UK) per well. The Guanidium-thiocyanate-phenol-chloroform extraction technique was used as previously described (Chomczynski and Sacchi, 1987). M-MLV reverse transcriptase and random hexamer oligonucleotides were used to synthesize cDNA from RNA (both from Promega, Southampton, UK) in a 25μl reaction. 1μl aliquots were amplified by PCR in 20μl reaction volumes on an ABI 7700 Sequence Detector using either a SYBR Green PCR mastermix or a Taqman mastermix were appropriate (Applied Biosytems, Warrington, UK). The fitness of GAPDH as a valid normalisation factor under different osmolarities has been previously established by our laboratory (Tew et al., 2009) (see Appendix 1). Relative expression levels were normalized to GAPDH and calculated using the $2^{-\triangle Ct}$ method (Livak and Schmittgen, 2001). Primers and probes for SOX9 and GAPDH were designed by Applied Biosystems Assays-by-Design and had the following sequences: SOX9 Forward; CGC-CGA-AGC-TCA-GCA-AGA, Reverse; CGC-TTC-TCG-CTC-TCG-TTCA, Probe; CAA-GCT-CTG-GAG-ACT-GC; GAPDH Forward; ACT-GGT-GTC-TTC-ACT-ACC-TTG-GA, Reverse; AGC-AGA-GAT-GAT-GAC-CCT-TTT-GG; Probe; AAG-TGA-GCC-CCA-GCC-TT. For determination of aggrecan, collagen II (COL2A1) and cyclo-oxygenase (COX-2), SYBR Green detection was used and primers were obtained from Eurogentec (Seraing, Belgium). The primer sequences for aggrecan were designed in Primer Express (Applied Biosystems) software and were: Forward; AGG-AGC-AGG-AGT-TTG-TCA-ACA; Reverse; CCC-TTC-GAT-GGT-CCT-GCT-AT. The sequences for COL2A1 (Taylor and Pinchbeck, 2008) and COX-2 (Figueiredo et al., 2009) have been previously reported. GAPDH primer sequences used in mRNA stability experiments have been reported formerly (Taylor and Pinchbeck, 2008). All primers used were predicted to cross exon boundaries. For decay experiments freshly isolated equine articular cartilage was grown in

monolayers and treated under experimental conditions for 2 hours before the addition of 1μM of the transcription inhibitor actinomycin D (Sigma- Aldrich, Dorset, UK). Decay of SOX9 mRNA was then measured following extraction of purified total mRNA at a number of time points 0-3 hours later. This was reverse transcribed and real-time PCR undertaken; decay curves were generated using GAPDH as a normalisation factor. Data was plotted on semi-log charts and exponential regression lines generated in Microsoft Excel. The slope (*m*) of the regression lines were used to calculate the mRNA half life ($t_{1/2}$) using the equation $t_{1/2} = ln\ (2)/m$.

Quantification of proteoglycan

GAG synthesis was quantified by measuring the incorporation of radioactive ^{35}S sulphate into GAGs. Cartilage explants were obtained from mature grossly normal metacarpophalangeal joints of horses following institutional ethical review. Full thickness cartilage was excised and cut into 3mm diameter explants from the entire surface of P1 from 3 donors. The explants were gently blotted on sterile gauze pads and weighed in pre-weighed sterile eppendorf tubes. They were then transferred into DMEM (Invitrogen, Paisley, UK), supplemented with 10% FCS, 100units/ml penicillin, 100μg/ml streptomycin (all from Invitrogen, Paisley, UK) and 500ng/ml amphotericin B (BioWhittaker, Lonza, USA) and maintained in 12-well culture plates for 48 h at 37°C in a 5% CO_2 incubator, as this has been shown to be sufficient time for synthetic activity to reach equilibrium after harvesting (Gray et al., 1989). To evaluate the effect of osmotic loading and MEK-ERK signalling on *de novo* GAG synthesis cartilage explants were labelled in DMEM containing 2μCi/μl of ^{35}S sulphate (MP Biomedicals Inc, Irvine , USA) at either 380 or 550mOsm and where appropriate with the MEK1/2 inhibitor U0126 (10μM) and cultured for 24 hours. Sulphate incorporation was determined following papain digestion of the explants as described previously (Homandberg et al., 1992b). Unincorporated radiolabel was separated from macromolecular products in all samples using PD-10 size exclusion columns (GE Healthcare Lifesciences, Amersham, UK) eluted in phosphate-buffered saline (Sigma-Aldrich, Dorset, UK) (Barker and Seedhom, 2001). The ^{35}S sulphate radioactivity was measured by liquid-scintillation counting (1410 liquid-scintillation counter; Wallac Oy, Turku, Finland)

of aliquots from void volume fractions. Total sulphate incorporation rate was calculated for the ^{35}S sulphate incorporation rate and normalized to wet weight. The sulphate incorporation rate is expressed as counts per minute per mg wet tissue.

Statistical analysis

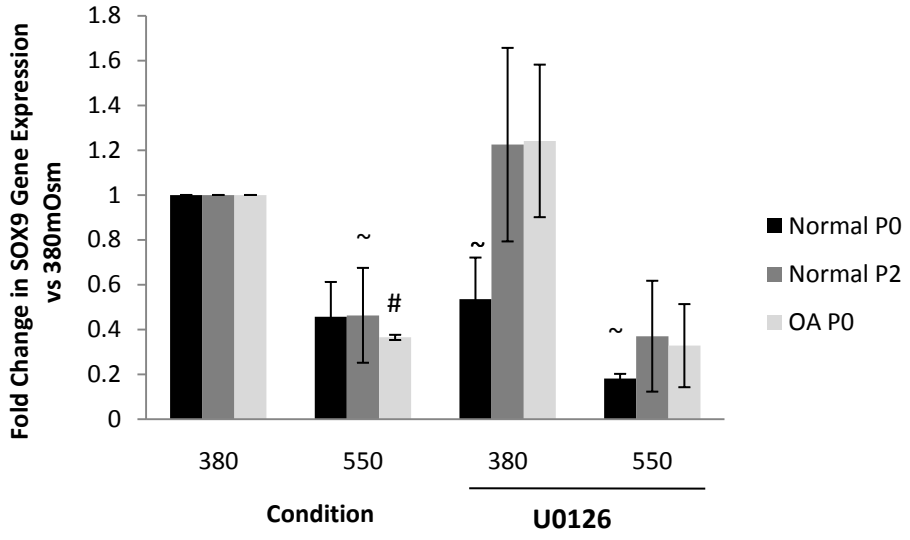
Statistically significant differences between gene expression values of control and treated cultures were analysed using mixed effects linear regression to allow for donors with significant biological variation. Significant changes in $t_{1/2}$ data was performed using paired student t-test. The analyses were undertaken using S-Plus, SPSS and Excel software.

Results

Effect of hyperosmotic loading on SOX9 and COX-2 gene expression

Equine articular chondrocytes grown or expanded in monolayer from normal or OA joints were exposed to static hyperosmolar loading of 550mOsm for a 5 hour period in order to determine the effect of hyperosmotic stress on SOX9 mRNA expression. Culture media with an osmolarity of 380mOsm was used as the control condition. The ROCK1/2 inhibitor Y27632 was present in all passaged cell cultures as it had previously been demonstrated that an increase in SOX9 mRNA in 550mOsm conditions was observed more consistently when the formation of actin stress fibres by the cells was prevented. (Tew et al., 2009). We wished to identify whether the presence of the ROCK inhibitor Y27632 was indeed required for the hyperosmotic induction of SOX9 in passaged chondrocytes. Monolayer culture of passaged HAC, for 3 donors was incubated at 380mOsm and 550mOsm for 5 hours in the presence or absence of the ROCK1/2 inhibitor Y27632 (10μM). It was identified that the inhibition of actin stress fibres has no significant effect on hyperosmolar induction of SOX9 in P2 HAC (data not shown).

1a



1b

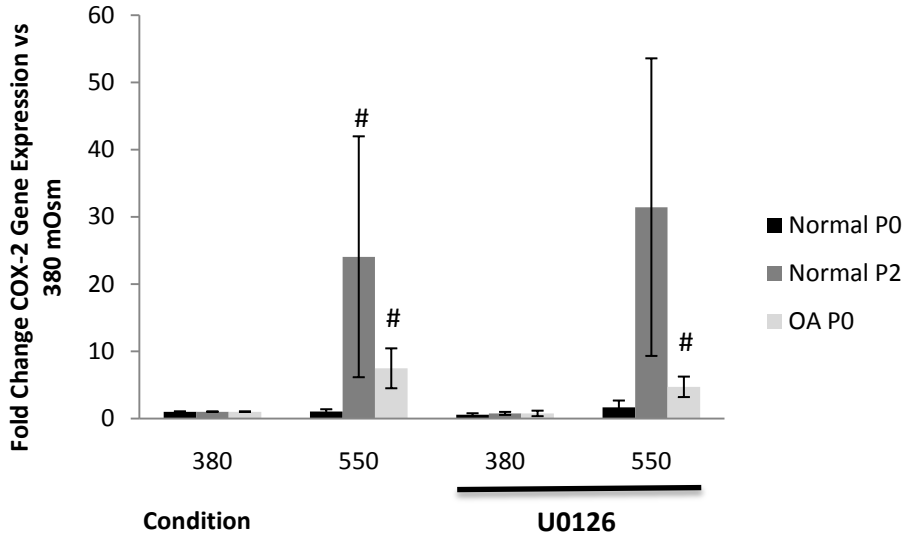


Figure 1: Effect of static hyperosmotic loading and the MEK1/2 inhibitor U0126 on SOX9 and COX-2 mRNA in equine articular chondrocytes Real time PCR analysis of (a) SOX9 mRNA levels and (b) COX-2 mRNA in monolayer culture of normal freshly isolated (P0), passaged normal (P2) and OA P0 chondrocytes cultured at 380 or 550mOsm in the presence or absence of the MEK1/2 inhibitor U0126 for 5 hours. In addition Y27632 (10μM) was present in cultures of normal P2. Data are represented as the fold change in expression compared to cells under 380mOsm conditions and without the inhibitor. Histograms represent means ± SEM. Data were evaluated using mixed effect linear regression and # ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as # $P<0.01$, ~$P<0.001$ (n=3).

There was a 50%±1.7 reduction in SOX9 mRNA in normal P0 chondrocytes subject to static hyperosmolar loading (Figure 1a); however, this reduction was not

statistically significant. No change in COX-2 gene expression was evident. In contrast, in P2 normal and P0 OA chondrocytes static hyperosmotic loading significantly reduced SOX9 mRNA (Figure 1a) (65%±2.0; p=0.0004, 55%±1.0; p=0.0096). Additionally COX-2 mRNA was increased in normal P2 and OA P0 chondrocytes under these conditions (24 fold, p=0.005; 7 fold, p=0.0054) (Figure 1b).

Examining the role of ERK in hyperosmotic loading

In normal P0 chondrocytes only, MEK1/2 inhibition with U0126 significantly reduced SOX9 mRNA under both normosmolar and hyperosmolar conditions (50% and 80% respectively, p=0.0003) (Figure 1a). Moreover MEK1/2 inhibition in OA P0 chondrocytes reduced COX-2 expression under static hyperosmotic conditions (2 fold, p=0.006) (Figure 1b).

Previously we found in HAC derived from OA tissue, that hyperosmotic conditions led to an increase in the half life of SOX9 mRNA, a process which is sensitive to p38 MAPK (Tew et al., 2009). We were therefore interested in identifying whether ERK1/2 affects this same mechanism in freshly isolated normal equine articular chondrocytes. Decay curves generated using mean values for all donors showed that culture in 550mOsm increased the $t_{1/2}$ of SOX9 mRNA (Figure 2). To further quantify this we calculated the $t_{½}$ for each donor individually and performed mixed effects linear regression analysis on the data (Table 1). At 380mOsm, the $t_{1/2}$ of SOX9 mRNA was 2.2 ±0.18hours; this was increased to 5.8±1.88 hours when the culture medium was 550mOsm. However statistical analysis identified that this increase did not reach statistical significance. The MEK1/2 inhibitor U0126 had no effect on the $t_{½}$ of SOX9. Taken together these data suggest counter-intuitively that while 550mOsm conditions increase the stability of SOX9 mRNA there was a paradoxical reduction in SOX9 gene expression. Moreover in normal P0 chondrocytes MEK-ERK signalling increases SOX9 and COX-2 mRNA.

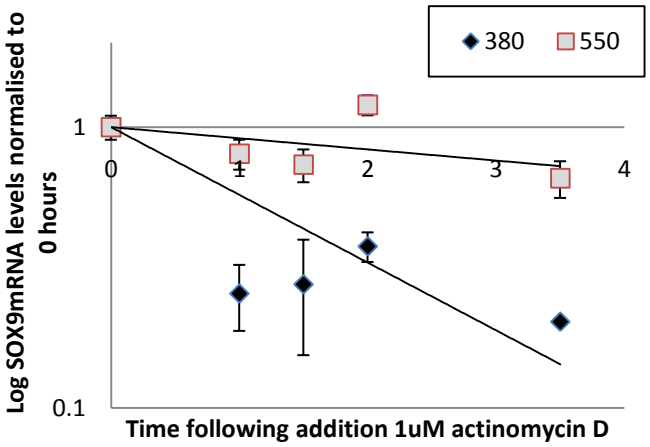


Figure 2: Decay curve for SOX9 in freshly isolated equine articular chondrocytes SOX9 decay in freshly isolated equine articular chondrocytes cultured at different osmolarities. Chondrocytes were cultured at 380 or 550 mOsm for 2 hours prior to the addition of actinomycin D. RNA was then extracted in triplicate at time periods over 3.5 hours for reverse transcription and analysed by real-time PCR. Data represents the means and standard errors of the fold changes in SOX9 mRNA levels compared to time point 0 for three donors

Osmolarity (mOsm)	Inhibitor U0126 (10µm)	Mean Half Life (Hours)	Standard Error
380	-	2.2	0.81
550	-	5.8	1.88
380	+	3.4	1.05
550	+	3.05	1.07

Table 1: Half life of SOX9 mRNA in freshly isolated chondrocytes cultured in osmotically defined media with, or without the MEK 1/2 inhibitor U0126 (10µM). Results demonstrated that although there was an increase in the $t_{½}$ in 550mOsm conditions this was not statistically significant compared to 380 mOsm tested by mixed effects linear regression (Data represents 7 donors).

Effect of hyperosmolar loading on ECM synthesis

We were interested in the effect of medium osmolarity on ECM production and whether MEK-ERK signalling had a role. Therefore we examined *de novo* GAG synthesis by ^{35}S sulphate incorporation in equine cartilage explants cultures. Following 24 hour culture at 550mOsm there was a significant increase in GAG synthesis compared to 380mOsm (19%, p=0.048). Interestingly the presence of the

MEK1/2 inhibitor U0126 in the cultures significantly reduced this effect at 550mOsm (32%; p=0.03) (Figure 3). These results suggest that hyperosmotic loading for 24 hours increases GAG synthesis and this is dependent on MEK-ERK signalling. Next to define further the downstream effects of static hyperosmolar loading on normal and OA chondrocytes, we investigated the expression of the cartilage matrix genes COL2A1 and aggrecan, known downstream targets of SOX9. In normal P0 chondrocytes during static hyperosmotic loading for a 5 hour period no difference in the expression of the two genes was evident. However there was a reduction in COL2A1 in normal P2 chondrocytes (3 fold, p=0.045) and an increase in aggrecan mRNA in OA P0 chondrocytes (3 fold, p=0.05).

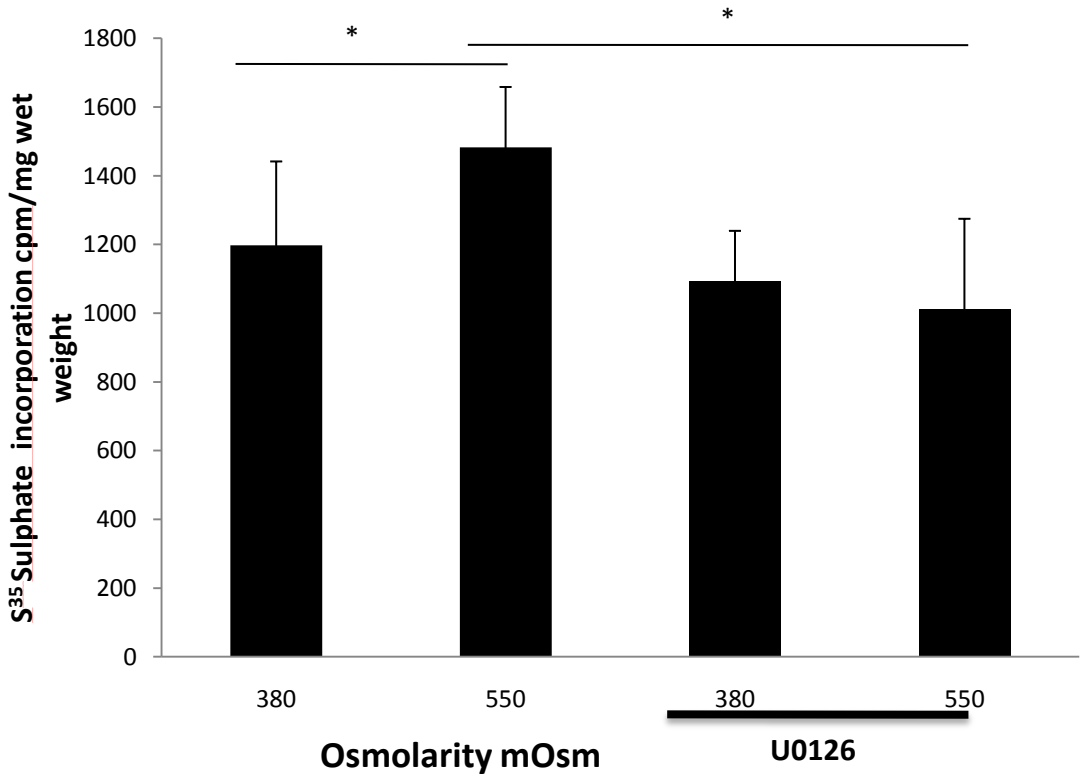


Figure 3: Sulphate incorporation rate 24 hours post hyperosmotic loading Radioactive ^{35}S sulphate incorporation rate was determined in normal equine cartilage explants obtained aseptically from the metacarpophalangeal joints of skeletally mature equines. Explants were cultured in 380mOsm or 550mOsm with or without the presence of the MEK/ERK inhibitor U0126 and labelled with ^{35}S sulphate for 24 hours. The incorporated radiolabel in the newly synthesized matrix macromolecules was then measured and normalized to wet weight of the explants. Histograms represent means ± SEM. Data were evaluated using mixed effect linear regression and * indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as $P<0.05$ (n=3).

Effect of cyclical hyperosmotic loading on SOX9 and COX-2 expression

It is known that *in vivo*, chondrocytes are subjected to periods of cyclical loading which will generate fluid flow and osmotic fluctuations within the tissue (Urban and Bayliss, 1989). Therefore we examined the effect of a cyclical application of hyperosmotic conditions on monolayer cultures of normal P0, normal P2 and OA P0 chondrocytes. Media was adjusted to 550mOsm or with periods of 380 or 550mOsm alternating every 30 (c30) or 60 (c60) minutes. At each frequency the final incubation period was under 550mOsm. In P0 normal chondrocytes dynamic hyperosmolar loading had no affect on SOX9 mRNA. However in normal P2 and OA P0 there was an increase in SOX9 mRNA of 2-3 folds. This was only statistically significant for OA P0 chondrocytes when the culture media was changed every 60 minutes (p=0.017) though a trend was evident for OA P0 at changes every 30 minutes (p=0.07). There was no increase in COX-2 during on-off loading for a 5 hour period in normal P0, normal P2 or OA P0 chondrocytes (Figure 4).

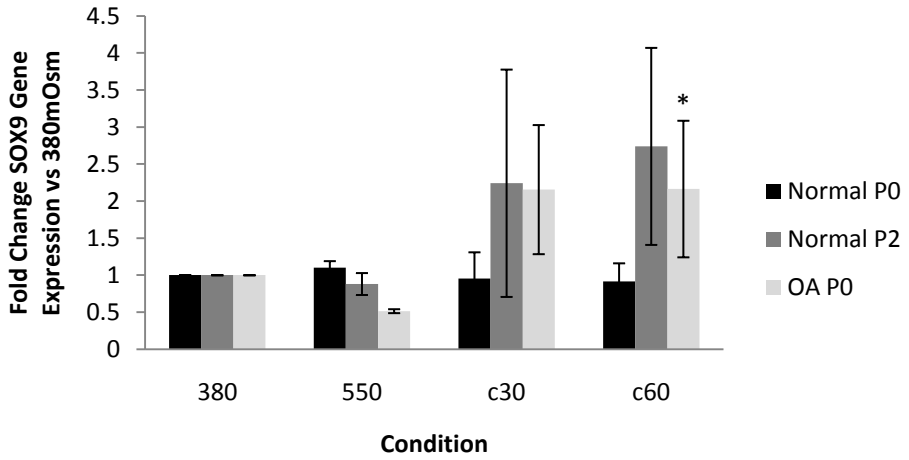


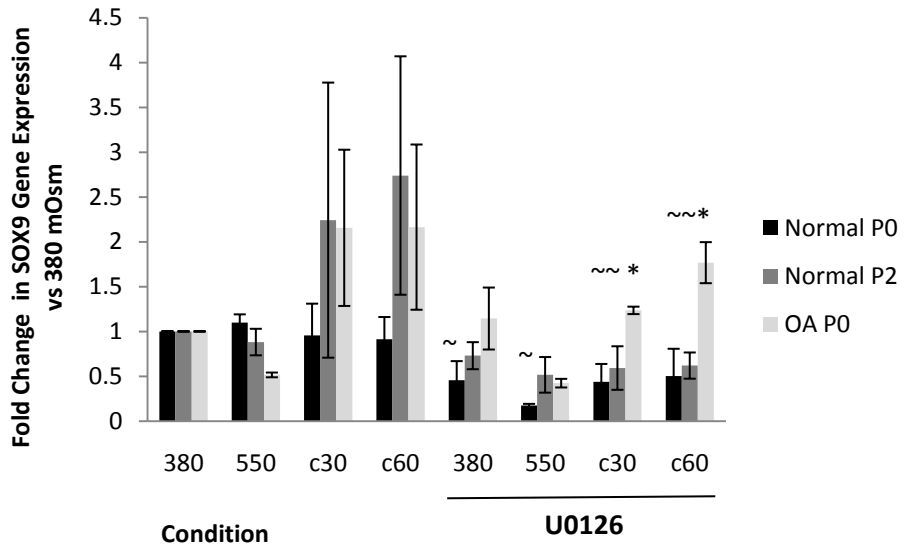
Figure 4: Effect of cyclical hyperosmotic loading on SOX9 mRNA in equine articular chondrocytes Real-time PCR analysis of SOX9 mRNA levels in monolayer culture of freshly isolate (P0), passaged normal and OA P0 equine articular chondrocytes incubated with cyclical application of 380mOsm and 550mOsm every 30 or 60 minutes for the 5 hour period. In control cultures of 380mOsm media was changed at the same frequencies to account for possible shear induced effects on the cells during the loading period. At each frequency, the final incubation period was under 550mOsm conditions. In addition Y27632 (10μM) was present in cultures of normal P2. Data are represented as the fold change in expression compared to cells under 380mOsm conditions and without the inhibitor. Histograms represent means ± SEM. Data were evaluated using mixed effect linear regression and * indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as $P<0.05$ (n=3).

Matrix gene expression (Col2A1 and aggrecan) was then investigated during cyclic hyperosmotic loading experiments. No change in matrix gene expression was evident in normal P0, P2 or OA P0 chondrocytes.

Effects of p38 MAPK and MEK1/2 inhibition on SOX9 and COX-2 gene expression in cyclic hyperosmotic loading

Having identified a role for MEK-ERK signalling in SOX9 expression in static hyperosmotically loaded normal P0 chondrocytes and following our previous findings that p38 MAPK activity is a requirement in HAC for hyperosmotic stimulation of SOX9 mRNA (Tew et al., 2009), further experiments were undertaken in order to determine if p38 MAPK or MEK-ERK signalling would affect SOX9 or COX-2 during cyclic hyperosmotic loading. Real time PCR determined that although cyclic hyperosmotic loading over a 5 hour in normal P0 chondrocytes demonstrated no affect of p38 MAPK inhibition, MEK1/2 inhibition significantly reduced SOX9 gene expression in all conditions (>50 %, $p<0.0005$) (Figure 5a). The presence of p38 MAPK or MEK1/2 inhibitors in cultures of both normal P2 and OA P0 chondrocytes prevented the increase in SOX9 mRNA evident at cyclic loading (normal P2 chondrocytes U0126; 77%; $p=0.001$, SB202190; 88%; $P=0.0001$ and OA P0 chondrocytes U0126; 50%; $p=0.044$, SB202190; 35%; $p=0.016$) (Figure 5a and 5b respectively). These findings indicate that the p38 MAPK and MEK-ERK signalling pathways are necessary for the elevation of SOX9 mRNA evident in cyclic hyperosmotic loading of normal P2 and OA P0 chondrocytes.

5a



5b

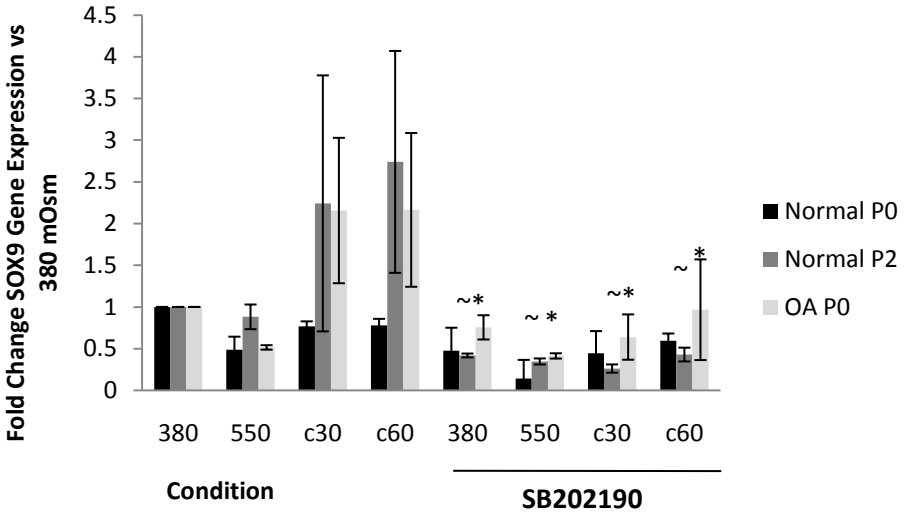


Figure 5: Elevated SOX9 mRNA associated with cyclical hyperosmotic loading of normal P0, normal P2 and OA P0 chondrocytes is reduced by the presence of either the MEK1/2 inhibitor U0126 or the p38 MAPK inhibitor SB202190. Real-time PCR analysis of SOX9 mRNA levels in monolayer culture of normal P0, P2 and OA P0 , with cyclical application of 380mOsm and 550mOsm every 30 or 60 minutes for the 5 hour period, with or without the presence of either (a) MEK1/2 inhibitor U0126(10μM) or (b) p38 MAPK inhibitor SB202190 (20μM). At each frequency, the final incubation period was under 550mOsm conditions. In addition Y27632 (10μM) was present in cultures of normal P2. Data are represented as the fold change in expression compared to cells under 380mOsm conditions and without the inhibitor. Histograms represent means ± SEM. Data were evaluated using mixed effect linear regression and * # ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as *P<0.05 # P<0.01, ~P<0.001 (n=3).

p38 MAPK inhibition reduced COX-2 in normal P0, normal P2 and OA P0 chondrocytes (p=0.0001, p=0.0001 and p=0.033 respectively) (Figure 6). In contrast the addition of MEK1/2 inhibitor had no effect on COX-2 mRNA (data not shown). These findings indicate that 550mOsm culture conditions have no effect on COX-2 mRNA in normal P0 chondrocytes irrespective of its application, however in normal P2 and OA P0 chondrocytes static but not dynamic hyperosmotic loading significantly increases COX-2 gene expression. In addition p38 MAPK signalling has a role in the regulation of COX-2 gene expression.

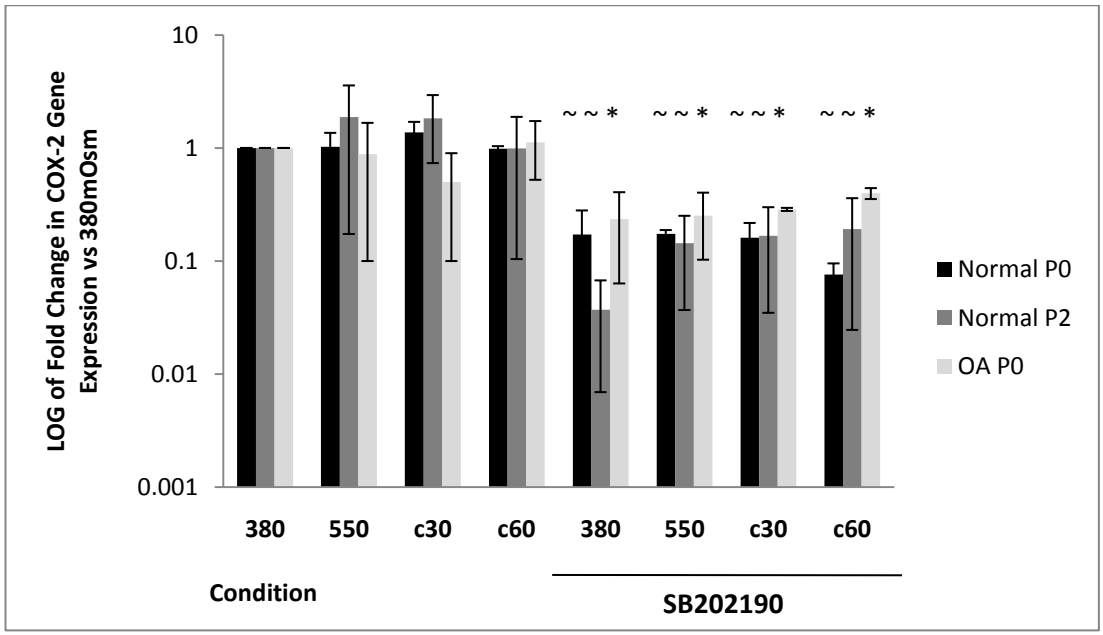


Figure 6 : COX-2 gene expression in cyclical hyperosmotic Real-time PCR analysis of SOX9 mRNA levels in monolayer culture of normal P0, P2 and OA P0 , with cyclical application of 380mOsm and 550mOsm every 30 or 60 minutes for the 5 hour period, with or without the presence of either the p38 MAPK inhibitor SB202190 (20μM). At each frequency, the final incubation period was under 550mOsm conditions. In addition Y27632 (10μM) was present in cultures of normal P2. Data are represented as the log of the fold change in expression compared to cells under 380mOsm conditions and without the inhibitor. Histograms represent means ± SEM. Data were evaluated using mixed effect linear regression and * ~ indicates significant difference relative to 380mOsm control. Statistical significance is defined for this study as * $P<0.05$, ~$P<0.001$ (n=3).

Discussion

In chondrocytes some of the changes in matrix expression in response to osmolarity have been well studied (Hall et al., 1991, Hopewell and Urban, 2003, Hung et al., 2003, Palmer et al., 2001, Schneiderman et al., 1986, Urban and Bayliss, 1989, Urban et al., 1993), however much less is known about these changes in OA chondrocytes. The expression of SOX9 is essential for the ability of the chondrocyte to produce a cartilage matrix (Stokes et al., 2001, Tew et al., 2005) and so we were interested in investigating the expression of SOX9 in normal and OA chondrocytes under osmotic loading, which is previously undocumented in equine tissue. Given the importance of SOX9 in the development and maintenance of the chondrocyte phenotype, its reduction in OA (Haag et al., 2008, Tew et al., 2005, Aigner et al., 2003, Tchetina et al., 2005) may contribute to the cartilage pathology. Mechanical stimulation of chondrocytes induces numerous physicochemical changes including alterations in osmotic pressure (Urban, 1994). This produces a number of physiological and biochemical responses resulting in changes in expression of matrix genes. The nature of the response, in part, depends on the nature of the mechanical stimulation. In general dynamic stimuli results in an anabolic response, whereas static compression more frequently inhibits chondrocyte activity (Gray et al., 1988, Sah et al., 1989, Schneiderman et al., 1986). The response of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes (Salter et al., 2002, Bush and Hall, 2001a) suggesting that altered sensing of the osmotic environment and inappropriate responses of the resident chondrocyte population may be important in disease progression.

Previous work has shown that static hyperosmotic loading for 5 hours of passaged HAC derived from OA joints of patients undergoing total knee arthroplasty results in an increase in SOX9 mRNA (Tew et al., 2009). In contrast here we were intrigued to find that in equine articular chondrocytes there was a reduction in SOX9 mRNA although this was only significant statistically in normal P2 and OA P0, not in normal P0 cells. However, similar to HAC there was also an increase in the stability of SOX9 mRNA in hyperosmotic conditions (Tew et al., 2009). This latter finding would suggest that more SOX9 mRNA should be present. In yeast hyperosmotic stress represses the transcription of HXT2 and HXT4 (Turkel, 1999) and the effect evident here may be due to a reduction in the transcription of SOX9 in equine

articular chondrocytes under hyperosmotic loading. Further studies using nuclear run-on assays (Gariglio et al., 1981), would need to be undertaken to investigate whether this is indeed the case. Surprisingly in normal equine articular chondrocytes there is no effect of p38 MAPK signalling on SOX9 mRNA. This would lead one to surmise that the p38 MAPK-dependant regulation of mRNA stability demonstrated in HAC derived from OA cartilage (Tew et al., 2009) is not likely to be occurring in equine normal cartilage and the former finding may be due to either species variation or disease.

Interestingly there is an increase in SOX9 during cyclic hyperosmotic loading in normal P2 and OA P0 but not normal P0 chondrocytes, which is in agreement with our findings in HAC OA P2 chondrocytes (See manuscript 1). These data suggest that there is only an effect on SOX9 mRNA of both static and cyclic hyperosmotic loading on chondrocytes with altered phenotype, from either culture dedifferentiation or phenotypic alteration from disease in equine tissue. Modifications of the articular chondrocyte phenotype are commonly observed in OA cartilage, including suppression of genes involved in the phenotypic stability of articular chondrocytes (Aigner et al., 2006, Aigner and Dudhia, 2003, Castagnola et al., 1988, Lemare et al., 1998), reduced ECM protein production (Yagi et al., 2005), proliferation (Aigner et al., 2001) and change in morphology (Tchetina et al., 2005). During expansion of normal chondrocytes and in OA chondrocytes there is a loss of the specific chondrocytic phenotype and a reversion to a more fibroblast-like phenotype (von der Mark et al., 1977, Mandl et al., 2004, Sandell and Aigner, 2001). This phenotypical change is accompanied by decreased gene expression of cartilage specific markers like COL2A1 and aggrecan. This process could also alter the response of chondrocytes to extracellular stimuli such as a change in osmolarity. In human normal chondrocytes cyclic stretch has an anabolic effect as shown by increases in aggrecan and matrix metalloproteinase 3 expression. However, this effect was not evident in OA chondrocytes, where no change in the expression of either gene was observed (Millward-Sadler et al., 2000). This difference might be attributed to a change in mechanotransduction pathways between normal and OA chondrocytes (Salter et al., 2002, Salter et al., 2004, Millward-Sadler and Salter, 2004). It would be interesting to investigate the effect of osmotic loading on SOX9 and matrix gene expression in normal HAC.

In normal equine cartilage explants we have demonstrated an increase in GAG synthesis in response to hyperosmotic loading. Furthermore this was dependant on MEK-ERK signalling. This is in agreement with a previous study undertaken in bovine chondrocytes encapsulated in alginate beads for 48 hours in which the increase in sulphate incorporation in response to hyperosmotic conditions was abrogated in the presence of the MEK inhibitor PD98059 (Hopewell and Urban, 2003). We examined aggrecan and COL2A1 levels in our cultures and found that hyperosmolarity had little effect on their expression in most experimental conditions. However, there was a reduction in COL2A1 in normal P2 chondrocytes agreeing with the majority of studies that have noted a decrease in ECM production under static hyperosmotic loading (Palmer et al., 2001, Hung et al., 2003, Urban and Bayliss, 1989, Schneiderman et al., 1986, Urban et al., 1993). Intriguingly there was an increase during static hyperosmolar loading in aggrecan mRNA in OA P0 chondrocytes, similar effects have been revealed in human and bovine intervertebral disc cells exposed to hyperosmotic conditions (Wuertz et al., 2007). This is in contrast to other studies in normal chondrocytes where an increase in osmolarity results in a reduction in aggrecan mRNA (Hung et al., 2003, Palmer et al., 2001, Guilak et al., 2002).

Many cells exist in an environment where osmolarity can fluctuate and have a variety of responses, countless of which appear to be controlled by a signalling network of protein kinases and transcription factors. In mammalian cells hypertonicity activates many MAP kinases including ERK1/2 and p38 MAPK. In yeast cells although ERK activity is not essential for the transcriptional regulation of BGT1 and SMIT, two genes that encode for osmolyte transporters (Kwon et al., 1995), inhibition of MEK1 down regulated TonE-mediated reporter gene expression (Nadkarni et al., 1999) and it has been proposed that the activation of ERK pathway in hyperosmotically stressed cells serves as a cell survival signal (Michea et al., 2000). Interestingly studies undertaken in rat nucleus pulposus cells, which produce an extracellular matrix similar to that of chondrocytes, have found that exposure to a hyperosmotic environment resulted in an increase in the transcription factor TonEBP with a subsequent activation of its target genes including aggrecan. This transactivation was sensitive to inhibition of ERK and p38 signalling (Tsai et al., 2006). Others have demonstrated that MEK-ERK signalling is activated in articular

chondrocytes experiencing normal osmotic conditions, exposed to fluid-flow leading to a down-regulation of aggrecan (Hung et al., 2000). In the present study the elevated response of SOX9 mRNA under cyclic hyperosmotic loading is dependent on p38 MAPK and MEK-ERK signalling which is comparable to findings in HAC (Manuscript 1). Although MEK-ERK signalling was not required for the reduction in SOX9 apparent in static hyperosmotic loading of normal P2 and OA P0 equine chondrocytes, in normal P0 chondrocytes blocking this pathway using the MEK1/2 inhibitor U0126 demonstrated that MEK-ERK signalling represses SOX9 expression in normal chondrocytes. Hyperosmotic stress has been previously shown to activate ERK in tissue culture (Terada et al., 1994, Kwon et al., 1995, Itoh et al., 1994). Furthermore in young murine primary chondrocytes there was an increased Sox9 expression, caused by FGF-2 stimulation which was inhibited by U0126 (Murakami et al., 2000a). These results demonstrate a similarity between the reactions of young murine costal chondrocytes and normal equine chondrocytes which were used in this study.

COX-2 has been shown to be regulated by hyperosmotic conditions in a number of cells including lipopolysaccharide-activated macrophages, (Zhang et al., 1995) renal medullary interstitial cells (Hao et al., 2000) and human umbilical vein endothelial cells (Arbabi et al., 2000). In 2-4 week old bovine articular chondrocytes, hyperosmotic-dependent potentiation of COX-2 occurred only in IL-1 stimulated bovine cartilage explants culture (Le et al., 2006). In the latter experiment there was an osmolarity dependant potentiation of COX-2 in IL-1 activated chondrocytes in cartilage explants culture, but without the presence of IL-1 no COX-2 protein was evident. Similarly normal mature unstimulated HAC did not contain detectable COX-2 mRNA (Geng et al., 1995). This is consistent with our findings in normal P0 equine chondrocytes subjected to static hyperosmotic loading and all chondrocyte types in cyclical hyperosmotic loading where no change in COX-2 mRNA is evident. In contrast our studies in static hyperosmotic loading in monolayer culture of normal P2 and OA P0 equine chondrocytes, an increase in COX-2 gene expression was apparent in the presence of hyperosmotic conditions alone. A previous study in dedifferentiated immortalised HAC demonstrated lower levels of COX-2 expression compared to differentiated immortalised HAC following alginate culture (Thomas et al., 2002). The association of COX-2 expression with

differentiation has also been reported in keratinocyte, adipocyte, and tracheal epithelial cells (Borglum et al., 1997, Leong et al., 1996, Hill et al., 1998). In our study in normal P2 and OA P0 chondrocytes there are higher levels of COX-2 mRNA compared to normal P0 and this may make them more responsive to static hyperosmotic loading (data not shown). In addition a recent study investigating ectopic expression of COX-2 induced differentiation in articular chondrocytes established that COX-2 over-expression caused a suppression of SOX9 expression (Lee et al., 2008). It is possible that in static hyperosmotic loading, where there is a reduction in SOX9, and cyclical hyperosmolar loading, where there is an increase in SOX9, that this has a feedback on COX-2 gene expression in a similar manner. In a recent study undertaken in renal medullary cells it was found that hypertonicity induces TNF converting enzyme (TACE)-mediated ectodomain shedding of pro-TGFα, which subsequently activates COX-2 expression via epidermal growth factor receptor (EGFR) and MAPKs. The expression of TGFα is increased in an experimental model of knee OA (Appleton et al., 2007a) and in a subset of human patients with OA (Appleton et al., 2007b). In our experiment it is possible that in OA chondrocytes, continual osmotic stress is inducing TACE which in turn activates ERK and p38 MAPK resulting in an increase in COX-2 expression. Nieminen et al (2005) (Nieminen et al., 2005) suggested that the activation of ERK1/2 and p38 MAPK pathways are two of the signalling cascades that mediate the up regulation of COX-2 expression in HAC exposed to IL-1. Inhibitors of p38 MAPK and ERK1/2 have been associated with a suppression of the hypertonicity stimulated COX-2 expression in cultured medullary epithelial cells (Yang et al., 2000). Other reports in human monocytes and RAW264 macrophages (Jones et al., 1999, Subbaramaiah et al., 2000, Caivano and Cohen, 2000, Dean et al., 1999, Shalom-Barak et al., 1998) have indicated that p38 MAPK and ERK1/2 pathways are involved in the cellular events leading to the up-regulation of COX-2 gene transcription. Our findings demonstrate that in the presence of the p38 MAPK inhibitor, SB202190 there is a reduction in COX-2 mRNA. These data suggest that p38 MAPK signalling may repress basal COX-2 expression. However with static hyperosmolar loading of OA P0 chondrocytes, there is a reduction in COX-2 in the presence of the MEK1/2 inhibitor U0126. Overall it appears that MEK1/2 inhibition has little effect on the expression of COX-2 in equine articular chondrocytes.

Conclusions

In this study we investigated how static and cyclical hyperosmotic loading of normal and OA equine chondrocytes regulates SOX9 and COX-2 gene expression and the role of the MEK-ERK and P38 MAPK signalling pathways. We have shown that in contrast to HAC there is a reduction in SOX9 mRNA in static hyperosmolar loading. The nature of the response to osmotic loading of SOX9 and COX-2 mRNA is dependent on the nature of the osmotic stimulation and on the chondrocyte phenotype. The response of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes suggesting that altering sensing of the osmotic environment and inappropriate responses of the resident cell population may be important in disease progression.

Acknowledgements

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Concluding Remarks

The work in this thesis supports the hypothesis that MEK-ERK and p38 MAPK signalling have roles, though by distinct mechanisms, in the control of SOX9 and COX-2 during osmotic loading. However, additional work needs to be undertaken in order to further elucidate the mechanisms by which the signalling pathways act in both normal and OA chondrocytes. It has been demonstrated here that hyperosmolarity stabilizes SOX9 mRNA in chondrocytes, irrespective of the species or phenotype and MEK-ERK signalling is not involved. In addition COX-2 is also regulated post transcriptionally in HAC in a similar way to SOX9. Supplementary work is required in order to establish if this is also through p38 MAPK-dependant regulation of its mRNA stability (Tew et al., 2009). A role for p38 MAPK in post transcriptional control of COX-2 mRNA levels is well established in response to a number of external stimuli including IL-1α, dexamethasone and IL-17 (Miyazawa et al., 1998, Lasa et al., 2000, Faour et al., 2001). Interestingly there is evidence of distinct species differences in the effect of static osmolar loading on SOX9 mRNA. In agreement with previous studies in HAC (Tew et al., 2009) hyperosmolar loading increased SOX9 mRNA, however in studies undertaken here in equine articular chondrocytes exposed to identical conditions intriguingly there was a reduction in SOX9 mRNA despite evidence of SOX9 mRNA stabilisation. Finally the response, in terms of the COX-2 and SOX9 gene expression due to osmotic loading of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes, and the OA chondrocytes show similarity to dedifferentiated passaged chondrocytes. This would suggest that altered sensing of the osmotic environment and inappropriate responses of the resident cell population may be important to cartilage degeneration in OA.

One of the most interesting findings from this thesis was that in equine chondrocytes although hyperosmolar conditions stabilize SOX9 and increase its half-life there is a reduction in SOX9 mRNA in static hyperosmolar loading. Further studies are required to identify the cause of these conflicting results. A nuclear run-on assay (Gariglio et al., 1981) would allow changes in transcription rates to be measured, which often differ from steady-state mRNA levels. The principle involved is that,

when a cell is lysed and the intact nuclei retrieved, the isolated nuclei contain transcription complexes which are stalled on the DNA template due to an acute loss of ribonucleotide substrates. Transcription has been halted at the point in time where the nuclei were removed. However, it but can be started up again *in vitro* with the addition of new ribonucleotides. The use of radiolabelled nucleotides, would allow transcription to finish, and be observed as well as identifying SOX9 mRNA transcripts produced from the stalled RNA polymerase reactions and thus the level of SOX9 being transcribed at the time the cells were lysed can be quantified. By comparing the amount of gene-specific radiolabelled RNA synthesized in one nuclei preparation with another you can get an idea of the transcriptional initiation events in the cells of interest under differing conditions. This would be the most reliable method to assess transcription of SOX9 directly although it would be time consuming and require the use of radioactivity and a large number of cells.

Previous studies in HAC demonstrated that in passaged chondrocytes the increase in SOX9 mRNA in hyperosmotic conditions was only observed when actin stress fibre formation was prevented (Tew and Hardingham, 2006). Therefore in both human and equine chondrocytes the effect of the addition of the ROCK inhibitor Y27632 to passage cell cultures was determined in 5 hour static hyperosmolar loading. Surprisingly there was no effect on SOX9 gene expression in hyperosmolar loading in the presence of the inhibitor in either species in comparison to cultures where there was no inhibitor present. Hence if more osmolar loading studies are undertaken utilizing passaged chondrocytes, actin stress fibre formation would not require prevention.

The result from cyclical hyperosmolar loading experiments demonstrated that a constant application of hyperosmolarity is not necessary for SOX9 mRNA induction. Indeed the cyclical application of hyperosmolar loading enhanced the increase in SOX9 further than static hyperosmolar loading alone in HAC and equine chondrocytes derived from either OA or passaged normal populations. Chondrocytes from different species derived from normal and OA joints are able to perceive periods of cyclical hyperosmotic stimulation which can enhance their production of factors such as SOX9. In passaged HAC derived from OA joints and equine articular chondrocytes derived from passaged normal and OA freshly isolated monolayer culture, induction of SOX9 mRNA was significantly higher than that seen following

static hyperosmotic exposure. However in normal equine chondrocytes there was no change in SOX9 mRNA in these conditions. Together these findings suggest that there is only an effect on SOX9 mRNA of cyclic hyperosmotic loading when chondrocytes have an altered phenotype. Whilst previous studies have indicated that the expression of many genes is dependent upon differentiation state in chondrocytes (Castagnola et al., 1988, Lemare et al., 1998, Thomas et al., 2002, Aigner et al., 2003) additional work needs to be undertaken in normal HAC in order to elucidate if the increase in SOX9 gene expression evident in cyclical hyperosmotic loading is indeed an adaptation by the chondrocyte to disease. Furthermore in order to produce and maintain a properly functional cartilage matrix, the chondrocyte displays a specific pattern of gene expression both during development and in the adult (Hering, 1999, Cancedda et al., 1995). It has previously been found that diseased chondrocytes alter their pattern of gene expression in response to changes in their surrounding matrix. The response of the chondrocyte may lead to long term changes in the phenotype of the cell and result in the inability to properly repair or maintain the ECM (Buckwalter et al., 2005).

The cyclical hyperosmotic loading experiments undertaken were not meant to precisely simulate the osmotic conditions experienced by a chondrocyte *in vivo*. It was identified that repeated applications of hyperosmotic media increased the production of SOX9 mRNA and it would therefore be fascinating to undertake further studies using a system in which cyclical changes could be undertaken at a higher frequency more relevant to normal loading patterns. Indeed Chao et al (Chao et al., 2006) developed a microfluidic system to deliver osmotic loading via composition modulated flow. The device is able to apply osmotic loading to a frequency of 0.1Hz with minimal fluid shear stress and the use of such a device would be beneficial in future work. The frequency of osmotic loading used in this experiment is still not entirely physiologically relevant as it equates to a step every 10 seconds. A frequency of more significance would be if the osmotic load was applied at a walking frequency of 1 Hz or a running frequency of 3 Hz.

From the studies in this thesis it has been established that MEK/ERK signalling plays an important part in the elevated response of SOX9 mRNA under cyclic hyperosmotic loading in OA derived HAC from human and equine as well in passaged normal equine articular chondrocytes. The dynamic hyperosmotic loading

of HAC is associated with the activation of ERK1/2 above that of both control and static hyperosmotic conditions. Although ERK, SOX9 and p38 MAPK activation was looked at in equine experiments no signal or a weak signal was produced which did not allow conclusions to be made. This was despite the use of higher concentrations of the antibodies, the use of more lysate and longer exposure time during enhanced chemiluminescence. Thus the poor results obtained from equine samples were probably due to a lack of antibody specificity as no antibodies that can be purchased for these proteins are validated against the horse. Although a search for equine studies undertaking western blotting, using the antibodies to p44/42 MAPK and SOX9 revealed no experiments have been published, there was evidence that p38 MAPK antibody was reactive against equine p38 MAPK in equine digital vein endothelial cells (Brooks et al., 2009). Future studies investigating signalling pathway activation would require equine specific antibodies to be produced. In contrast the activation of p38 MAPK is greatest under static hyperosmotic loading. Generally p38 MAPK signalling increases SOX9 and COX-2 mRNA under all conditions in all chondrocyte phenotypes. This was consistent with previous studies where SOX9 mRNA levels were found to be controlled through p38 MAPK-dependant regulation of its mRNA stability (Tew et al., 2009) and provides a possibility that COX-2 mRNA stability is controlled in a similar manner. However the exception to this is in normal equine chondrocytes where p38 MAPK signalling has no effect on SOX9 mRNA indicating that p38 MAPK signalling does not have a role in SOX9 gene expression in normal equine chondrocytes in our system. The p38 MAPK inhibitor SB202190 did have an effect on COX-2 gene expression in the horse and others have also used this inhibitor with effect in equine studies (Eckert et al., 2007, Eckert et al., 2009), demonstrating that the inhibitor does react with the equine p38 MAPK signalling pathway. Therefore is the p38 MAPK-dependant regulation of SOX9 mRNA stability reliant on the phenotype of the tissue and was the effect previously demonstrated in HAC derived from OA joints due to an altered phenotype evident in OA. Additional work is required to establish how the stabilisation of SOX9 in hyperosmotic conditions of normal equine chondrocytes demonstrated here is controlled.

MAPK pathways are major information highways from the cell surface to the nucleus. The output of these pathways is transduced via MAPK family members that phosphorylate and regulate a wide array of substrates including transcription factors and other protein kinases. Small cell permeable inhibitors of protein kinases have been used widely to investigate the physiological roles of protein kinases, as they can be used simply and rapidly to block specific pathways in normal cells. Considerable caution in using small molecule inhibitors of protein kinases to assess the physiological roles of these enzymes is required. As there are more than 500 protein kinases encoded by the human genome, selectivity is critical. A number of studies have been undertaken to examine the specificity of these inhibitors (Davies et al., 2000, Bain et al., 2007, Bain et al., 2003). U0126 targets MEK1 and the closely related MEK2 and has been exploited in thousands of studies. It is a non-competitive inhibitor that interacts with the inactive unphosphorylated kinase more strongly than the active phosphorylated equivalent and thus exerts its effects by preventing the activation of MEK1/2 and not by blocking its activity (Davies et al., 2000). In additionU0126 has been reported to inhibit MEK5 with a similar potency to MEK1, (Kamakura et al., 1999). SB202190 has been used to assess the physiological roles of p38α and p38β MAPKs. Recently studies have identified other protein kinases that they inhibit with similar (Cyclin G-associated kinase; GAK and casein kinase; CK1) or greater (RIP2) potency (Godl et al., 2003). Therefore there is a danger with the use of either of these inhibitors that observed effects on cells result from the inhibition of targets distinct from those investigated here. Additional studies are required in order to confirm the effects of the specific pathways investigated here. One of the simplest methods to do this is the use of two structurally unrelated inhibitors, whose specificities have been tested towards a wide range of protein kinases *in vitro*. For ERK signalling pathways PD 184352 (Squires et al., 2002) has been identified as having no effect on ERK5 when used at1-2μM (Bain et al., 2007). Thus a study using both these inhibitors would be useful. For p38 MAPK studies in addition to SB202190 BIRB 0796 could be used in parallel. This interacts with p38α in a manner distinct from that exhibited by SB202190 and its binding induces a slow conformational change that locks the protein into an inactive conformation (Pargellis et al., 2002).

From this thesis it is apparent that in normosmotic conditions the effect of the MEK-ERK signalling on SOX9 mRNA is variable and dependant on the differentiation state and species of origin of the chondrocytes used. In order to clarify the role of MEK-ERK signalling in experiments here, future studies could use alternative methods to modulate MAPK activities. The use of dominant negative or constitutively active mutants of MAP kinase subtypes is an alternative and possibly more physiological way to modulate MAPK activities. These consist of mutations, engineered in the protein of interest that either activate or abolish its function as well as either activating or inhibiting the function of simultaneously expressed wild type protein. For example, mutating Lys71 and Lys52 to Arg in ERK-1 and -2, respectively, generates dominant negative kinase forms (Robbins et al., 1993). Infection of primary chondrocytes with lentivirus (Yin et al., 2009), adenovirus or retrovirus carrying mutant MAP kinase cDNA resulted in approx 80% of cells over-expressing the mutant (Chun, 2004). A further method that could be used to modulate ERK1/2 activity would be the use of Small interfering RNA (siRNA). These serve as a guide for cleavage of homologous mRNA. Degradation of a targeted mRNA would lead to specific suppression of the gene of interest, here ERK1 or 2.

Additionally studies require to be undertaken in systems that are more relevant to biological situations. Future studies could be undertaken in explants of articular cartilage. The experiments in this thesis could be applied to explants cultures derived from normal and OA donors. It is also possible to undertake mRNA decay studies on explants (McQuillan et al., 1986, Maurer and Wray, 1997). Alternatively experiments could be performed in culture conditions which allow a more usual chondrocyte phenotypic expression. In the some studies in this thesis passaged chondrocytes were used. This allowed a sufficient number of cells to be produced for high density seeding of plates. However the use of cells at passage 2 and no greater passage number was an attempt to reduce the effects of phenotypic change evident in cells from monolayers subject to repeated passage, which leads to a change in morphology and in gene expression patterns (Benya et al., 1978, Stokes et al., 2002, Aulthouse et al., 1989, Lin et al., 2008). However it is well known that even during early passage there is a great alteration in the chondrocyte phenotype (Lin et al.,

2008, Cournil-Henrionnet et al., 2008). In the future osmolarity studies could be undertaken within 3D suspensions such as agarose, alginate beads or on a hydrogel substrate as chondrocyte phenotype can be reexpressed in these systems (Watt and Dudhia, 1988, Guo et al., 1989, Reginato et al., 1994). One potential problem with the use of these systems is that it is more difficult to extract the protein for protein quantification, although this has been achieved in previous studies of bovine chondrocytes in alginate culture (Mok et al., 1994). Finally one factor that was not studied in this thesis was the nature of the substance used to undertake osmolar loading experiments. Although some groups have demonstrated the use of both sucrose and sodium chloride as chemicals to produce the osmotically defined media, similar results with each have been found (Chao et al., 2006, Hung et al., 2003, Urban et al., 1993, Tew et al., 2009). Therefore in the experiments in this thesis sodium chloride was used to produce osmotically defined media and the use of saline alone has been demonstrated in many previous chondrocyte osmolar loading studies (Borghetti et al., 1995, Palmer et al., 2001, Bush and Hall, 2001b, Schneiderman et al., 1986).

In this thesis it has been demonstrated for the first time that in chondrocytes COX-2 mRNA is upregulated in hyperosmotic conditions alone. This was evident in human and equine OA and normal equine passaged cells subject to static hyperosmotic loading. However, interestingly no effect was evident in cyclic hyperosmolar loading of equine chondrocytes which could be a species variation. Although in bovine articular chondrocytes, hyperosmotic-dependent potentiation of COX-2 has been demonstrated it occurred only in the presence of IL-1 (Le et al., 2006). The difference between normal and OA or passaged chondrocytes in COX-2 mRNA induced by hyperosmolarity, suggests an increased sensitivity in dedifferentiated chondrocytes. In a recent study undertaken in renal medullary cells it was found that hypertonicity induces TNFα converting enzyme (TACE)-mediated ectodomain shedding of pro-TGFα, which subsequently activates COX-2 expression via epidermal growth factor receptor (EGFR) and MAPKs. The expression of TGFα was increased in an experimental model of knee OA (Appleton et al., 2007a) and in a subset of human patients with OA (Appleton et al., 2007b). In our experiment it is possible that in OA chondrocytes, continual osmotic stress is inducing TACE which in turn activates MAPKs resulting in an increase in COX-2 expression. Although

COX-2 protein was demonstrated in HAC OA chondrocytes, no difference in COX-2 protein production was evident when monolayer cultures were subjected to hyperosmotic load. This latter finding may be due to already elevated levels of COX-2 protein in diseased tissues. This has been demonstrated in rat where COX-2 is detected in arthritic limbs and but is absent from normal limbs (Kang et al., 1996). Similarly normal mature unstimulated HAC did not contain detectable COX-2 mRNA (Geng et al., 1995).

The role of the ERK and p38 MAPK signalling pathways in the response of chondrocytes to periods of osmotic stress is complex. p38 is the mammalian homologue of the yeast hyperosmolarity glycerol (HOG) proline directed kinase, which controls osmotically regulated genes involved in the protection against osmotic and other stresses (Han et al., 1994). It is possible that signal transduction pathways initiated by osmotic stress in chondrocytes converge on this pathway resulting in downstream effects. Studies in this thesis indicate a major role for p38 signalling in the hyperosmotic induction of COX-2 gene expression in both normal and OA chondrocytes and this is species independent (Yang et al., 2000). Post transcriptional control of COX-2 mRNA levels is well established and has also been implicated in this thesis. There is evidence for a role for p38 MAPK in stabilising COX2 mRNA in response to a number of external stimului including IL-1α, dexamethasone and IL-17 (Miyazawa et al., 1998, Lasa et al., 2000, Faour et al., 2001). In contrast Thomas et al (Thomas et al., 2002) found that p38 MAPK had no effect on COX-2 half-life in chondrocyte cell lines. It would be interesting to investigate whether increased stability of COX-2 mRNA evident under hyperosmotic conditions is acting through p38 MAPK mediated post transcriptional regulation similar to that described previously in SOX9 (Tew and Hardingham, 2006).

The role of ERK in our system is more complex. In normosmolar conditions in HAC MEK-ERK signalling results in a reduction in COX-2 and this may indicate that constant low level ERK activity has a repressive role on COX-2 mRNA. However, the variable effect of this signalling on the up regulation of COX-2 under cyclical osmolar loading in passaged HAC requires further investigation as these findings are in contrast to our studies in both freshly isolated HAC and all equine cells, where MEK-ERK signalling generally had no affect on COX-2 mRNA. Further studies are required in this field in order to establish what role ERK signalling has in the

hyperosmotic increase in COX-2 mRNA in chondrocytes. Clinical studies have documented the efficacy of COX-2 inhibitors in OA (Cannon and Breedveld, 2001). However there are certain gastrointestinal, renal and cardiovascular side effects with COX-2 specific drugs. Thus the elucidation of COX-2 transcriptional activation in human chondrocytes in response to physical or chemical stimuli such as osmotic stress may aid in the identification of alternative therapeutic targets.

Hyperosmotic conditions have been demonstrated to affect articular cartilage ECM production (Urban et al., 1993, Urban and Bayliss, 1989, Palmer et al., 2001, Schneiderman et al., 1986, Chao et al., 2006, Hopewell and Urban, 2003). In addition it has established that dynamic osmotic loading modulates chondrocyte signalling and gene expression differently than static loading (Gray et al., 1988, Sah et al., 1989, Schneiderman et al., 1986). The results from this thesis also indicated that both proteoglycan synthesis and matrix gene regulation can be effected by hyperosmolarity. In addition hyperosmolarity affects SOX9 gene expression. Results from this thesis do not indicate a specific correlation, under hyperosmotic loading, between chondrocyte matrix gene expression and SOX9 gene expression. This is interesting as previously SOX9 retroviral transduction of chondrocytes has been shown to up-regulate chondrocyte matrix protein genes (Li et al., 2004). The expression of COL2A1 has been previously identified to be SOX9 dependant (Lefebvre et al., 1997) however a lack of correlation between SOX9 and COL2A1 has been identified previously (Gebhard et al., 2003, Aigner et al., 2003, Brew, 2009). This suggests that whilst SOX9 is necessary for cartilage formation (Lefebvre and de Crombrugghe, 1998) there may be other mechanisms that regulate COL2A1 transcription. Thus the control of the downstream effects of hyperosmotic loading is complex. Several authors have noted differing responses of chondrocytes following exposure to hyperosmotic conditions. In contrast to previous experiments in bovine explants cultures (Urban et al., 1993) and osmotic solute loaded HAC (Schneiderman et al., 1986, Urban and Bayliss, 1989), results from equine explants in this thesis indicated an increase, though moderate, in ^{35}S-sulphate incorporation rates in hyperosmotic conditions. This was dependant on MEK-ERK signalling and was in agreement with a previous study undertaken in bovine chondrocytes encapsulated in alginate beads for 48 hours in which the increase in sulphate incorporation in response to hyperosmotic conditions was abrogated in the presence

of the MEK inhibitor PD 098059 (Hopewell and Urban, 2003). Additionally, no change was identified in matrix gene expression in any isolated equine chondrocytes exposed to hyperosmolar loading in this thesis. Future studies would be necessary to explore these findings further in order to elucidate whether this is a species variation and if this is due to differential SOX9 expression in the horse or due to post transcriptional control as elucidated in HAC previously (Tew et al., 2009). In HAC derived from OA tissue there was a difference in the matrix gene expression of aggrecan and COL2A1 between static and cyclical hyperosmolar loading. Static hyperosmotic loading reduced matrix gene expression whereas cyclical hyperosmotic loading produced no change. This is in contrast to Palmer et al (Palmer et al., 2001) who demonstrated that in contrast to static hyperosmolar loading, dynamic hyperosmolar loading stimulated aggrecan gene expression. There are dramatic alterations in the chondrocytes surroundings as a result of cell isolation, which may affect matrix gene expression and hence matrix synthesis. For instance the change in ionic environment, the loss of chondrocyte attachment to matrix molecules, gradients of nutrients such as oxygen are disturbed, access to exogenous growth factors is enhanced and mechanical forces disappear (Sommarin et al., 1989, Urban et al., 1993, Newman and Watt, 1988, Archer et al., 1990). All these factors could contribute to differences in matrix synthesis and therefore future work under taken in explants cultures would be beneficial.

The effect of hyperosmotic loading in normal and OA chondrocytes on the regulation of two genes identified as being regulated by hyperosmotic conditions; SOX9 and COX-2, has been examined in this thesis. These responses are not unique to chondrocytes and could be used as a basis to further elucidate the roles of hyperosmolarity in the regulation of these genes in other tissues. In addition the osmotic responsiveness of cells is complex and involves the participation of multiple MAPKs (Burg, 1995). The regulation of SOX9 and COX-2 has wider implications than in cartilage alone. SOX9 has multiple functions in vertebrate development. In addition to chondrocyte differentiation it is involved in the cellular differentiation of sertoli cells (Moniot et al., 2009), neural crest cells (Stolt and Wegner, 2009), neural retina cells (Yokoi et al., 2009), prostate gland (Thomsen et al., 2008) and the heart (Lincoln et al., 2007). COX-2 is a highly inducible gene product expressed in response to a variety of stimuli, and is the rate limiting step in the production of

prostaglandins in some circumstances. Hyperosmotic conditions have been demonstrated to stimulate COX-2 expression in cultured medullary epithelial cells (Yang et al., 1999), colonic epithelium (Arbabi et al., 2001) and kupffer cells (Zhang et al., 1995). As it has been demonstrated in this thesis that hyperosmotic conditions can stimulate COX-2 dependant on the cell phenotype this may have consequences in the study of other disease processes. For instance in colonic epithelium it has been predicted that an increase in luminal osmolarity of the colon can induce COX-2 and thereby promote a neoplastic phenotype (Arbabi et al., 2000).

This thesis has demonstrated the response to osmotic loading of SOX9 and COX-2 mRNA is dependent on the nature of the osmotic stimulation and on the chondrocyte phenotype. The response of chondrocytes from OA cartilage is significantly different from that of normal chondrocytes suggesting that altering sensing of the osmotic environment and inappropriate responses of the resident cell population may be important in disease progression.

Appendix 1

Identification and selection of endogenous controls for relative quantification in osmolar loading studies of chondrocytes

RT-PCR is used as a method for the quantification of mRNA control. A critical stage in relative quantification design is the selection of an appropriate internal standard, sometimes known as endogenous controls (Thellin et al., 1999). All relative RT-PCR require internal standards, mainly housekeeping genes, so called because their synthesis occurs in all nucleated cell types since they are necessary for the cell survival. The synthesis of those molecules is often considered as being less erratic in comparison to that of others and, by their commonplace use, their expressions are considered as constant and secure. However numerous studies have indicated that even in these genes, their expression does vary in given situations between different tissues and between the same tissue under different conditions (Huitorel and Pantaloni, 1985, Chang et al., 1998, Hobbs et al., 1993).

RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. This is especially relevant when the samples have been obtained from different individuals, and will result in the misinterpretation of the expression profiles of the target genes. Therefore endogenous control expression levels must be the same in all samples in the study. It is therefore important to determine if the study treatment is affecting the expression level of the candidate endogenous genes. Consequently, an important aspect of experimental design in this thesis is determining an appropriate internal standard for chondrocyte studies, that is expressed at a constant level and which is unaffected by the changes in osmolarity.

In previous studies in chondrocytes looking at gene expression in osmolarity experiments GAPDH has been used (Aigner and Dudhia, 2003, Tew et al., 2007, Hung et al., 2003, Palmer et al., 2001). Therefore, prior to commencement of gene expression studies the validation of GAPDH as an appropriate internal control was investigated.

A human endogenous control plate, part number 4396929 (Applied Biosystems, Warrington, UK) was used to undertake this study. The plate evaluates the

expression of thirty two select housekeeping genes in total RNA samples using RT-PCR.

The following genes were evaluated;

ACTB	POP4	PSMC4	PPIA	IP08	RPS17	GUSB	TBP
PGK1	GAPDH	ELF1	CDKN1B	TFRC	PUM1	MRPL19	CDKN1A
UBC	B2M	RPL37A	EIF2B1	POLR2A	YWHAZ	PES1	ABL1
CASC3	RPLP0	HPRT1	MTATP6	GADD45A	HMBS	RPL30	18S

Samples of cDNA from human articular chondrocytes exposed to 380mOsm and 550mOsm with and without the MEK/ERK inhibitor U0126 were used in duplicate. Total RNA was prepared from monolayers in culture plates using Tri Reagent (Ambion, Warrington, UK). The Guanidium-thiocyanate-phenol-choloform extraction technique was used as previously described (Chomczynski and Sacchi, 1987). M-MLV reverse transcriptase and random hexamer oligonucleotides were used to synthesize cDNA from RNA (both from Promega, Southampton, UK) .Aliquots were amplified by PCR in 20µl reaction volumes on an ABI 7700 Sequence Detector using a Taqman gene expression mastermix (Applied Biosytems, Warrington, UK).

Data was analyzed by first determining the average CT of each sample for each control gene. A ratio was then made by successively dividing each sample by the first for each control gene. This data was then manipulated in order to run it through the GeNorm programme (www.medgen.ugent.be/~jvdesomp/genorm/). GeNorm is a collection of 'Visual Basis for Applications' (VBA) macros for Microsoft Excel to determine the most stable reference internal control genes from a set of tested candidate reference genes in a given sample panel. From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes. The underlying principles and formulas have been described (Vandesompele et al., 2002).

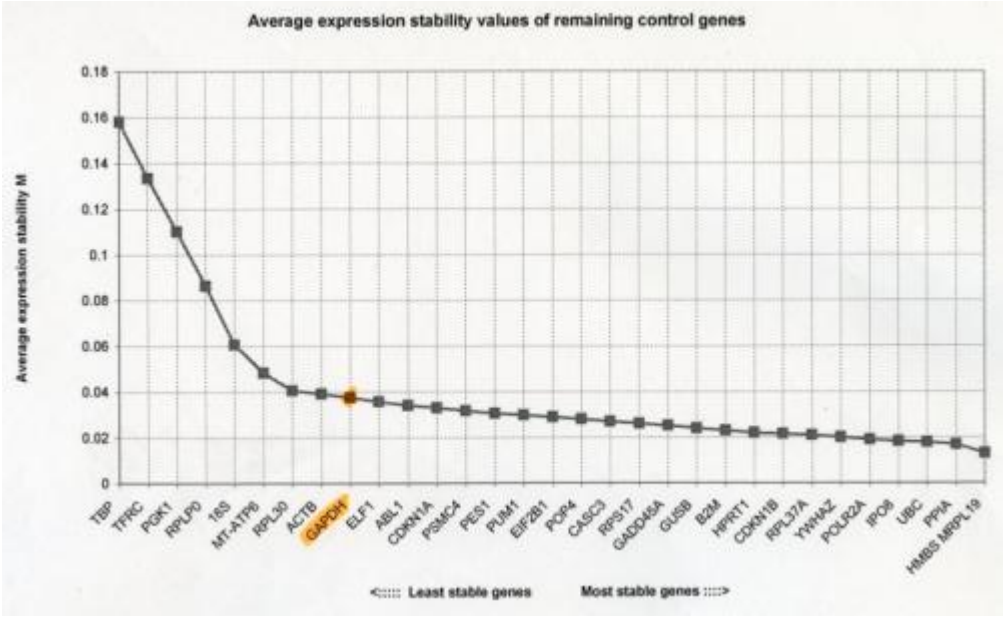


Figure 1: Average expression stability values (M) of the candidate reference genes. Average expression stability measure (M) of control genes during stepwise exclusion of least stable reference genes. M represents from the least stable (left) to the most stable (right), analysed by the geNorm programme.

The results are expressed as 'Average Expression Stability' (M). Within this system the least stable gene has the highest M value and the most stable gene has the lowest M value. To assess the validity of the established gene-stability measure, that is, that genes with the lowest M values have the most stable expression, the gene specific variation for each control gene is determined as the variation coefficient of the expression levels after normalization. This coefficient should be minimal for proper housekeeping genes. It has been proposed that M values inside the ranges $M \leq 1$, are acceptable (Hellemans et al., 2007). Interestingly 18S which has been used in a wide variety of studies in various tissues (Gorzelniak et al., 2001, Liu et al., 2005) had a relatively high value of 0.06 and expressed the 5^{th} worst gene stability. The findings

confirmed that with an M value of 0.035, GAPDH was a suitable normalisation factor for the osmolarity studies in chondrocytes. The majority of studies quantifying gene expression in connective tissues also use GAPDH as the reference gene (Ayers et al., 2007).

Despite many qRT-PCR studies having reported the use of a single endogenous control gene (Suzuki et al., 2000), a normalisation strategy based on a single housekeeping gene may lead to erogenous errors (Vandesompele et al., 2002, Tricarico et al., 2002). Hence the use of a panel of reference genes has been proposed (Vandesompele et al., 2002, Thellin et al., 1999). The geometric mean of multiple carefully selected housekeeping genes would then be calculated. However as many gene expression studies in cartilage osmolarity studies have used GAPDH as a single reference gene (Tew et al., 2009, Hung et al., 2003, Palmer et al., 2001) and following the validation using the endogenous control plate of GAPDH in this thesis GAPDH was used as a single endogenous control gene for this study.

Appendix 2

Equine SOX9 vector production

Determination of mRNA half-life is important to our understanding of gene expression and mechanisms involved in the regulation of the level of transcripts in response to environmental changes. In addition, the stability of mRNA may determine how rapidly the synthesis of the encoded protein can be shut down after transcription ceases (Yang et al., 2003). One method of determining the stability of mRNA is through RNA samples removed from cells treated with transcriptional inhibitors such as actinomycin D (Sobell, 1985). In order to determine the half-life of SOX9 mRNA in the horse, a plasmid vector containing equine SOX9 sequence was produced. This was to produce a calibration curve from known dilutions using qRT-PCR in order to calculate SOX9 mRNA copy number. Copy numbers could then be normalised to input RNA concentrations.

Method

Amplification and purification of the DNA sequence to be cloned was undertaken in order to produce a DNA fragment (for insertion into a plasmid) of equine SOX9. Three donors were initially used. First strand cDNA was synthesized as previously described (Tew et al., 2009). In brief, M-MLV reverse transcriptase and random hexamer oligonucleotides were used to synthesize cDNA from RNA obtained from normal equine chondrocytes (Promega, Southampton, UK). A region of the SOX9 within the coding regions of exon 1 and exon2 between 99bp and 157bp (accession number AF322898) was amplified using the following equine SOX9 primers; forward; CGCCGAGCTCAGCAAGA, reverse; CGCTTCTCGCTCTCGTTCA

(Eurogentec, Hampshire, UK) using Taq DNA Polymerase (Sigma-Aldrich, Dorset, UK).

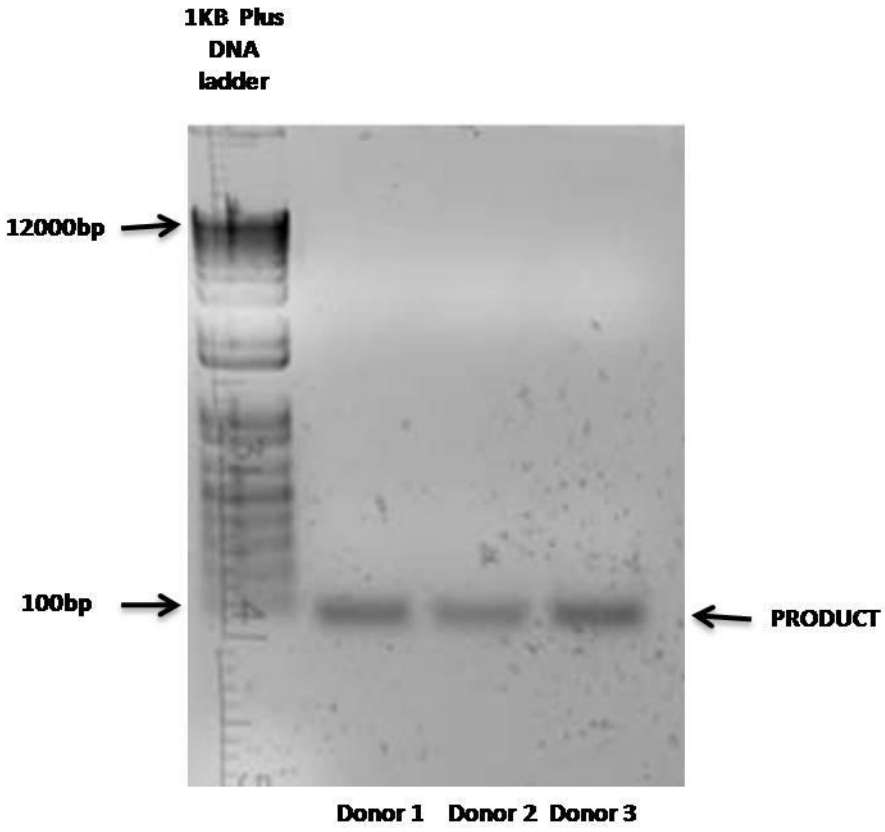
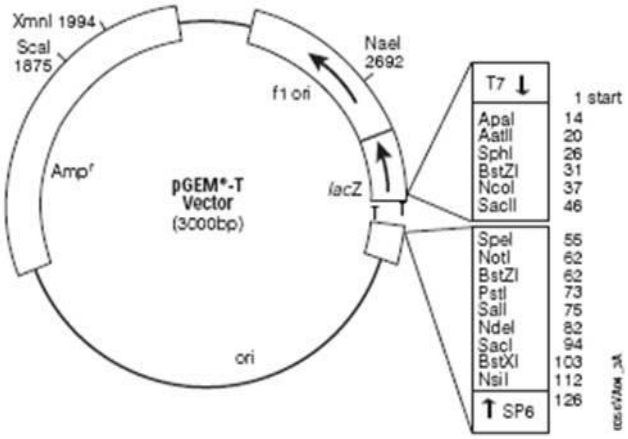


Figure 1: 1% agarose mini gel showing amplification products

The PCR products produced were then examined using agarose gel electrophoresis. Bands of the correct expected product size (58 bp) were found and were excised from the gel (Figure 1). DNA was purified from the gel using a Qiagen QIAquick Gel Extraction Kit (Qiagen LTD, Sussex, UK) and eluted in 30μl of Qiagen EB Buffer. 10 μl of this DNA solution was then ligated into the pGEM®-T Easy Vector (Promega, Southampton, UK) using T4 DNA Ligase (Promega) overnight at 4^{o}C. The ligation product was then transformed into One Shot®TOP10 Chemically Competent E.Coli (from Invitrogen, Paisley, UK) following the manufacturer's procedures. Transformed cells were mixed with Isopropyl β-D-1-

thiogalactopyranoside (IPTG) and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) (Sigma-Aldrich, Dorset, UK), applied onto LB agar plates containing 50μg/ml ampicillin (Sigma-Aldrich, Dorset, UK), and incubated overnight at 37°C. Blue-white screening was used to detect the successful ligations and a number of white colonies were selected. Each colony selected was incubated for overnight at 37°C in a Stuart Orbital Incubator 5150 in 3ml LB broth starter culture (Sigma-Aldrich, Dorset, UK) containing 3μl of ampicillin. Plasmid DNA was purified from the cultures using a Qiagen QIAprep Spin Miniprep Kit Qiagen LTD, Sussex, UK). Further investigations of the resulting purified colonies were performed by digesting the plasmid DNA with the EcoR1 restriction enzyme (Roche Hertfordshire, UK). Webcutter (www.rna.lundberg.gu.se/cutter2) was used to ascertain that EcoR1 did not cut through the projected sequence so should only cut within the vector, either side of the insert.

5.B. pGEM®-T Vector Map and Sequence Reference Points



pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

Note: Inserts can be sequenced using the SP6 Promoter Primer (Cat.# Q5011), T7 Promoter Primer (Cat.# Q5021), pUC/M13 Forward Primer (Cat.# Q5601), or pUC/M13 Reverse Primer (Cat.# Q5421).

Figure 2: pGEM-T vector map and reference points

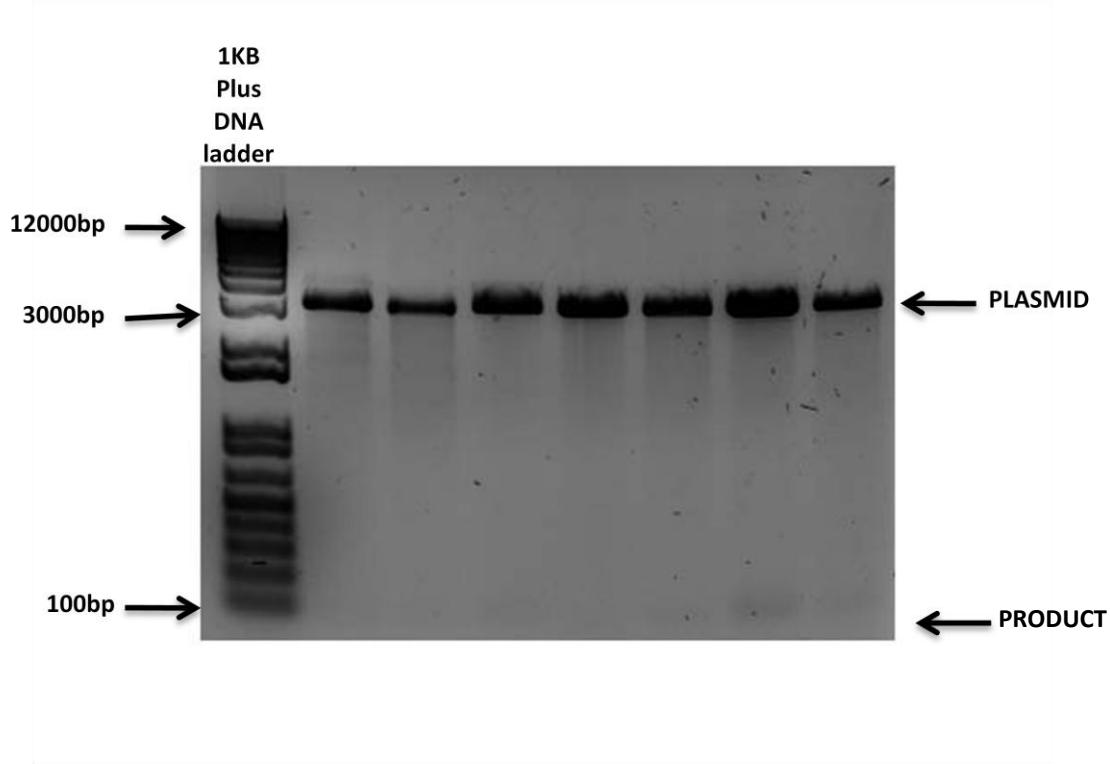


Figure 3: 1% agarose mini gel following EcoR1 restriction

Digests were examined on 1% agarose gels (Figure 3). Product was identified at just below 100bp. This correlated with 58bp from the SOX9 sequence and additionally a few base pairs either side of the sequence which would correlate to the site of restriction by digestion by Eco R1.Samples of the purified plasmid containing the required sequence of SOX9 were sent to Macrogen, Korea for sequencing with T7 promoter primer, designed for sequencing inserts cloned in the pGEM®-T Easy Vector (Promega, Southampton, UK). Sequence profiles returned from Macrogen were run through ClustalW (a general purpose multiple sequence alignment program for DNA or proteins (www.ebi.ac.uk/clusatlw/)) in order to identify the required sequence. Samples containing the SOX9 sequence in the correct orientation, between the 2 primers described previously here, were identified. A standard curve was created using the pGEM-T Easy-equine SOX9 identified as containing the SOX9 sequence in the correct orientation. Here LOG_{10} of copy number was plotted against CT; R^2 was 0.9859. In order to calculate the half life of equine SOX9 mRNA in

decay studies copy numbers were normalised to input RNA concentrations. However in the experiments undertaken in 12 well plates the RNA concentrations in freshly isolated cultures were consistently too low when measured using a NanoDrop 8000 spectrophotometer, to allow the use of this method. Therefore in order to calculate the half life experiments were undertaken using GAPDH as the non-target message normalization factor. The use of GAPDH as a normalisation factor has been validated in a wide variety of decay studies (Morris et al., 2008, Laroia et al., 2002).

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