BMP-7: Role and Regulation in Osteoarthritis.

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Abstract

Osteoarthritis (OA) is a disease characterised by changes in the structure and function of articular joints, leading to pain and loss of mobility. Bone Morphogenetic protein 7 (BMP-7), a member of the transforming growth factor ß superfamily, has been shown to promote anabolic events within articular cartilage, and confer protection from OA associated destruction in a number of animal models. It has been shown that a disease-associated loss of BMP-7 in OA may contribute to the joint destruction. The mechanism associated with the loss of BMP-7 has yet to be fully elucidated. Recently, small non-coding RNAs (microRNAs) that participate in post-transcriptional gene regulation, have been identified as a potential dysregulated mechanisms in OA. It was therefore hypothesised that disease-associated alterations in these microRNAs could lead to subsequent changes in the expression of BMP-7 and its signalling family. The aims of this thesis were to investigate the expression of BMP-7 and other associated BMP signalling molecules and identify any microRNAs that may regulate these transcripts. Furthermore, the study aimed to elucidate the molecular mechanisms by which BMP-7 is able to confer protection in cartilage.

The studies presented in this thesis show that both articular chondrocytes and the synovial membrane can express very low levels of BMP-7 transcript in a subset of patients. In juxtaposition, protein can be clearly detected in both articular chondrocytes and synovial membrane. Interrogation of the BMP-7 signalling family transcripts revealed that all members are detectable in OA cartilage. This expression was independent of the eroded nature of the cartilage. Evaluation of the circulating microRNAs that were predicted to target the BMP-7 pathway revealed that several miRNAs (including miR24-3p) were altered in the plasma of OA patients. Interestingly, miR24-3p was able to target BMP receptors ALK2 and BMPR1B. Moreover, there was a significant negative correlation between the expression of miR24-3p and the ALK2 receptor in OA patients. Thus suggesting there is a role for this miRNA in the negative regulation of BMP-7 signalling in OA cartilage.

To complement the work evaluating the endogenous signalling pathway, studies were also undertaken to investigate the impact of exogenous BMP-7 stimulation on chondrocytes. BMP-7 was able to promote its own transcriptional expression in a patient specific manner and induce expression of IL-1ß in all of the donors investigated. In addition to the induction of IL-1ß, BMP-7 was also able to up-regulated the IL-1ß antagonist, IL-1Ra. Taken together this data suggests a role for BMP-7 in the regulation of the inflammatory mediator IL-1ß. Finally, BMP-7 was able to up-regulate several pro-inflammatory cytokines and chemokines in both primary OA chondrocytes and *in vitro* differentiated macrophages.

In summation, the work presented in this thesis suggests that BMP-7 may be contributing to the promotion of inflammation and subsequent repair as part of the cartilage homeostatic mechanisms. Further to this, miR24 has been highlighted as a regulator of cartilage homeostasis via the direct targeting of ALK2. Changes in the expression of this miRNA over the course of OA disease progression may be involved in driving disease pathogenesis. Therefore understanding the targets for this disease-associated miRNA may help in the development of disease modifying therapies.

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Author's Declaration

I declare that this thesis is the result of my own work. No part of this thesis has been submitted for any other degree at The University of Glasgow, or any other institution.

Louise Agnes Bennett

Abbreviations

A	ACLT	Anterior Cruciate Ligament Transection
	ACR	American College of Rheumatology
	AGO2	Argonaute 2
	ALK	Activin Like Kinase
	ARE	AU Rich Element
	AS	Ankylosing Spondylytis
B	BMI	Body Mass Index
	BMP	Bone Morphogenetic Protein
	BMPR	Bone Morphogenetic Protein Receptor
	BRF1	Butyrate Response Factor 1
	BSA	Bovine Serum Albumin
C	cDNA	Complementary DeoxyriboNeucleic Acid
	CM	Control Mimic
	COMP	Cartilage Oligomeric Matrix Protein
	CT	Cycle Threshold
	CVD	CardioVascular Disease
D	DAMPS	Damage-Associated Molecular Patterns
	DGCR8	Digeorge Syndrome Critical Region Gene 8
	DLC	Distal Lateral Condyle
	DMC	Distal Medial Condyle
	DMEM	Dulbecco's Modified Eagle Medium
	DMM	Destabilisation of the Medial Meniscus
	DNA	DeoxyriboNeucleic Acid
	DPP	Decapentaplegic
E	ECM	ExtraCellular Matrix
	EDTA	EthyleneDiamineTetraacetic Acid
	ELISA	Enzyme-Linked Immunosorbent Assay
	EMT	Epithelial Mesenchymal Transition
	EULAR	European League Against Rheumatism

F	FBS	Foetal Bovine Serum
	FGF-18	Fibroblast Growth Factor-18
	FLS	Fibroblast-Like Synoviocytes
	FOX	FOrkhead Box
	FST	Follistatin
G	GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
	GBB	Glass Bottom Boat
	GDF	Growth Differentiation Factor
	GREM	Gremlin
H	HA	Hyaluronic Acid
	HAS	Hyaluronic Acid Synthase
	HEK	Human Embryonic Kidney
	HRP	Horse Radish Peroxidase
	Hr	Hour
I	ID4	Inhibitor of Differentiation 4
	IGFBP-5	Insulin Like Growth Factor Binding Protein 5
	IGF1	Insulin Like Growth Factor 1
	IHC	Immunohisto Chemistry
	IL-1β	InterLeukin 1 - β
	IL-1Rα	InterLeukin-1 Receptor Antagonist
	IL-1R	InterLeukin-1 Receptor
	IL-4	InterLeukin-4
	IL-6	InterLeukin-6
	IL-8	InterLeukin-8
	IL-10	InterLeukin-10
	IL-11	InterLeukin-11
	IL-13	InterLeukin-13
	IL-17	InterLeukin-17
	IL-18	InterLeukin-18
	IL-21	InterLeukin-21
	IPFP	InfraPatellar Fat Pad

K	KDa	KiloDalton
	KO	Knock Out
L	LncRNA	Long non-coding RiboNucleicAcid
	LTP	Lateral Tibial Plateau
M	MAPK	Mitogen Activated Protein Kinase
	MCP-1	Monocyte Chemotactic Protein 1
	miRNA/miR	Micro RiboNucleic Acid
	Min	Minute
	MMP	Matrix MetalloProteinase
	MRE	MicroRNA Response Element
	mRNA	Messenger RiboNucleic Acid
	MSC	Mesenchymal Stem Cell
	MTP	Medial Tibial Plateau
N	NCBI	National Centre for Biotechnology Information
	ncRNA	Non coding RiboNucleic Acid
	NGF	Nerve Growth Factor
	NHS	National Health Service
	NICE	National Institute of Health and Care Excellence
	NO	Nitric Oxide
	NTC	Non Template Control
O	OA	OsteoArthritis
P	PBMC	Peripheral Blood Mononuclear Cell
	PBS	Phosphate Buffered Saline
	PCR	Polymerase Chain Reaction
	PDGF	Platelet Derived Growth Factor
	PLC	Posterior Lateral Condyle
	PMC	Posterior Medial Condyle
	PPARA	Peroxisome Proliferator-Activated Receptor Alpha
	PsA	Psoriatic Arthritis

	P/S	Penicillin/Streptomycin
Q	qPCR	Quantitative Polymerase Chain Reaction
R	RA	Rheumtoid Arthritis
	RACE	Rapid Amplification of cDNA Ends
	RIPA	RadioImmunoPrecipitation Assay Buffer
	RISC	RNA Induced Silencing Complex
	RNA	RiboNucleic Acid
	RPMI	Roswell Park Memorial Institute Medium
	RT	Room Temperature
S	SASP	Senescene-Associated Secretory Phenotype
	SD	Standard Deviation
	SF	Synovial Fluid
	SLE	Systemic Lupus Erythematosus
	SMADS	SMA/Mother Against Decapentaplegic
	SMC	Smooth Muscle Cell
	SNP	Single Nucleotide Polymorphisms
T	TAE	Tris –base Acetic Acid
	TBST	Tris Buffered Saline and Tween
	TGFß	Transforming Growth Factor ß
	TKR	Total Knee Replacement
	TLR	Toll Like Receptor
	TMB	TretraMethylBenzidine
	TNFα	Tumour Necrosis Factor α
	Trb3	Tribbles –Like Protein 3
	TTP	TrisTetraProline
U	UKR	Unilateral Knee replacement
	UNG	Uracil N-Glycosylase
	USAGI	Uterine Sensitisation–Assocaited Gene-1
	UTR	Untranslated region

V	VEGF	Vascular Endothelial Growth Factor
	vSMC	Vascular Smooth Muscle Cell
W	WB	Western Blotting
	WOMAC	Western Ontario and McMaster universities Arthritis Index
	24M	miR24 3p Mimic
	342M	miR342 3p Mimic

1 Introduction

1.1 Osteoarthritis

1.1.1 General Overview

Osteoarthritis (OA) is the most common form of arthritis (2,3) with hip and knee OA being one of the leading causes of global disability (3). Arthritis Research UK estimate that at least one in six people are affected by the disease and the American Arthritis Society state that there are more than 10 million people suffering from OA in the USA. This disease imposes a colossal strain on the government due to reduced mobility, which can affect an individual's ability to work. This effect can be quantified as loss of economic production annually, where in the UK is it estimated that due to OA the loss is over £3.2 billion. The National Health Service (NHS) also suffers economically with the prescription of non-steroidal, ant-inflammatory drugs and surgeries relating to OA contributing an estimated £900 million to the national budget annually. The strain on the NHS is not limited to finance, as there is also strain on staff and resources from the increasing numbers needing treatment and care for OA. This, in part, can be attributed to the aging population of the UK and to the increasing problem of global obesity. In 1990, the median age of the population was 36 years (with half the population being below and half being above this age). This number has been increasing and it is currently estimated that by 2035 the median age will be 42 years (Office of National Statistics, 2012). It can be theorised that the number of OA sufferers will only increase in accordance with the aging population. Obesity is a major health problem across the UK. It is estimated that across Scotland, England, Ireland and Wales 58 -65% of the population are overweight and of these 22-28% are clinically obese(5). Thus with this current obesity level and the aging population the prevalence of this disease is likely set to increase and as a result will become an unmanageable social and economic burden. Therefore increased research and better treatment strategies are needed in the management of the disease along with new therapies that can prevent or delay the need for surgical intervention and potentially reduce the disability caused by this disease.

1.1.2 Articulating and Non Articulating Joints.

The human body has 360 joints which can be classified into three types; ones that cannot move at all- synarthroses (i.e. the joints in the skull), joints that have a small range of motion amphiarthroses (i.e. vertebral discs) and joints that can be moved freely with a wide range of motion- diarthroses (i.e. the hip). The variation in the movement of each classification of joint can be attributed to the surrounding tissue structure; fixed joints are fibrous, joints with a limited movement are cartilaginous and joints with the most motion are synovial. Synovial joints include the shoulder, elbow, hip and knee. The elbow and the knee are examples of a hinge joint (can move primarily in one plane) whereas the shoulder and hip are ball and socket joints (can move in more than one plane). All of these joints were designed to allow effortless, pain free movement.

According to the National Institute of Arthritis and Musculoskeletal and Skin Disease the knee is one of the major weight bearing joints within the body and is the most common site of OA. It consists of two bones, the femur and the tibia. At the lower end of the femur there are 2 rounded protrusions of bone known as condyles. The inner is known as the medial condyle and the outer is the lateral condyle. The condyles of the femur rest on the tibial plateau and aid the articulation of the joint. When the leg is in extension, it is the distal sections of the femoral condyles that make contact with the tibial plateau, whereas in flexion it is the posterior femoral condyles that make contact. The condyles are covered in cartilage, a substance made of extracellular matrix (ECM) that facilitates articulation of the bones during movement. Other components of the joint, such as the synovial fluid along with the menisci, absorb shock and help to facilitate movement- distributing load evenly throughout the joint.

1.1.2.1 Cartilage

Articular cartilage is a specialised tissue that covers the surfaces of bones that form joints. The main function of the cartilage is to facilitate the movement and to distribute load, enabling the joint to withstand extreme biomechanical stress on a daily basis. The cartilage covering bones is commonly 2-4 mm thick and is composed of a specific type of cartilage, hyaline cartilage. This tissue is unique

in that it has no blood vessels, nerves or lymphatics and has only one cell type present, chondrocytes (section 1.1.2.2). The ECM makes up the majority of the cartilage (Section 1.1.2.3), comprising collagens and proteoglycans, along with some other molecules such as non-collagenous proteins and glycoproteins.

1.1.2.2 Chondrocytes

Chondrocytes are the cellular component of the cartilage and are the only cells resident in the tissue. They function to maintain the ECM via secretion of anabolic and catabolic factors within their vicinity and account for about 1-2% of the cartilage volume (8). Chondrogenesis is the differentiation of Chondrocytes from precursor mesenchymal stem cells (6). Following on from chondrogenesis the chondrocytes can either remain in a resting state in the articular cartilage where they maintain the ECM or they go through chondrocyte maturation, which occurs over several steps and results in new bone formation at the endochondral growth plate. Firstly, mesenchymal stem cell differentiate into slowly proliferating periarticular chondrocytes and then into fast proliferating, column chondrocyte (10) both of which can produce type II collagen. If the cells remain in the slowly proliferating state they will be articular chondrocytes. These steps are thought to be under the control of the transcription factor Sox5/6/9. From here, non articular chondrocytes exit the cell cycle and become hypertrophic and begin to produce the less structurally useful type X collagen and matrix metalloproteinases (MMPs) which break down the matrix. Vascular endothelial growth factor (VEGF) which promotes angiogenesis is also produced at this point. Through these factors the hypertrophic chondrocytes help to facilitate the formation of new bone before becoming apoptotic (12,13). Chondrocyte apoptosis is essential in endochondral bone formation, as when these cells die, osteoblasts (bone forming cells) occupy the space that is left behind and begin to lay down new bone(10,14).

1.1.2.3 The Extracellular Matrix Composition

The three major components of the ECM are water, collagen and proteoglycan. Water accounts for 80% of the total cartilage wet weight (6). The abundance of water from the deep layer to the superficial layer increases from 65% to around 80% (17). Water does not move easily though the cartilage and requires either a

pressure gradient across the tissue or compression of the matrix to facilitate this movement. Through this resistance to water flow and resultant pressure of water in the matrix, the articular cartilage is able to resist biomechanical stress greater than that of individuals own body weight. There are 7 collagens known to be present in the cartilage, however, it is type II collagen that accounts for 95% of the ECM collagen. The other collagens (types I, IV, V, VI, IX and XI) make up the remaining 5%. Collagen forms a triple helix structure that provides the sheer and tensile properties of this tissue. Proteoglycans make around 10-15% of the wet weight of the cartilage and include the macromolecules, aggrecan, decorin, biglycan and fibromodulin. They are all protein monomers that have been heavily glycosylated, with the largest of the group being aggrecan. Specifically the proteoglycan aggrecan can be found in the spaces between the collagen fibrils and is responsible for the osmotic properties that aid the cartilage in withstanding compressive forces (6).

1.1.2.4 The Extracellular Matrix Structure and Organisation

The cartilage is composed of 4 zones: the calcified zone directly next to the subchondral bone, the deep zone, the middle zone and the superficial tangential zone at the surface of the tissue (6). The size and structural arrangement of the zones has been shown to be significant in the functional properties of the cartilage (8). The superficial tangential zone primarily protects the deeper zones from sheer stress and makes up between 10 -20% of the cartilage. The cells of the superficial zone have been shown to produce less collagen and proteoglycans than the cells of the middle and deep zones, however, the ECM that they do produce tends to be collagen based rather than proteoglycan (8). The superficial zone also has the highest concentrations of water and fibronectin, aiding its ability to resist sheer and compressive forces. The chondrocytes in this zone are flattened and parallel to the articular surface. In this way they are in line with the tightly packed collagen fibres that are mainly composed of type II and type IX collagen.

The middle zone is the intermediate between the deep and superficial zones and contains higher concentrations of proteoglycans and the collagen fibres are also denser. The chondrocytes in this zone are sparse compared to the superficial layer and are more spherical in shape. They also possess a greater capacity to

produce ECM macromolecules as they have increased numbers of protein processing organelles such as golgi apparatus and endoplasmic reticulum. The fibrils produced in this zone are thicker than those of the superficial zone.

The deep zone gives the most protection from compressive forces, facilitated by arranging the very thick collagen fibrils in a perpendicular manner to the articular surface. This zone has the highest proteoglycan concentration and the lowest water concentration aiding its protective properties from compressive forces. The chondrocytes remain spherical in shape but begin to form columns. An anatomical feature called the tidemark separates the deep zone from the calcified zone. The calcified zone acts to secure the cartilage onto the subchondral bone. There are few chondrocytes in this zone and those that are present are hypertrophic and aid the formation of new bone.

In addition to the zonal classification of cartilage, the ECM in each zone can be further classified into regions. This is based on the matrix composition, organisation, proximity of the matrix to the chondrocytes and diameter of the fibrils. These three regions are the pericellular matrix, the territorial matrix and the interterritorial matrix.

The pericellular matrix binds to the chondrocytes and is predominantly made from proteoglycans and non-collagenous matrix proteins. It is thought that this matrix may play a role in signal transduction during load bearing episodes (6). Chondrocytes can produce other factors that are important in the organisation of the pericellular matrix by facilitating extracellular binding of macromolecules to the chondrocytes, such as glycoprotein CD44 (19). It has also been shown that CD44 can also act as a receptor for hyaluronic acid which acts as a scaffold allowing aggrecan aggregates to form, furthermore its receptor, CD44, is involved in anchoring these aggregates to the chondrocytes (20-22).

The territorial matrix, composed of thin collagen fibrils, surrounds chondrocytes and the pericellular matrix . It has been suggested that the territorial matrix provides protection from mechanical forces and may also add to the overall load bearing capacity of the cartilage. In the deep zone, where the chondrocytes can be found in columns, the territorial matrix surrounds the whole column instead of the individual cells. The interterritorial matrix accounts for the majority of

the ECM and is composed of the largest collagen fibrils. In the superficial zone the fibrils are arrange parallel to the articular surface whereas the arrangement is diagonal in the middle zone and perpendicular to the articular surface in the deep zone.

1.1.2.5 Synovium

The synovium is a soft tissue that lines the spaces of the diarthrodial joints (23). It is made up of 2 sections, the intimal lining layer and a subintima layer (23). In health the intimal lining layer is comprised of fibroblast like synoviocytes (FLS) and is generally 1-2 cells thick and therefore is regarded as a relatively low cellular abundance tissue(23). The subintima or sublining layer contains mainly blood vessels, adipose cells, FLS and macrophages. The synovium is thought to be a main source of chondrocyte nutrition providing essential molecules such as glucose (25). A vast proportion of the articular surface lies within 50mm of the synovium, thus allowing nutrients from the blood vessel in the synovium to pass to the chondrocytes within the matrix via the synovial fluid. With regards to the areas of cartilage that are not in close contact with the synovium, movement of the fluid caused by articulation of the joint will allow the nutrients to reach these areas (23). The synovial fibroblast appears to be the main source of hyaluronan, which further to its role in aggrecan aggregation outlined previously (section 1.1.2.2), can also inhibit adhesion of the synovial surface by retaining the fluid volume in the joint (28).

1.1.2.6 Synovial Fluid

Synovial fluid (SF) is an essential part of the joint, facilitating 'frictionless' movement of the bones. Human synovial fluid was first analysed by Ropes, Rossmeisl and Bauer in 1940. From their study, it was concluded that the synovial fluid was a relatively cell-poor, clear/pale yellow and viscous liquid (29). They also reported that the most abundant cell present was the mononuclear phagocyte and that albumin and globulin were also present in the fluid (29). Our understanding of synovial fluid composition has become more comprehensive over time. It is now evident that most of the same proteins found in plasma are present in SF, including glucose and uric acid. Furthermore, additional proteins that are known to help with lubrication of the joint include,

proteoglycan and hyaluronic acid (which as previously mentioned can be secreted by the synovium) are also found in the S.F (32). In disease the balance of the S.F constituents can alter; for example the presence of lactic acid can be used to diagnose septic arthritis. Similarly lactate dehydrogenase can be used as an indicator of rheumatoid arthritis, gout or infectious arthritis. Rheumatoid factor, an antibody to immunoglobulins within the joint, is present in the synovial fluid of more than half of the patients diagnosed with RA (33). However, currently there are no OA specific alterations in the S.F.

1.1.3 OA Disease Classification

There are no current identifiable biomarkers for OA diagnosis or classification. Generally symptoms of OA, for example joint stiffness and pain, are commonly the first diagnosable signs of disease (34). However, by this point irreversible damage has often already occurred in the joint, as disease onset is likely to considerably predate the onset of these symptoms.

To date there are only two real classifications of OA: primary and secondary. Secondary OA, as the name suggests, is a secondary consequence of an initial trauma or primary disease leading to OA. Here the aetiology of the disease can normally be ascertained, due to a specific event that generally results in mechanical distortion of the joint and subsequent erosion. Unlike secondary OA the aetiology of primary OA remains elusive. In the hand, primary OA has been further subcategorised depending on the joints affected and the damage associated: nodal OA, erosive OA and inflammatory erosive OA. When the disease manifests as nodes over the interphalangeal joints of the hand it is referred to as nodal OA, however if there is erosion of the interphalangeal joints this is termed erosive OA. Finally erosive inflammatory OA has characteristic inflammatory flares and erosion of the joint (36). Therefore, these subcategories encompass a substantial range of disease phenotypes and hence provide no insight into how to stratify patients for treatments. Despite these subclassifications for hand OA, there is still very limited classification of primary OA in the larger joints.

1.1.4 OA Disease Pathogenesis

Osteoarthritis is the most common form of arthritis with disease incidence on the rise. It has long been considered a degenerative disease of the joint associated with the elderly and very limited treatment options. Arthritis Research UK states that there are currently 8.75 million people in the UK seeking or having sought treatment for OA (38) with over half of these cases relating to the knee (38). Furthermore, as previously stated OA has a substantial impact on the UK economy as 2.36 million of these individuals are of working age. This problem is expected to grow as it is estimated that by 2020 the disease incidence will hit 6.5 million with knee OA alone (38).

OA is a heterogeneous disease and is now being recognised as a group of diseases, all with a common manifestation of symptoms (44,45). Historically, due to a lack of knowledge, OA has been considered a non-inflammatory, mechanical disorder associated with age, thus giving rise to the 'wear and tear' theory of disease progression. Over the past decade this has changed and there has been considerable effort to better understand and define this disease, leading to an acceptance of the role of inflammation in the disease process.

OA is characterised by joint destruction and remodelling which is a manifestation of cartilage degradation, osteophyte formation and bone remodelling (47) (Figure 1.1). It is now also recognised that synovitis also plays a role in the disease process (48,49)(Figure 1.1). This destruction, remodelling and inflammation is thought to develop over many years, with several risk factors including, age, obesity, trauma and genetic susceptibility (51) being attributed to the etiology of the disease.

1.1.4.1 Bone remodelling in OA

In 1986 Rose and Radin suggested that subchondral bone changes initiate cartilage damage (54), thus being one of the first to highlight bone remodelling as one of the earliest features of the osteoarthritic joint (55,56). These changes were highlighted in the Dunkin-Hartley strain of guinea pig that spontaneously develop OA in a fashion that is biochemically, histologically and radiographically similar to that of humans (55). These guinea pigs had significant increased bone

density and subchondral bone thickness at both 24 and 36 weeks when compared to the non-OA control (Bristol strain 2 Guinea pigs). It was also noted that these changes in the bone preceded any changes in the cartilage in the Dunkin-Hartley guinea pigs (55). Similar remodelling of the bone has also been noted in human studies. Using macroradiography thickening of the subchondral cortical plate and the subadjacent horizontal trabeculae as been observed prior to joint space narrowing in the hand (57).

Osteophytes, another feature of bone remodelling in OA, are fibrocartilage-capped bony outgrowths. There are three types of osteophyte; two which are associated with tendon and ligament insertion sites and a third which is found in OA, the osteochondrophye, and is commonly located at the junction between the bone and the cartilage(58) . Osteophytes have been identified as being chonrdocytic in origin expressing chondrocyte specific transcription factors SOX-9 and RUNX2 (59). The development of osteophytes in mice can be seen in as little as 7 days post OA inductive surgery (59) and in humans is often seen before any radiographic evidence of joint space narrowing (58). Osteophytes can have severe clinical impact and can be a primary cause of pain or restricted movement, as has been highlighted in a study looking at osteophyte formation at the medial tibial condyle in knee OA (60). It should be noted that for some individuals the presence of osteophytes does not cause pain or impingement of the joint (58) and for others they have been reported to improve symptoms by increasing the surface area of the articulating joint and spreading the load through the joint more evenly (58).

1.1.4.2 Cartilage degradation in OA

During the disease process chondrocytes attempt to repair damaged cartilage (caused by an initial insult or stimuli in the case of secondary OA) by up-regulating anabolic processes that will increase ECM synthesis. However, the cells will also begin to form clusters and differentiate into hypertrophic chondrocytes due to the damage. The hypertrophic cells can produce MMP-13 consequently adding to the destruction of the cartilage and further stimulating catabolic responses from the chondrocytes. At this point, the cartilage can become fully disrupted and the chondrocytes become apoptotic (53). Degradation of the cartilage can occur not only via the up-regulation of matrix

degrading enzymes such as MMPs, but also A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS). These mediators are able to breakdown matrix macromolecules, enabling turnover of the ECM, which in a homeostatic environment is of great importance. In disease, however, this is not the case: the matrix is not replenished at the same rate it is being broken down and this ultimately leads to the ECM structure becoming weak and disorganized, aiding erosion. Fragments of the cartilage may also be lost into the synovial fluid of the joint where they may activate immune cells, causing an unwanted inflammatory reaction that can perpetuate disease (62) (1.1.7). MMPs have been shown to be a major contributor to cartilage erosion. MMP-3 knock out (KO) mice are protected from cartilage damage in murine models of OA and are also less likely to develop severe spontaneous OA in old age, however, it is also acknowledged that other MMPs are also important in disease development (35). MMP-13 has been highlighted as a potent degenerative enzyme in the disease process, with co-localisation of both MMP-13 and disease associated type I collagen in OA tissue (65). Piecha *et al* have shown that selective MMP-13 inhibition can prevent collagen degradation by up to 80% in human cartilage explants (67). Furthermore MMP-13 appears to be directly up-regulated via IL-1ß, a prominent pro-inflammatory cytokine found in OA (1).

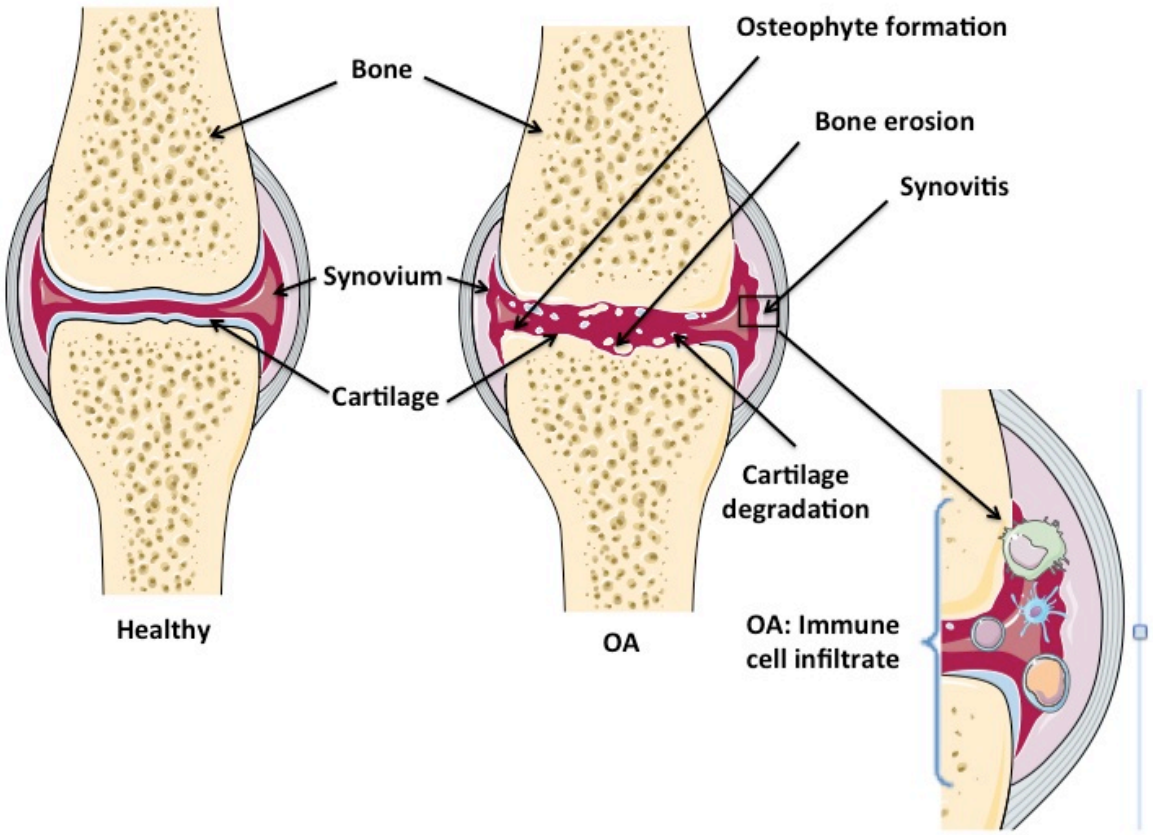


Figure 1.1 General Joint Changes Observed in OA.
Comparison of healthy and OA joint highlighting the anatomical changes in OA pathology that contribute to symptomatic disease; cartilage degradation, bone erosion, synovitis and osteophyte formation.

1.1.5 Pain in OA

Pain is one of the primary symptoms that result in disability in OA, yet there is commonly little correlation between the severity of joint pain experienced and the radiographic evidence of structural damage (4,39). Generally pain is regarded as beneficial, as when we sense pain the action that is responsible for this can be terminated. However, if a more dramatic injury or chronic inflammation occurs, then this will not only produce pain through the local stimulation of the nociceptors but also peripheral stimulation outwith the affected area. The peripheral stimulation can result in innocuous stimuli such as walking or standing resulting in pain, as is seen in OA. A release of inflammatory mediators is associated with more extensive damage that may account for the peripheral sensitisation and may also help to explain why pain does not always remain localised in those suffering from OA (4). Pain studies suggest that the source of pain can be of synovial and/or bone origin (4), as even though cartilage erosion is one of the key characteristics of OA, the cartilage is not innervated and is therefore not likely to be a source of pain.

Nerve growth factor (NGF) is a protein that has been associated with increased pain in OA. It has been found that those with OA have increased expression of NGF in the synovial fibroblasts and to a lesser extent the synovial macrophages (6). Additionally, chondrocytes have also been shown to up-regulate NGF upon stimulation with the prominent OA cytokine IL-1β and upon mechanical stress (9). NGF has been implicated in the sensitisation of peripheral neurons in a disease setting, thus lowering the pain threshold in those with OA (11). A proof-of-concept clinical trial has been conducted showing that by blocking NGF with a monoclonal antibody, Tanezumab, patients reported a significant reduction in pain when walking compared to the placebo (14). Therefore, it would appear that NGF is highly associated with disease and may play a prominent role in the pain sensation associated with OA.

Cytokines have also been implicated in pain, with specific focus on TNFα as a driver of peripheral pain within the joint. A positive correlation between synovial TNFα and the western Ontario and McMaster Universities Arthritis Index (WOMAC) pain measurement has been established (15). Circulating c-reactive protein and IL-6 in the blood have also been associated with worsening of knee pain over 5 years thus again highlight the potential contribution both growth factors and cytokines can have in the perpetuation of pain in OA (16).

It is now also believed that central pain, through the spinal cord and brain, may be playing a role in OA(11). It has been shown that elevated central pain can be reversed following total knee replacement (TKR). Data demonstrated that patients with OA were shown to have reduced pain thresholds when compared to a control group. However, following joint replacement, the pain threshold was returned to normal(18), thus suggesting that central pain is dependent on the presence of a peripheral stimuli in those with OA (18,77).

1.1.6 OA Comparison with Other Forms of Arthritis

Joint destruction is not an OA specific phenomenon and is key in the development and pathogenesis of many diseases such as rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic Lupus Erythematosus (SLE) and psoriatic arthritis. OA has commonly been used as a control disease to RA and other joint related autoimmune diseases, as it was considered to be non-inflammatory. Recent advances however, now suggest that this is not the case and that inflammation is involved in OA pathogenesis too. To this end, the way in which the comparison is interpreted must change, as the inflammatory phenotype in OA can no longer be ignored.

1.1.7 Inflammation in OA

Our understanding of the immune system and dysregulation in autoimmune disease is a currently expanding area, with the role of immune mediators in OA now being a more prominent focus of this field. In 1990 a study was carried out to determine if the inflammation was associated with early or moderate OA. Using biopsies from individuals with arthroscopic evidence of OA it was determined that 55% of those presented had synovitis, suggesting there may be

an inflammatory component driving OA in a subset of patients (79). The cause of OA associated inflammation remains elusive, however, some theories as to the initiation of this response have been put forward. The most accepted hypothesis has been summarised in a review by Berenbaum in 2013. Here it is suggested that cartilage fragments (created through either trauma or age related joint erosion) become dislodged and are detected as danger associated molecular patterns (DAMPs) by the synovial cells through Toll Like Receptors (TLRs). The immune system then recognises these fragments as danger signals which results in the initiation of an immune response (80) and ultimately inflammation. This inflammation leads to vasodilation and permeabilisation of blood vessels facilitating increased blood flow to the necessary area, increased immune cell infiltration and migration of monocytes and granulocytes (83).

Cytokines produced by cells at the site of injury (and also by recruited members of the immune system) normally function to initiate, maintain and terminate the immune response. They are secreted in a highly controlled manner in order to ensure that immunity is carried out in a regulated fashion. Current dogma suggests that a major aspect of cartilage destruction in OA is associated with an imbalance of catabolic and anabolic cytokines produced by chondrocytes (Table 1.1). Of these cytokines associated with OA, IL-1β is one of the most studied. IL-1β is produced as an inactive protein and is cleaved to its active form by the interleukin-1 converting enzyme, caspase-1 (85). Upon stimulation with IL-1β *in vitro,* chondrocytes have been shown to switch from normal ECM collagen production (type II) to the production of type I and type III collagen. This promotes the production of inferior pseudo-cartilage that is ineffective at withstanding load and movement (24,26). The IL-1β mediated dampening of type II collagen production is thought to be facilitated by repression of the transcription factor SOX9, which has been shown to be involved in cell-specific production of type II collagen (27). Thus, IL-1β does not only positively regulate catabolic factors, but also negatively regulates anabolic factors. Interestingly, chondrocytes that are clustered due to stress are more likely to express IL-1, cleavage enzyme caspase-1 and the IL-1 receptor (IL-1R). Additionally these cells have been reported to produce concentrations of IL-1β capable of inducing MMP expression aiding degradation (1).

IL-1β and TNFα have been implicated in the recruitment of other pro-inflammatory cells and extending the inflammatory damage in OA. They have been shown to induce both nitric oxide (NO) and prostaglandin E_2 as well as IL-6 and IL-8 (1). TNFα expression in chondrocytes is increased immediately after impact, thus may be involved in the initial inflammatory insult in the joint in secondary OA resulting from trauma (30). The role of these cytokines in OA pathogenesis is further supported by the neutralisation of both TNFα and IL-1β inhibiting the induction of other pro- inflammatory mediators from fibroblasts (31).

The synovial macrophage is considered to play a prominent role in the perpetuation of inflammation in OA (31). It has more recently been suggested that macrophages contribute to the increased level of IL-1β and TNFα that can subsequently stimulate the production of other pro-inflammatory cytokines and MMPs from synovial fibroblasts (31). If the CD14+ macrophages of the synovium are depleted, then fibroblast production of OA associated cytokines (i.e. IL-1, TNFα, IL-6 and IL-8) and monocyte chemoattractant protein 1 (MCP-1) along with MMP-1 and 3 are reduced. Further studies also report that synovial lining macrophages not only induce MMP-1 and MMP-3, but also MMP-2 and MMP-9. Moreover, the expression of these MMPs was down-regulated in a mouse model of OA when the synovial lining macrophages were depleted (35). Further to the macrophages role in perpetuating degradation and inflammatory mediators, they have also been shown to have an impact on osteophyte formation in OA. Studies have shown that if synovial lining macrophages are depleted, osteophyte size is decreased by 84% (37).

Anabolic	Catabolic	Anti-catabolic
IGF-1	IL-1β	IL-4
TGFβ 1,3	TNFα	IL-10
FGF 2,18	IL-8	IL-11
BMP 2, 7	IL-18	IL-13
PDGF	IL-6	1L-1ra
	NO	
Reference (39-42)		

Table 1.1 Overview of Anabolic, Catabolic and Anti-Catabolic factors that are Prominent in OA.

1.1.8 Angiogenesis in OA

Angiogenesis, the formation of new capillary blood vessels from the already established vascular network, is known to be essential for several processes in the body such as growth, development and tissue repair. The articular cartilage is not vascularised and is therefore a hypoxic tissue. This is most likely due to the biomechanical stress which does not make the joint the ideal location for capillary vessels (43). In a diseased environment such as an OA joint, this has been shown to change (43) with an increase in vascular networks contributing to and exacerbating the disease process. The contribution of angiogenesis to the disease process has also been noted in other disease states such as cancer, facilitating tumour growth via promotion of proliferation, and chronic inflammation, providing an entry route for increased numbers of cells to infiltrate the affected area - perpetuating the inflammatory process (46) .

There are several sites in the joint that appear to be subject to increased angiogenic activity: the synovium, osteochondral junction, articular cartilage and menisci (43). With regards to the synovium, the extent of angiogenesis has been reported to be comparable to that seen in RA (50) and positively correlates with infiltration of macrophages (52) . Macrophages are known to contribute to angiogenesis through the production of the angiogenic factor, vascular endothelial growth factor (VEGF), which promotes angiogenic activity in other cells such as fibroblasts and endothelial cells. Under normal physiological condition, chondrocytes have been shown to produce antiangiogenic factors (61), however, it is thought that OA associated inflammation may promote the expression of VEGF from the chondrocytes and hence angiogenesis. It has been shown that the vascularisation of the articular cartilage is associated with a loss of proteoglycans and a shift in collagen production towards the structurally inferior type I and X collagen (43,63). This phenotypic change in collagen and loss of aggrecan can disrupt and weaken the ECM, leaving the cartilage more permeable to neovascularisation (64).

1.1.9 Risk Factors in OA development

1.1.9.1 Mechanical Loading

OA has long been regarded as a mechanical disease that occurs due to wear and tear overtime and is therefore considered an inevitable process of ageing. The mechanical theory of OA aetiology relates to the disease being driven by movement and physical force on the joint. Mechanical stress is essential for homeostatic matrix turnover in the articular cartilage. Studies carried out in 1986 by Slowman and Brandt show that proteoglycan content is higher in areas that have been exposed to normal mechanical load compared to areas that are completely unloaded (66). However, mechanical injury has been shown to decrease the biosynthetic capacity and viability of the chondrocytes (39). It is not only the cells surrounding the area affected by the mechanical stress, as nitric oxide (NO) released from the damaged cells has been shown to cause apoptosis in cells distant from this area (39). Due to chondrocytes being sparsely scattered throughout the cartilage, a decrease in the biosynthetic ability or survival of these cells could have a profound effect on the ECM in their immediate vicinity. Furthermore increased expression of MMP-1, MMP-3, MMP-8, MMP-9 and MMP-13 have been observed in the remaining viable chondrocytes after impact, which together with a loss of anabolic capabilities could aid in the breakdown of local cartilage.

Secondary OA associated with sporting injuries is generally connected with prolonged mechanical stress. It has been suggested that injury to the knee, either to the anterior cruciate ligament or meniscus, in those under thirty, does not result in radiographic signs of OA for at least 15 years post injury. However, in those over thirty this can lead to radiographic signs of OA within 5 years of injury (68). This suggests that age related changes may contribute to OA progression in secondary OA. Furthermore the number of injured sites in the joint can also play a significant role in the development of OA. If an isolated injury is incurred in the anterior cruciate ligament then the prevalence of OA is between 0-13%. However, if the injury occurs at both the anterior cruciate ligament and the meniscus then the prevalence dramatically increases to between 21 -48% (69).

1.1.9.2 Obesity

Obesity is also thought to contribute to disease development. As weight increases, the load that runs through both the hip and knee also increases, resulting in an unmanageable load and ultimately erosion of the joint. In this way obesity has been strongly linked to OA. Studies have been conducted demonstrating that weight is the most modifiable factor that contributes to the development of OA, with weight loss correlating with a reduction in cartilage volume loss (70). A total body weight loss of 1kg has been calculated to result in a decrease of 2.2kg load through the knee (72). Therefore, there is clear evidence that weight gain can contribute to mechanical load and that weight loss may be an effective means of preventing further disease progression (73). However, it should be noted that others have suggested that modulations in weight, i.e normal Body Mass Index (BMI), to high BMI back to normal BMI, may be more detrimental than a constant high BMI. It has been suggested that the joint may adapt to cope with the increased load and that changes to this may cause more harm and increase the need for arthroplasty later in life (71).

Obesity does not only increase the load that is exerted on the joint, but may also contribute to disease at the molecular level by altering adipokines; metabolic factors produce by fat tissue (74). The infrapatellar fat pad (IPFP) in the joint capsule of the knee could contribute to the dysregulated secretion of adipokines in OA specifically related to the knee (75). It has been suggested that the IPFP may be a more potent source of adipokines than subcutaneous adipose tissue. When compared directly it has been shown that the IPFP from obese women suffering from OA produce more of the adipokine, adiponectin, when compared to the subcuanteous fat control. Additionally, another adipokine, leptin has been associated with cartilage destruction. Data shows that leptin can up-regulate MMP1 and MMP-13 and furthermore, that this up-regulation can be partially inhibited using an anti-leptin antibody (76). Thus taken together this highlights a role for the IPFP in contributing to inflammation and joint destruction OA patients (108).

1.1.9.3 Age Related Alterations

OA has been classically defined as a disease of the elderly as symptoms tend to manifest in those over 50 years of age. It has been found that OA related molecular factors may be slightly decreased in aged cartilage, however when age matched OA cartilage is examined these factors are dramatically reduced (78). Suggesting that the extreme symptoms observed in OA cannot be attributed solely to the natural process of aging, even though it is likely to be a predisposing factor that contributes to disease aetiology.

Loeser reviewed the age related changes that may contribute to the development of OA in 2012 (80). It is suggested that a decrease in general physical activity that could lead to sarcopenia (loss of skeletal muscle and strength) and increased fat mass. This may then result in muscle weakness, increased joint loading and an increase in adipokine expression from the fat cells, all of which have been linked to OA. An increase in pro-inflammatory mediator secretion is also described, which may contribute to the catabolic destruction of the joint. Furthermore an age related decrease in growth hormones and sex steroids has been linked to a decrease in anabolic factors within the joint. A study was carried out in the Netherlands looking at determinants in OA disease onset in a cohort of individuals over 90 years of age. It was shown that 16% of the participants were free from OA; attributes such as being male, having a normal BMI and an absence of familial predisposition for OA were associated with disease prevention in the aged cohort (82).

Importantly, this disease is now being regarded as a multifactorial disease with many contributing factors such as injury, age, obesity, genetics and inflammation. Therefore, as alluded to, there are several age related changes that may contribute to OA, however, they alone could not be solely responsible for pathological change.

1.1.10 Clinical assessment of OA

Assessment of OA is not easy as there are no biomarkers or tests available for diagnosis. Orthopaedic specialists have to rely on patient questionnaires, along

with x-ray and MRI scoring for diagnosis and assessment of disease. A range of these questionnaires and scoring systems are detailed below.

1.1.10.1 WOMAC

The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score is an assessment of the patients perceived pain, stiffness and physical function. In accordance with the American college of rheumatology (ACR) this questionnaire is comprised of 24 questions which the patients are asked to respond to with the following answers: none, mild, severe or extreme. The first 5 questions refer to pain, asking the individual to rate their pain when walking, using stairs, in bed, sitting and standing. Stiffness is then assessed when the patient first wakes and then later on in the day. Finally, 17 questions regarding physical function are addressed. The answers can then be culminated in an overall score to assess disease severity. One of the major draw backs of this survey is the reliance of the patient to answer the questions, therefore circumstances at the time of questionnaire completion may have an affect on the answers given.

1.1.10.2 Kellgren-Lawrence score

The Kellgren-Lawrence scoring system for diagnosis of OA from radiographic images was first proposed by Kellgren in 1957. This is a non invasive system of determining joint damage in patients with OA and is therefore the most informative tool in the diagnosis and assessment of OA. The modified scoring system is again measured on a 5 point scale (84,86):

Grade 0: No radiographic signs of OA.

Grade 1: Doubtful narrowing of joint space and possible osteophytic lipping.

Grade 2: Definite osteophytes and possible narrowing of joint space.

Grade 3: Moderate multiple osteophytes, definite narrowing of joint space and some sclerosis and possible deformity of bone ends.

Grade 4: Large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone ends.

1.1.10.3 Outerbridge Classification

The Outerbridge classification, used to assess cartilage breakdown, was first described by Outerbridge in 1961(87). This analysis of disease progression can only occur if the cartilage surface is exposed during surgery and therefore is not a useful clinical tool for disease assessment. However, it can be very informative for the *ex vivo* assessment of tissue obtained during surgery. This modified Outerbridge classification consists of a 5 point grading system (87-89);

Grade 0: Normal cartilage.

Grade I: Cartilage with softening and swelling.

Grade II: A partial-thickness defect with fissures on the surface that do not reach subchondral bone or exceed 1.5 cm in diameter

Grade III: Fissuring to the level of subchondral bone in an area with a diameter more than 1.5 cm

Grade IV: Exposed subchondral bone.

1.1.11 Treatment of OA

1.1.11.1 Analgesics

Analgesics / painkillers are currently the first line therapy for those suffering from OA, as pain is one of the most common symptoms of the disease. The National Institute of Health and Care Excellence (NICE) guidelines for OA state that OA related pain should initially be treated with over the counter analgesics such as paracetamol. If this is not sufficient to relieve pain, then it is suggested non steroidal anti-inflammatories such as ibuprofen, or selective COX2 inhibitors such as celecobix, should be given. If pain worsens (or is still not controlled with these measures) then drugs from the opioid family can be given. Generally, starting with co-codamol (paracetamol/codeine) and increasing to more potent formulations (i.e morphine) if the pain is deemed severe enough. These are currently the only form of medication that are prescribed for OA and can only abate symptoms for a short period of time. They are unable to halt or reverse the underlying structural damage causing pain in OA.

1.1.11.2 Exercise

The American College of Rheumatology (ACR), along with the European league Against Rheumatism (EULAR), also suggest that exercise should be prescribed in order to combat OA progression. Exercising the surrounding muscles is known to provide support to the damaged joint (90) and is also undeniably associated with weight loss. Therefore with the strong link between obesity and OA, exercise may in turn help to alleviate some of the load through the joint (91,92). Exercise prescriptions have however come under scrutiny in the past as they are often vague, leaving the patient unsure of the levels of activity they should engage in (93).

1.1.11.3 Surgery

When symptoms become severe and an individual's quality of life is significantly affected the physicians may then consider joint replacement surgery. This involves complete removal of the joint (including both eroded and non-eroded sections) and replacing it with a metal/plastic prosthesis. In the majority of cases this restores quality of life and greatly reduces pain. The prosthesis has a finite life span and generally has to be replaced after 15 -20 years. The prosthetic joint can only be revised a limited number of times, thus the total therapeutic duration for joint replacements is limited. For this reason, delaying the need for arthroplasty as long as possible is essential. Other drawbacks to the procedure include the risk of infection from the surgery and the potential that the prosthesis will not improve the symptoms of disease. Studies have shown that 10-20% of patients undergoing total knee joint replacement surgery (TKR) will be dissatisfied with the results of their surgery (94).

1.1.11.4 Stem Cell Therapy

Mesenchymal stem cells (MSC) are a potential new therapy for OA. MSCs were first described as a potential reparative mechanism in OA by Murphy *et al* in 2003 (95). The study used a combined anterior cruciate ligament transection (ACLT) and medial meniscectomy in goats to mimic OA. MSCs were injected into the intra-articular space and left for 20 weeks before the animals were sacrificed and analysed. The results were encouraging, showing regeneration of the meniscus and a reduction in OA related cartilage and bone changes (95). This

study has lead to further work being carried out with regards to the therapeutic potential of these cells in OA. As of June 2014 there have been 5 other studies looking at the effect of MSC therapy in an osteoarthritic setting. These studies have looked at a variety of species including porcine, rabbit and guinea pig using a variety of approaches to investigate disease; partial thickness cartilage defect, osteochondral defect, spontaneous OA and ACLT models. All of the studies that have been conducted using animal models of disease have reported positive outcomes and support the use of MSCs as a potential therapy for OA (96). Following on from the success of MSCs in animal models there have been several clinical trials, all of which have reported significantly improved outcomes for patients. The injections of the cells appeared to improve a variety of symptoms including pain, range of movement and walking time, along with reported cartilage and meniscus growth in some trials (110).

An alternative way of potentially utilising MSCs comes from the procedure known as micro-fracture. This technique, where small holes are made in the damaged area, releasing bone marrow (potentially containing MSCs) into the eroded area, is currently being used in order to slow down cartilage damage. The bone marrow released clots and creates a plug in the affected area. New cartilage is thought to be synthesised from the bone marrow cells (MSCs) however, the resulting new cartilage is the less resilient fibrocartilage instead of the normal hyaline cartilage. Thus, this pseudo cartilage is likely to erode faster, meaning that surgical intervention is almost inevitable. This therapy however may buy time, which maybe extremely beneficial for younger individuals who are unsuitable for joint replacement surgery because of their age.

1.1.11.5 Anabolic Agents

Anabolic therapies are urgently required for the treatment of OA as these offer a modality that will not just deal with the symptoms of OA, but will help to combat the underlying disease pathology or at least delay the degradation of the joint.

Hyaluronic acid (HA) is an anabolic agent that has been recommended by both EULAR (97) and ACR (98) for the treatment of OA under certain circumstances. It is thought that the therapeutic benefits of HA are down to several mechanisms

including increased proteoglycan synthesis and decreased protease expression, hence helping to maintain the cartilage integrity. HA has also been shown to reduce pro-inflammatory mediators and have an effect on immune cells, thus reducing overall inflammation. However, there is controversy over it's effectiveness as a treatment, with concerns over both efficacy and safety profile (99).

Growth factors are also being investigated as potential treatments for OA, such as TGFβ, BMP-7, platelet derived growth factor (PDGF), Insulin-like growth factor-1 (IGF-1), and fibroblast growth factor 18 (FGF-18) (99). To date most studies have focused on the therapeutic potential of BMP-7 (section 1.2.1) and FGF-18. It was found that FGF-18 could promote repair in damaged chondrocytes (100). Additionally, results from a randomised, double blind, placebo-controlled trial revealed that FGF-18 was able to reduce cartilage loss and prevent joint space narrowing, thus meeting the secondary end points of the trial. There were also no local or systemic safety concerns raised from the study, therefore it shows promise as a potential treatment for OA (101).

1.2 Bone Morphogenetic Protein -7

Marshall Urist was the first person to discover BMPs within the bone in 1965. Urist elegantly demonstrated that extracts from the matrix of the bone contained mediators that were capable of inducing ectopic bone formation in soft tissues of rabbit and rats (102). Later, it was more specifically discovered that BMP-7 played a role in bone development via the differentiation of bone forming osteoblasts from stem cells (143,152).

The names given to this protein, bone morphogenetic protein and osteogenic protein, are derived from where it was first discovered and therefore do not fully encompass the vast range of functions this protein is now known to have. With BMP-7 being shown to be critical in the growth, development, maintenance and repair of other organs, tissues and cells including: Kidney, eye, cartilage, vasculature, adipose tissue and stem cells (115).

1.2.1 Bone Morphogenetic Proteins

The transforming growth factor- β (TGF β) superfamily are a large family of structurally related regulatory proteins. The superfamily can be subdivided into four groups: the decapentaplegic-Vg-related subfamily (including Bone Morphogenetic Proteins (BMPs) and growth differentiation factor (GDFs), the activin/inhibin subfamily, the TGF β subfamily and the divergent member subfamily. All of the ligands form either a homodimer or a heterodimer complex, initiating signalling via activation of heterodimeric receptor complex containing serine threonine kinase regions, which can recruit Smad proteins within the cell. BMPs are a subset of the TGF β superfamily and as previously stated were first discovered by Marshall Urist in the 1960's. The BMP subfamily also includes the growth differentiation factors (GDFs) due to their structural similarity. With this taken into account, 22 BMPs have been identified; however, of these 22 only 21 belong to the TGF β superfamily, BMP-1 is classified as a metalloprotease. An extensive list of BMP family members can be found in Table 1.2 (excluding the GDFs), together with an overview of their function.

BMP	Function	Ref.
BMP-2 (BMP-2α)	• Induction of cartilage and bone formation and osteoblast differentiation • Heart formation	(117) (119)
BMP-3 (Osteogenin)	• Antagonist of osteogenic BMPs in skeletal development	(119,)
BMP-3b (GDF-10)	• Involved in adipocyte differentiation. • Inhibition of osteoblast differentiation.	(106,107)
BMP-4 (BMP-2b)	• Embryonic formation of limbs, teeth and bones.	(117)
BMP-5	• Role in cartilage development	(117)
BMP-6 (Vrg-1)	• Developed joint homeostasis. • Nervous system development	(117, 119)
BMP-7 (OP-1)	• Involved in kidney development and homeostasis. • Involved in cartilage repair and homeostasis • Role in osteoblast differentiation. • Eye formation	(117, 119)
BMP-8 (BMP-8a/OP-2)	• Involved in bone and cartilage homeostasis.	(117)
BMP-8b (OP-3)	• Spermatogenesis	(119)
BMP-9 (GDF-2)	• Promotes MSC differentiation into chondrocytes	(117)
BMP-10	• Involved in embryonic heart development.	(117)
BMP-11 (GDF-11)	• Embryonic development	(119)
BMP-12 (GDF-7/CDMP-3)	• Tendon and ligament development	(119)
BMP-13 (GDF-6/CDMP-2)	• Joint and bone formation	(119)
BMP-14 (GDF-5/CDMP-1)	• Skeletal repair and regeneration	(119)
BMP-1	• Thought to play a role in early embryogenesis.	(117)

Table 1.2 Overview of the BMP signalling family members and their functions

BMPs have analogues found in invertebrates, such as the decapentaplegic (Dpp) and 60A/Glassbottom boat (Gbb) found in *Drosophila*. These are of interest as Dpp has been shown to be structurally similar to BMP-2 and BMP-4 and with Gbb being similar to BMP-6 and BMP-7 (103). It has been reported that BMP-2 and Dpp have 75% sequence identity and BMP-7 and Gbb have 70% sequence identity (104). Given these similarities, it is not completely surprising that Dpp and Gbb can induce bone and cartilage formation in mammals (104). BMPs can exert one of three actions upon secretion from the cell. They can act locally at the site of secretion, they can be bound and sequestered by antagonists or they can bind ECM components to enhance BMP mediated effects (105).

1.2.1.1 Structure

There is strong homology between the members of the TGFβ /BMP signalling family. This homology can be attributed to a variety of common features that are shared by this family of proteins. These features facilitate processes such as secretion, folding and stability of the protein as well as ensuring effective signalling. BMP proteins are first expressed as large precursor molecules that contain 3 distinct sites: a single peptide domain, a pro-domain and a mature peptide domain (Figure 1.3). The precursor peptide needs to be cleaved at RXXR sites by a cleavage enzyme before the protein is in a mature and active conformation; a dimer composed of 2 disulphide linked monomer units. Following the first enzymatic cleavage the complex is regarded as unstable and a likely target for degradation and only upon the second cleavage and release of the mature motif is the protein regarded as stable (109). After the cleavage of the single peptide domain, the rest of the peptide undergoes glycosylation and dimerization (Figure 1.2), either as a homodimer with the same BMP or a heterodimer with another BMP. A second processing event then occurs where the pro-domain is cleaved releasing the active mature peptide (109,117). The pro-protein convertase furin (also known as paired basic amino acid cleaving enzyme – PACE) has been shown to cleave the pro-domain of the protein (111). It has also become apparent (in the context of BMP-4) that the cleavage of the pro-domain is essential for the mature peptide to be able to leave the endoplasmic reticulum. The pro-domain of BMP-7 may act in a similar fashion as, after the pro-domain is cleaved it facilitates BMP-7 solubilization by shielding the exposed hydrophobic regions on the surface of the protein (112).

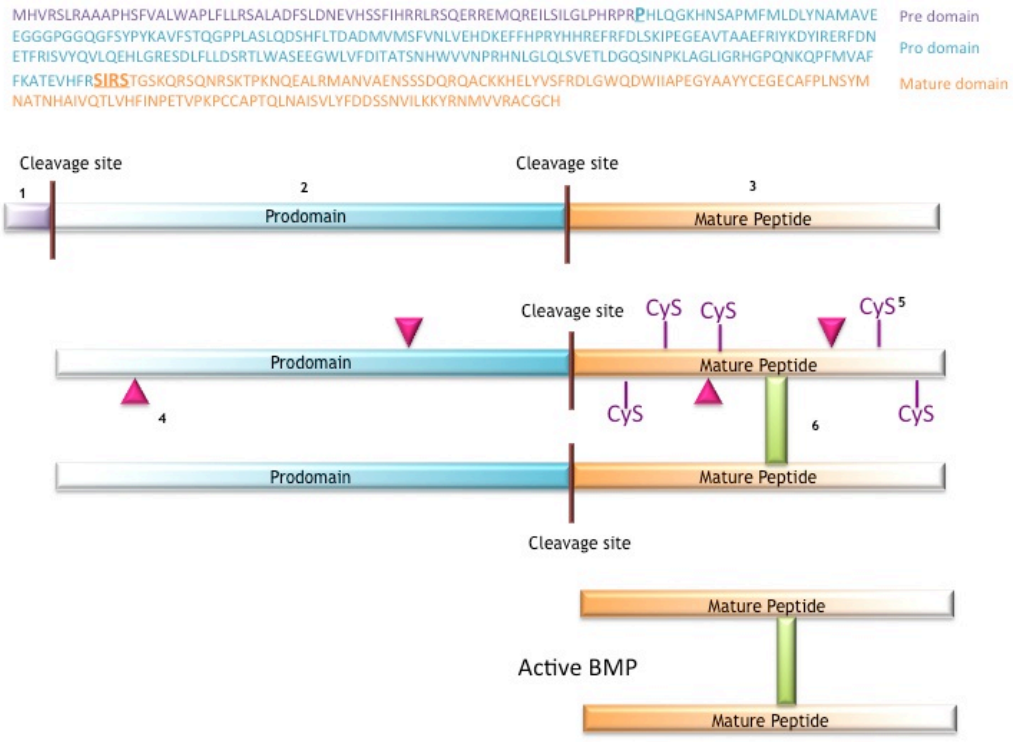


Figure 1.2 Structure, Post-Translational Modifications and Cleavage Sites for BMP-7. The structure of BMP-7 along with post transcriptional modifications along with the amino acid sequence for the BMP-7 pre, pro and mature, (106). 1) Amino acid terminal sequences; facilitates excretion of the protein from the endoplasmic reticulum. The amino acid sequence for this portion of the protein has been shown in purple. Both the red line and the P on the amino acid sequence highlight the cleavage site for the removal of this section. 2) Pro-domain latency- associated peptide; allows appropriate folding, increases solubility and helps to facilitate quaternary interactions. This domain shown in blue and is cleaved from the mature peptide, highlighted gain by the red line on the diagram and in the amino acid sequence at SIRS. 3) Mature signalling peptide; Mediates the effector functions of BMP-7, shown in the amino acid sequence in orange. 4) Glycosylation sites (pink triangles); four are present on the BMP-7 protein and are thought to be used for correct folding, protein-protein interactions and secretion of the peptide after sorting in the Golgi. 5) Intra-peptide disulphide bonds; six conserved cysteine residues (CyS) essential in the formation of the cysteine knot tertiary structure involved in the stabilisation of BMP-7. 6) Inter-chain disulphide bonds; facilitate homodimer and heterodimer formation. The full-length protein is first cleaved at the end of the amino acid terminal, a second cleavage then takes places releasing the mature protein from the pro-domain.

Further studies on the structure and processing of BMP-7 by Gregory *et al*, have shown that after the second cleavage the pro-peptide remains non-covalently associated with the mature protein, which is considered to facilitate binding to fibrillin. This is similar to TGFß1, where latency is conferred via ß1 latency associated peptide remaining non-covalently associated to the mature peptide after cleavage (112). This non-covalent association also allows interaction with latent TGFß binding protein 1, which facilitates secretion of the protein into the matrix. The mature BMP protein also contains seven cysteines (Figure 1.2), six of which are involved in the formation of a cysteine knot in the protein, whilst the seventh is used as a dimerization point with another BMP monomer (117).

1.2.1.2 Signalling Cascade

BMPs signal through a type I and type II serine threonine kinase receptor complex. Three type I BMP receptors have been identified: Activin-like Kinase 2 (ALK2), ALK3 and ALK6 (BMPR1B). All of the receptors have been shown to bind BMPs, with BMP-2 and BMP-4 preferentially binding ALK3 and ALK6, whereas BMP-7 preferentially binds ALK2 and ALK6. The type II receptors, bone morphogenetic protein receptor II (BMPRII), activin receptor II (ActRII) and activin receptor IIb (ActRIIb) all recognise BMPs (113).

BMP signalling has been depicted (Figure 1.3) highlighting that BMP binding to the type II receptor, which is constitutively active, causes recruitment and phosphorylation of the type I receptor. The activation of the type I receptor results in the phosphorylation and release of receptor regulated Smads (R-Smads), which are then free to form a complex with Smad4. This complex then translocates to the nucleus to modulate gene transcription via specific regulatory elements (105). This is considered the canonical signalling pathway (Figure 1.3). Smads can be divided into two categories based on R-Smad activation. Ligands binding to activin/nodal/TGFß type I receptors recruit Smads 2 and 3, whereas BMP type I receptor binding is associated with Smads 1/5/8 (114). Receptor binding can also result in the activation of a Smad independent non-canonical pathway (Figure 1.3), which induces the mitogen activated protein kinase (MAPK) pathway (116).

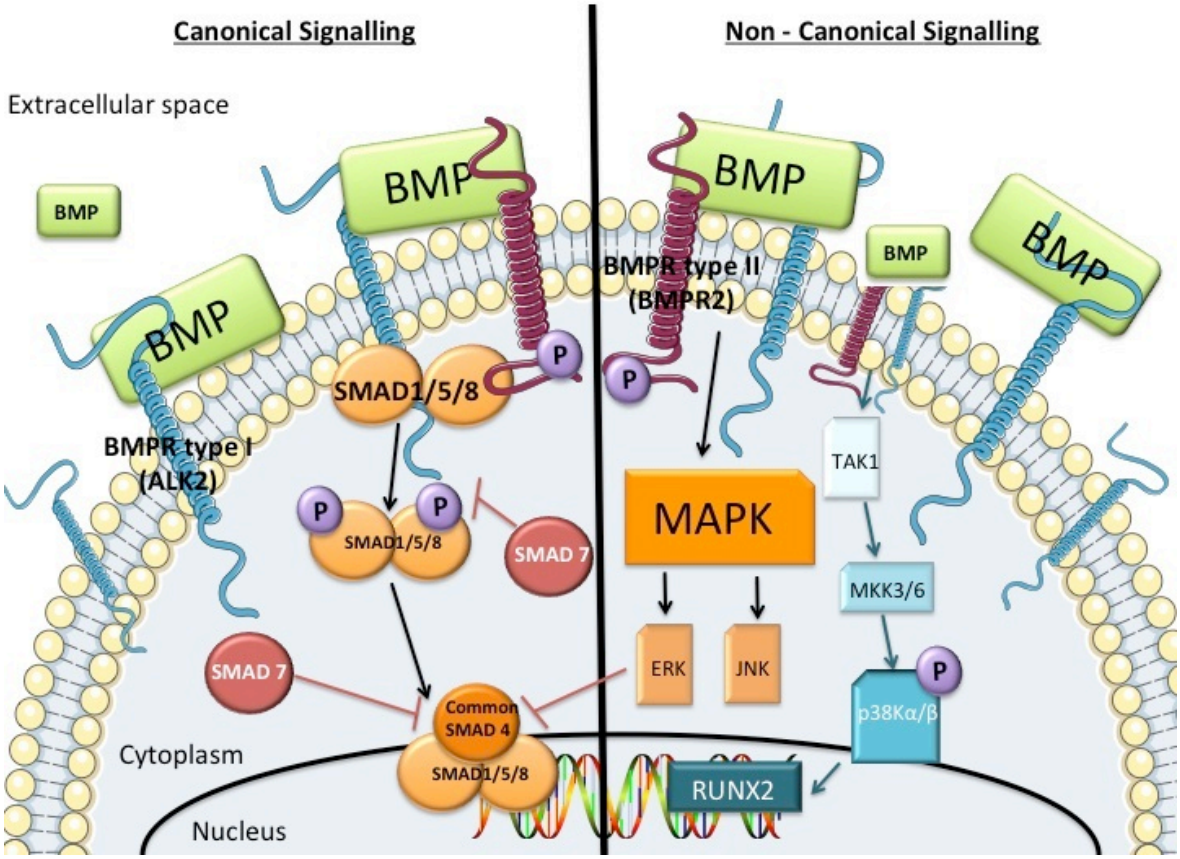


Figure 1.3 BMP Canonical SMAD Signalling and Non Canonical SMAD Independent Signalling.
Canonical signalling (136) : BMP-7 binds to the Type II BMP receptors which is constitutively active. The type I receptor is then phosphorylated and intracellular mediators SMAD1/5 and 8 move to the activated type I receptor where they are also phosphorylated. From here they join with common SMAD4 and translocate to the nucleus modulating gene transcription. Non- Canonical signalling (136): The activated receptors in this case activate the MAPK signalling cascade, this can ultimately lead to the activation of chondrocyte associated transcription factor RUNX2 via the specific activation of TAK1, MKK3/6 and P38K.

Following receptor phosphorylation after ligand binding, TAK1 and MKK3/6 are activated, these then phosphorylate p38α/β, which in turn activate the runt related transcription factor RUNX2 (116).

There is promiscuity in the ligand receptor interactions in the BMP signalling family, with more than one BMP being capable of binding each receptor. This would suggest that ligand binding to a receptor might not be the only signal necessary to initiate a specific response from the cascade. It has been suggested that the R-Smads that partners with the type I receptor may confer specificity. There is data to support this hypothesis which relies on only one R-Smad pathway being regulated by each receptor ligand interaction (105,135).

1.2.1.3 BMP Regulation

Interaction between BMPs and their inhibitors is essential for embryonic development and the appropriate signalling that is required for cellular processes in which BMPs are involved (118).

There are several groups of endogenous antagonists that target the BMP signalling cascade. These include the CAN (ceberus and DAN) family proteins, the twisted gastrulation proteins and the chordin family (120). Noggin is a member of the chordin family and is a specific inhibitor that has been implicated in neuronal differentiation by blocking BMP signalling (121). BMP-2 and BMP-4 have been shown to have the highest affinity for noggin, whereas BMP-7 binds with a lesser affinity to the inhibitor and TGF β does not interact with it at all (122). Due to the generation of crystal structures, it is now known that noggin binding blocks the sites required for interaction with both the type I and type II BMP receptors (118).

Follistatin (FST) is another BMP inhibitor that can directly interact with BMPs. However, unlike noggin it does not prevent BMP binding to its receptors (123). Instead it is thought that conformational changes elicited by FST binding prevents receptor activation (123). FST has been shown to bind both BMP-2 and BMP-7 and this binding has been shown to be reversible (124). Animal models have shown that FST is up-regulated in OA-like cartilage at the transcript level

(125). Similarly, BMP inhibitor Gremlin (grem), a member of the CAN family of BMP inhibitors, has also been shown to be up-regulated in OA (125).This antagonist has recently been identified as a marker for osteochondroreticular stem cells in the bone marrow, a cell population that can be utilised for bone remodelling and fracture repair (126). Grem has been shown to bind both BMP-2 and BMP-4 over BMP-7, and prevents signalling in three ways. Firstly, by direct binding of the BMPs, secondly, by blocking BMP secretion and finally, by increasing BMP endocytosis (127).

Cytokines and growth factors can influence the expression of grem and FST in chondrocytes. Specifically, various anti-inflammatory cytokines have been shown to inhibit the expression of FST including IL-4, IL-13 (125), whilst BMP2 and 4 have been shown to induce grem (128).

1.2.2 BMP-7 KO Mice

BMP-7's role in foetal development has been investigated in mice. It has been shown that BMP-7 knock out mice die postnatally with several developmental abnormalities, including: defects in kidney development, abnormalities in eye development, ineffective skeletal patterning, impaired corticogenesis, decreased brown fat and diminished Langerhans cell number (115). This suggests that BMP-7 signalling plays a comprehensive role in the development of many systems throughout embryogenesis, however, these mice are of restricted use for studying the deletion of the gene *in vivo*. To this end, BMP-7 mutant mice were created using deletions of either the first exon (129) or the sixth and seventh exon of the gene (130). Both groups found that by mutating BMP-7, mice had severe abnormalities in kidney and eye development (129,130) along with skeletal patterning defects in the rib cage, skull and hind limbs (130). This was further validated by an accidental deletion of BMP-7 that was found in the F2 offspring of bcl2α transgenic mice. These pups again died postnatally and displayed defects in skeletal, kidney and eye development. Conditional limb specific knock out mice have been generated using a flox-cre system whereby the mice with a floxed BMP-7 allele were bred with mice containing the cre recombinase in the paired related homeobox 1 gene (PRX1::cre mice). It has been shown that the conditional knock down of BMP-7 had no effect on limb

growth or bone mass and it also had no effect on cartilage formation or fracture healing (131). It has been suggested that other BMP's may be able to compensate for the absence of BMP-7 in the limb (131). The idea of the BMP family members being able to compensate for one another has been elegantly shown in kidney development (132). BMP-7 deletion has previously been reported to produced catastrophic abnormalities in the kidney, however, if BMP-4 is expressed under the control of the BMP-7 promoter, this can prevent these BMP-7 associated kidney abnormalities, highlighting the ability of this family to compensate for the loss in expression of one of its members (132). It is also interesting to note that BMP-7 and BMP-4 are structurally divergent but appear to be interchangeable in kidney development. It was less surprising that BMP-6, which is more structurally comparable to BMP-7, is also interchangeable in the context of kidney development. The interchangeable nature of the BMPs appears to be tissue specific as BMP-4 is unable to rescue eye defects known to be related to an absence of BMP-7(132). The concept of redundancy with regards to BMP-7 signalling has been further supported by two studies demonstrating that the BMP-7 null phenotype is exacerbated in mice lacking the closely related BMP-5 or BMP-6 (132). BMP-7 null and conditional knock out mice have added valuable knowledge to the literature, confirming BMP-7 role in development and showcasing complex interplay between the different BMPs in specific tissues.

1.2.3 Role of BMP-7 in the Body

1.2.3.1 Kidney

Postnatally, BMP-7 is most abundantly expressed in the kidney (133), with murine embryo studies naming the kidney as the main site of synthesis for BMP-7 (134). BMP-7 homozygote mutant mice have helped to demonstrate the proteins' fundamental role in kidney development in mammals (129). A lack of BMP-7 in the developing kidney has been shown to result in a severe lack of architectural organisation and limited epithelial and mesenchymal continuity (129). At this anatomical location, it has been implicated in the maintenance of nephron progenitors and also in the sensitization of these cells to ureteric bud-derived differentiation signals (129). Furthermore, BMP-7 may have therapeutic potential for the treatment of kidney disease. In 2003, BMP-7 was shown to reverse epithelial –mesenchymal transition (EMT) in renal tubular epithelial

cells, which ultimately leads to chronic renal failure (137). The data presented defines a role for BMP-7 in reintroducing E-cadherin, an epithelial adhesion molecule, that is dampened in the presence of disease derived TGFß1 (137). Thus BMP-7 has been shown to be a potential therapeutic candidate for renal failure. Further work with the BMP-7 enhancer, kielin/chordin – like protein 1 has also highlighted a role for BMP-7 in protection from renal fibrosis (138). If mice lack this enhancer they become more susceptible to fibrosis in the kidney. Additionally, inhibition of a renal BMP antagonist, uterine sensitisation-assocaited gene-1 (USAG1), has been shown to be protective in renal injury (129). Taken together, this evidence showcases a strong role for BMP-7 in the maintenance of renal homeostasis and protection against renal disease.

1.2.3.2 Vasculature

BMP-7 expression has been reported in the vasculature with a distinct decrease in protein expression being noted in several disease states that are associated with calcification, such as chronic kidney failure. It is thought that BMP-7 more specifically prevents the trans-differentiation of the smooth muscle cells (SMC) into an osteoblast like phenotype that can result in vascular calcification. BMP-7 treatment has been shown to limit osteoblastogenesis via the down regulation of osteoblast function marker osteocalcin, thus preventing calcification (133). Further to this, Dorai *et al* suggest an anti-inflammatory role for BMP-7 in the vasculature as well as showing that BMP-7 is an essential regulator of its own superfamily member, TGFß, via up-regulation of inhibitory Smads (139).

1.2.3.3 Stem Cell Regulation.

Due to BMP-7s extensive role in embryogenesis, it may not be too surprising that it has been reported to successfully expand hematopoietic stem cells. Yeu-Han *et al* demonstrated that umbilical cord derived stem cells were significantly expanded *in vitro* when cultured in the presence of BMP-7, but not BMP-2 (140). It was also found that the BMP-7 mediated expansion had no effect on the colony-forming ability of the cells or on the lineage of the cells comprising the population (including $CD3^+$ T cells, $CD19^+$ B cells, $CD33^+$ myeloid cells or $CD71^+$erythroid cells) (140). Immuno-deficient mice were used to investigate the engraftment of the stem cells *in vivo*, demonstrating that the BMP-7 expanded

population had increased $CD45^+$ cells in both the bone marrow and the spleen, therefore demonstrating the improved reconstitutional properties of the BMP-7 expanded cells (140). BMP-7 is also reported to play a role in human MSC differentiation into osteoblasts (the cells responsible for bone formation)(141,142). BMP-7 has been shown to alter gene expression during early osteoblastic differentiation, up-regulating a number of osteogenic marker genes and down regulating genes associated with the cell cycle – thus facilitating the differentiation of the MSCs into osteoblasts (143).

The data generated in the vasculature, reporting that BMP-7 inhibits osteoblastogenesis (section 1.2.3.1), along with the data regarding BMP-7 function in the bone, reporting the induction of osteoblastogenesis, suggest that BMP-7's function is cell specific.

1.2.3.4 Immune Cell Regulation

BMP-7 has been linked to the activation of immune cells including, B cells and macrophages. It has been shown that the human monocytic leukemia cell line (THP-1) can be polarised into an anti-inflammatory M2 macrophage-like phenotype upon stimulation with BMP-7 (144). In this context, BMP-7 drove increased expression of anti-inflammatory cytokines such as IL-10 and IL-Rα along with decreased expression of pro-inflammatory cytokines IL-6 and TNFα. Furthermore, Rocher *et al* describe a BMP-7 mediated increased in BMP receptor BMPR2 (144). Evaluation of the underlying mechanism of this unique polarisation revealed that BMP-7 phosphorylated SMAD and PI3K, leading to subsequent activation of Akt and mTOR (145). Therefore, the PI3K-Akt-mTOR pathway has been implicated in THP-1 (monocyte) polarization, however this has yet to be confirmed in primary cells.

BMP-7 has also been shown to have a role in in B cell functioning. Numerous BMPs have been shown to inhibit IgG, IgA and IgM production in both memory and naïve B cells (146). It has been surmised that the inhibition of IgG may be attributed to a BMP-7 mediated inhibition of proliferation and increased cell death. It was noted that this was a BMP-7 specific mechanism as even though BMP2/4 and 6 were all able to dampen IgG, IgM and IgA release from B cells, only BMP-7 had any effect on apoptosis (146).

These data, taken together highlight a role for BMP-7 in the regulation of the immune system, mainly in macrophage and B cell functioning. This role is just beginning to be uncovered and there are still significant gaps in our understanding of BMP-7's full role within this complex system.

1.2.4 BMP-7's Role in the Joint and OA

Various studies have attempted to identify patterns of expression for BMP-7 in a normal and an osteoarthritic joint (predominantly in the cartilage). In 2000, Chubinskaya *et.al* were the first to demonstrate that BMP-7 was expressed by human articular chondrocytes (147) using nested primers and *In Situ* hybridization. Quantification of the RT PCR suggested that there were increased levels of transcript in OA chondrocytes compared to newborn cartilage and adult articular cartilage (148). There is some controversy in the literature, regarding BMP-7 expression in human chondrocytes as it has also been reported that BMP-7 transcript is only detectable in foetal cartilage and not in OA or normal adult tissue (149). The discrepancies between these two studies may lie within the methods used for mRNA detection, as Chubinskaya detected the transcript using methods optimised for low-level transcript detection. Therefore, it is plausible that *Chen et al* have failed to locate the transcript as the methods implemented were not sensitive enough for this purpose.

Building on the work demonstrating detectable BMP-7 in chondrocytes, Chubinskaya *et.al*. also revealed that there is a negative correlation between age and BMP-7 expression in the cartilage. This was reported at both a transcript and protein level (148). This correlation potentially suggests that an age related decrease in BMP-7 in chondrocytes may predispose some individuals to OA via an alteration in the homeostatic balance between anabolic and catabolic pathways (148). Furthermore, it has been reported that BMP-7 protein expression is also decreased in OA, contrary to the previous finding by Chubinskaya (150). Levels of the BMP-7 transcript were also reported to decrease with increasing cartilage erosion i.e. OA severity (150). However, it must be noted that the control cartilage used in this study was reported to be of Collins grade II-III, which would typically be regarded as eroded. Therefore, this questions the interpretation of

these results as the cartilage has not been compared to a true healthy control tissue.

In 2006, Chubinskaya *et al* reported that OA synovial fluid contained in the region of 50ng/ml of BMP-7 - a similar concentration to control groups (151). Whilst other data suggests that OA synovial fluid contains a lower concentration of BMP-7, approximately 10pg/ml (153). This data must be interpreted carefully, as the concentrations at which BMP-7 was reported were at times below the limit of detection for the assay (Human BMP-7 Quantakine ELISA kit, R & D Systems). BMP-7 protein expression has also been reported in RA synovial fluid at higher concentrations than in the OA and non arthritic joints, with RA synovial fluid containing around 100ng/ml BMP-7 (twice as much as the OA fluid) (151). It is possible that this increase in readout from the ELISA analysis could be due to non-specific binding of rheumatoid factor in the RA synovial fluid, as the methods do not state if an appropriate block was carried out to prevent this interference. Additionally, a positive correlation between BMP-7 protein and severity of OA in both plasma and synovial fluid in patients has been reported (154). However, this correlation opposes that which was found in the cartilage, whereby BMP-7 protein is decreased with increasing OA severity(150).

Moreover, studies have also investigated the consequence of activating BMP-7 signalling in chondrocytes; Chubinskaya *et al* used a gene array to describe BMP-7s potential role in cartilage homeostasis (155). The transcriptional signature revealed that several genes were under the control of BMP-7 including: chondrocyte cytoskeleton constituents, matrix production proteins and catabolic factors such as matrix degrading enzymes and cell death components (155). Further to this, there is also evidence to suggest that BMP-7 is able to upregulate hyaluronic acid synthase (HAS), hyaluronic acid (HA) and CD44, all of which are components in extracellular matrix production in primary human chondrocytes (156). Additionally, BMP-7 can increase proteoglycan expression in articular chondrocytes and further to this at low concentrations it can also reverse the inhibitory effects of IL-1 β on proteoglycan expression (157).

1.2.4.1 BMP-7's Therapeutic Potential in OA: Animal Models

BMP-7 has been shown to be chondroprotective in several animal models of OA including, a rat inflammatory model, a sheep impact model and a rabbit mechanical model of arthritis.

Inflammatory arthritis was emulated by intra-articular injection of zymosan into the joint of rats (158). It was shown that BMP-7 prevents the zymosan driven loss of type II collagen, as well as preventing the expression of MMP-3. Overall joint swelling was significantly decreased in rats receiving the higher doses of BMP-7 compared to the control and the low dose group conferring a dose dependent anti-inflammatory aspect to BMP-7 therapy. Moreover, IL-1ß concentrations were also decreased following the BMP-7 treatment (158). The data also goes on to suggests that therapeutic BMP-7 up-regulates endogenous BMP-7 at the protein level. However, the expression of the BMP-7 transcript following the treatment was not investigated and therefore it cannot be confirmed if the increase was due to an increase in BMP-7 from the chondrocytes or synoviocytes or just due to remnants of the therapeutic protein administered.

A mechanical model of arthritis known as the anterior cruciate ligament transection (ACLT) has also been utilised to mimic the mechanical stress exerted on the joint in OA. The anterior cruciate ligament is surgically cut, causing destabilisation and eventually erosion of the joint due to uneven mechanical load. Hayashi used this model in 2010 to demonstrate BMP-7s protective effect in this OA like disease. BMP-7 was administered therapeutically via intra-articular injection on a weekly basis from 4 weeks post transection of the ACLT to 12 weeks, at which point the animals were sacrificed (159). It was found that BMP-7 was effective in protecting the cartilage at macroscopic levels when the tissue was scored according to the Osteoarthritis Research Society International (OARSI) cartilage grading system. Furthermore, micro computed tomography was used to demonstrate that BMP-7 had no adverse effects on etopic bone formation, a side effect which was expected due to BMP-7 ability to promote bone growth via the promotion of osteoblast differentaion (143).

An impact injury model was used in sheep to establish the efficacy of intra articular BMP-7 and to explore the time window for therapeutic intervention

following injury (160). It was concluded that the chondroprotection conferred from BMP-7 is markedly increased if the intervention is received immediately after the injury. Additionally, BMP-7 was able to significantly decrease the number of apoptotic cells, this was most significant if treatment was received on the same day as the as the injury was sustained. A more modest reduction in apoptosis was seen in the group that received intervention three weeks post-surgery, however, this was still regarded as significant. It must be taken into account that administration of any therapeutic agent at the time of injury is unlikely as it can take months for an individual with joint damage to be referred to specialist orthopaedic department and to undergo the necessary examinations to identify the problem. Therefore, in this respect, treatment 3 months post injury is a more accurate reflection on the likely timescale for NHS patients (160).

All three animal models demonstrate a chondroprotective effect of BMP-7 in OA like disease, despite all three having differing aetiology. This provides data on which to drive forward BMP-7 as a potential disease-modifying agent in the treatment of OA.

1.2.5 Clinical Applications of BMP-7

1.2.5.1 OA: Phase I Clinical Trial

A phase I safety and tolerability study has been carried out for BMP-7 (161). The study found that there were no dose limiting toxicities and no difference in adverse events compared to placebo (161). Due to BMP-7s involvement in bone formation, it was essential to ensure that ectopic bone formation (osteophytes) was not increased in the BMP-7 treated cohort. Radiographic analysis in the follow up report showed no such effects in the BMP-7 treated individuals (161). In addition to evaluating safety and tolerability, patient outcome was also assessed. To do this, investigators measured pain and functional improvement using both the WOMAC scoring system and improvements in the OARSI responder criteria. Optimal WOMAC measured responses (50 -70% improvements) were observed in those patients that received high dose BMP-7. The OARSI responder criteria, which assess pain, was also improved with BMP-7 treatment (161). In summation, using the evaluation techniques in this study BMP-7 treated

individuals reported a self-assessed improved response with regards to their OA symptoms after BMP-7 treatment.

1.2.5.2 Fractures

The levels of BMP-7 are significantly decreased in non-union fractures when compared to normal callus tissue (162). In addition to BMP-7, other members of the BMP family are also dysregulated in non-unionised tissue, including a decrease in BMP8 and an increase in BMP4, BMP5 and BMP-6. BMP-7 is a licenced agent for the treatment of non-unionised fractures (163). Clinical trials have demonstrated that recombinant BMP-7 inserted in a rod with a type I collagen carrier has a comparable outcome to bone autograft and is therefore successful in treating non unionised fractures (164). The mechanism underlying this ability of BMP-7 in facture healing is thought to be related to its role in proliferation and osteogenic differentiation of MSCs (165) (section 1.2.3.1), which results in faster healing and increased mechanical strength of the bone (166).

1.3 Gene Regulation

Genes can be regulated in many ways including at the epigenetic level. These epigenetic mechanisms can include histone modification (methylation, acetylation), AU rich elements for degradation motif binding, non-coding RNAs (section 1.3) and methylation of the gene promoters. In health, all of these regulatory mechanisms enable homeostasis to be achieved, however, in disease they may become dysregulated and contribute to pathology.

1.3.1 AU Rich Elements

AU rich elements (AREs) are sequences in the mRNA rich in the bases adenine and uridine, that signal transcripts for degradation(167). These regions are commonly found on the 3'UTR of many transcripts and are bound by AU binding proteins such as tristetraprolin (TTP), butyrate response factor 1 (BRF1) and butyrate response factor 2 (BRF2), which are all zinc finger proteins. Recognition of the mRNA via AUUUA motif by AU binding proteins, leads to the activation of the mRNA decay machinery and thus the destruction of the transcript. Under normal physiological conditions this mechanism can act to maintain homeostasis, however, disease associated dysregulation can be catastrophic and has been implicated in several cancers and autoimmune disease processes (167). Furthermore, a role for 3'UTR regulation in autoimmune disease was highlighted using TNFα transgene mice (168,169), showing that specific modification of the 3' UTR, altering the AU binding sequences, can change the expression pattern of TNFα that results in chronic inflammatory polyarthritis (168,185). It was later confirmed that these mutations in the 3'UTR of the TNFα transcript were interfering with TTP regulation of the mRNA (170) and had a functional consequence that manifested in inflammatory arthritis (171).

1.3.2 Methylation

DNA methylation is another form of transcript regulation and occurs when a methyl group is added to the 5^{th} carbon of a cytosine nucleotide in the gene promoters in the CpG islands region, which is present in 60% of gene promoters (172). The modification is regarded as highly stable and can be detected in easily

accessible fluids such as plasma, saliva and urine (172). If too much methylation occurs, this can be referred to as hypermethylation and conversely, if there is not enough methylation then this is hypomethylation, both of which have been associated with a vast number of pathologies (173). Moreover, hypomethylation has been associated with facilitation of catabolic processes, whereas hypermethylation has been connected to the prevention of anabolic action in OA (172).

Methylation of the BMP-7 promoter has been proposed as a mechanism for the age related decrease in the protein that is reported in the literature (174). Furthermore, inhibition of promoter methylation in chondrocyte cultures has led to an increase in BMP-7. Thus hypermethylation may predispose some individuals to OA by preventing BMP-7s homeostatic actions. This age related phenomenon of increased methylation is consistent with that found in the epidermis where it has been shown that there are significant age-associated changes in the methylation (175).

1.4 MicroRNAs

1.4.1 Non-Coding RNAs

For years it was believed that the genome encoded only proteins, hence the central dogma of protein synthesis: DNA leads to RNA, RNA leads to protein. Whilst this does indeed hold true, it was believed that the remaining DNA that did not code protein was "junk DNA" that higher eukaryotes had evolved to no longer need. This however, is not the case and these genomic regions, referred to as non-coding regions, can be transcribed into non-coding RNA (ncRNA) with the resultant RNA having a range of biological functions. In 1961 Francois Jacob and Jacques Monod published a review article describing some of these functions (176). They looked at the regulation of protein synthesis and concluded that the mechanisms of control were inhibitory, operated at the genetic level and the mechanism itself was genetic in origin (176). They were describing epigenetic regulation of coding RNA, however, they were not the 1st to come across this type of RNA regulation - Waddington published a book in the 1940s, *Organisers and Genes,* that coined the term epigenetics in reference to this RNA regulation. Waddington was fundamental in merging the fields of genetics and embryology (with outstanding success) and challenged the current thinking of protein regulation at the time (177).

Over the past decade, the general understanding with regards to genetic regulation has vastly improved, with miRNA's being a major focus. More recently, long non-coding RNA (lncRNA) have been brought to the forefront as the latest form of functional RNA. These lncRNA are defined as noncoding regions of over 200 nucleotides in length. The function of many of these lncRNAs have yet to be elucidated but, it is thought that they may fit into one of 4 functional categories; signal, decoy, guide and scaffold (192). If the lncRNA is thought to be in the signal category it may play a role as a molecular signal that can be used as an indicator of gene activity, whereas the decoy classification is for lncRNA that act as a sponge, competing for binding of other regulators such as miRNA. The third classification, termed as guide lncRNA, are thought to aid chromatin modification by guiding ribonuclear proteins to their targets. Finally,

scaffold lncRNA can act as stabilising structures at certain points along a gene(192) facilitating gene transcription.

1.4.2 miRNA Discovery

MicroRNAs (miRNAs/miRs) are short non-coding RNA sequences, commonly around 22 nucleotides in length, that are able to target specific mRNA transcripts for degradation based on complementary base pairing, thus modifying the expression of the target transcript. In 1981 miRNAs were first discovered in worms, more specifically *C.Elegans*. Their function was deciphered and it was reported that the identified RNA was able to decrease the expression of the lin4 protein via imperfect binding to the 3' untranslated region (UTR) of the lin4 mRNA (178). This research led to the identification of a second *C.Elegans* miRNA, Let 7(179). However, this miRNA was well conserved between flies and human and thus research on miRNAs moved into higher species (180). It is now well documented that miRNAs display great evolutionary conservation and our knowledge regarding these small RNAs has expanded rapidly over the past two decades. There are currently over 2000 identified miRNA (180) and *in silico* analysis predicts that combined, they target over 50% of the human genome.

The levels of expression of miRNAs in a cell varies greatly, from as little as 10 copies up to 80,000 copies per cell (180). MiRNA can be transcribed from introns that have no protein coding function (intergenic miRs) or can be located in the introns of protein coding regions (intragenic miRs). Further to this a smaller proportion are also located within exons (180).The expression of miRNAs are also tightly regulated and often miRNAs can be involved in complex networks of regulation. For example, if a miRNA caused repression of a target gene, this could lead to subsequent dowstream alterations in the expression of the miRNA itself (181).

1.4.3 miRNA Biogenesis and Function.

The synthesis of miRNAs can be broken down into 4 main stages: transcription, processing, maturation and strand selection by the RISC complex (181). Initially, RNA polymerase II transcribes pri- miRNA from the genomic DNA. The pri-miRNA has a specific tertiary structure (containing either one or several hairpin loops) and the transcript is capped at the 5'UTR with a 7 methyl guanosine cap, and polyadenylated at the 3'UTR (180). The pri-MiRNA is processed into smaller pre-miRNA fragments by drosha, a RNase II type molecule, along with its co-factor digeorge syndrome critical region gene 8 (DGCR8). From here, the pre-miRNA is actively exported to the cytoplasm by exportin 5, which utilises nuclear pores for transport (180,181). A second RNase III type protein, dicer, then generates a double stranded duplex miRNA from the pre-miRNA generating a mature miRNA. From here, one strand (the guide strand) will be selected for incorporation into the RNA–induced silencing complex (RISC) and the other strand, the passenger strand, will be degraded.

The mature single stranded miRNA can then lead the RISC complex to the target mRNA (182). The seed region of the miRNA normally binds to a complementary microRNA response element (MRE) sequence in the 3' UTR of the mRNA, however, binding to the 5' UTR or even the protein coding region itself has been noted. The miRNA seed regions that binds to the mRNAs are often between positions 2-8 of the mature miRNA sequence (183). Furthermore, seed length has been linked to specificity of miRNA binding, i.e. the longer the seed sequence the more specific the binding. After binding, there are two mechanisms for protein synthesis interference, the mRNA can be translationally repressed, mainly through the interruption of either the initiation or elongation stages of protein synthesis. Alternatively, the mRNA is degraded and this commonly occurs via the removal of the polyA tail via de-adenylase activity (184). The proposed mechanism for the binding of a miRNA to a target mRNA is based on complementarity, of seed regions. It has been established that if the miRNA and the target mRNA have near complete complementarity then the mRNA is targeted for degradation. However, if there is only partial complementarity then the mRNA may be translationally repressed. A study carried out in 2006 questioned the reliability of the 3'UTR seed regions as a predictor of mRNA-

miRNA interaction (185). Based on this study (and subsequent work) it is now hypothesised that the complementarity of the seed region along with seed region accessibility and perhaps co-expression of other RNAs or nuclear/cytosolic proteins might be more indicative of miRNA binding to a target. Therefore, this ultimately suggests that the binding of the miRNA to a mRNA is context specific and not solely based on complementary (185).

1.4.4 miRNA in OA

MiRNA dysregulation has been strongly associated with OA, with the first systematic profile miRNA discrepancies identifying 17 miRNA that were differentially expressed between OA and healthy cartilage (186). A review published in 2015 went on to further profile all of the miRNA that are predicted to be dysregulated in OA, creating an extensive list of cartilage derived disease associated miRNAs (Figure 1.4). The exact role that these modulators play in disease has not been fully elucidated.

Further to miRNA being associated with molecular pathways and cell differentiation within the joint environment, they have also been associated with aging and mechanical loading (187) (known to be risk factors for OA). To date, four miRNA have been reported to be deregulated with ageing, resulting in changes to both collagen and ADAMTS production: miR320c, miR199a-3p, miR193b and miR21 (187). A further two miRNA, miR365 and miR222, have been associated with mechanical loading (187). MiR365 is a mechano-responsive miRNA that plays a role in chondrocyte cell proliferation and differentiation (188) and is up-regulated in OA (189).

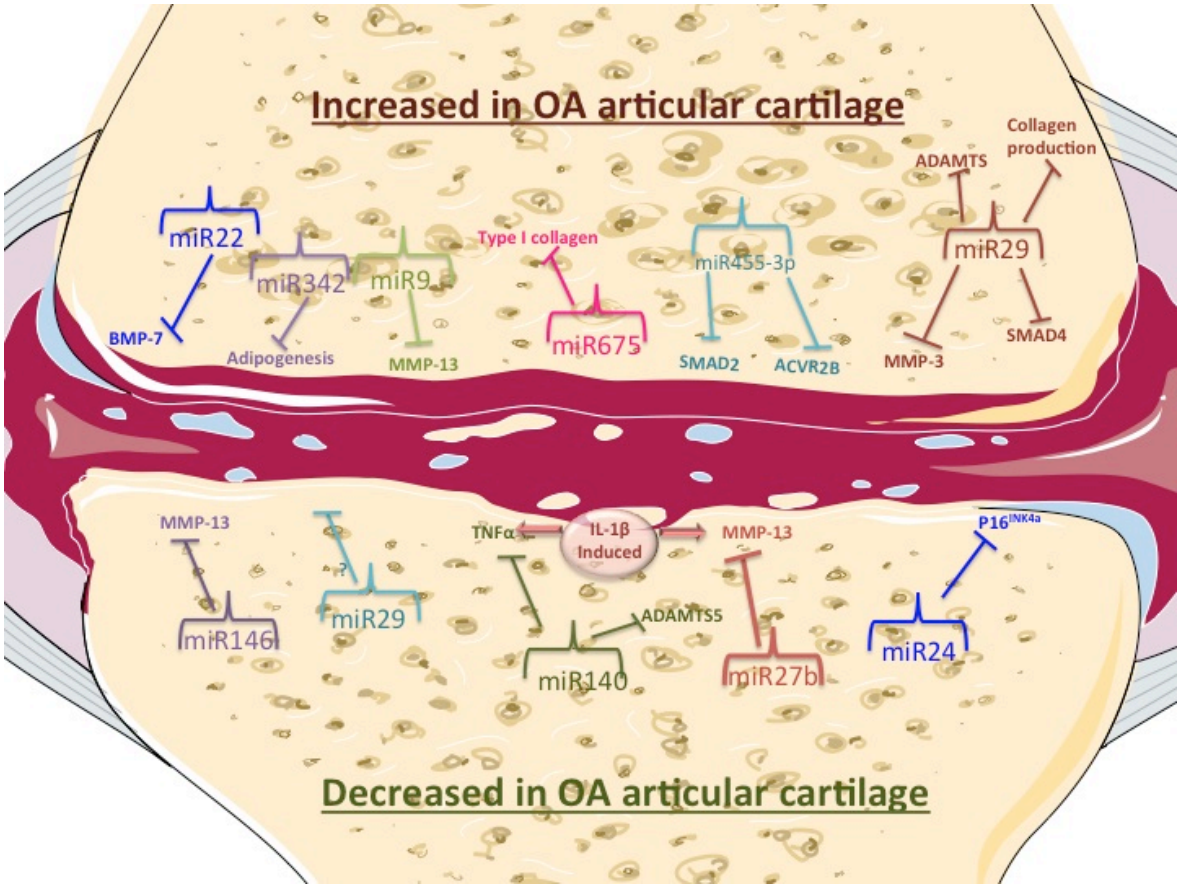


Figure 1.4 OA Associated miRNA Alterations in the Joint.

miRNA that are thought to be altered in the articular cartilage during OA pathology and their predicted mRNA targets **(187,194,202)**.

1.4.4.1 miR140

Of all the miRNA that have been shown to be dysregulated in OA miRNA 140 is one of the most intensely studied. Mice lacking miR140 have a subtle skeletal phenotype; slightly shorter body length and reduced body weight, along with abnormalities in the skull (a shorter snout and domed shaped skull). Furthermore, these mice also have articular cartilage abnormalities. Work has been carried out to show that miR140 is essential for chondrogenesis, with levels of the miRNA increasing significantly between the precursor human mesenchymal stem cells and differentiated chondrocytes (190). This increase in expression was observed over chondrogenesis and was consistent with other increased chondrocytic markers such as aggrecan, type II collagen and SOX9 (190). It has been further suggested miR140 expression is decreased when SOX9 is deleted, demonstrating that transcription factor SOX9 controls the expression of miR140 (191). However, this requirement for miR140 in the cartilage postnatally is not the same in embryogenesis, as miR140 knock out mice have normal cartilage at birth. As the mice age, the miR140 knock out (but not the wild type) develop spontaneous OA like symptoms; by 12 months they show severe structural damage to the joint with almost a complete loss of cartilage (191). Therefore, this miRNA appears to be required for the regulation and homeostasis of the cartilage and not the development. Furthermore, this miRNA has also been shown to confer protection against OA by transgenic overexpression (191).

The role of miR140 may extend past cellular differentiation. Miyaki *et al* went on to show that miR140 is negatively regulated by the OA dominant cytokine IL-1ß. Conversely, if human chondrocytes were transfected with a mimic of miR140, a suppression of IL-1ß induced ADAMTS5 was observed along with a reversal of IL-1ß induced aggrecan repression (190).

Taken together, these data suggest a role for miR140 in cartilage homeostasis and protection from OA , furthermore dysregulation of this miRNA may be a contributing factor to OA pathogenesis. In humans it has been robustly shown that miR140 is decreased in the cartilage of OA patients compared to normal controls (190,193).

1.4.4.2 miR22

To date there is only one miRNA that has been shown to regulate BMP-7: miR22 (194). MiR22 was shown to be increased in OA cartilage and was highlighted as a regulator of not only BMP-7, but also of the peroxisome proliferator-activated receptor alpha (PPARA) protein, known to be involved in lipid metabolism. *In vitro* validation demonstrated that both BMP-7 and PPARA are directly regulated by miR22. Via this direct regulation it is also thought that miR22 can indirectly modify the expression of catabolic modulators IL-1ß and MMP-13 (194). Furthermore, over expression of miR22 in chondrocytes can cause an increase in both IL-1ß and MMP-13. Conversely, transfection with 50nM miR22 inhibitor can lead to increased expression of both BMP-7 and PPARA and a down-regulation in IL-1ß and MMP-13 (194). Data from the sample cohort revealed a robust correlation co-efficient between miR22 and both BMP-7 and PPARA in the healthy and OA cartilage, thus revealing a pathological role for this miRNA in OA.

1.4.5 MicroRNA 24

MiRNA 24 has been previously linked to OA, with a significant decreased in expression being noted in OA cartilage (195). MiR24 belongs to a miRNA cluster that is transcribed in tandem. There are two clusters in which miR24 can belong, cluster 1: miR24a~27a~24-2 located on chromosome 9q22 and cluster 2: miR23b~27b~24-1, located on chromosome 19p13. These clusters are both very similar as both miR23a and miR27a only differ by one base pair form miR23b and miR27b respectively (196) and miR24 is identical in both clusters. Due to the sequence similarity, these miRNAs are predicted to share many common targets. However, they are located on different chromosomes and therefore their regulation is likely to differ, with multiple factors within the local environment most likely determining which of the clusters are activated at a given time (196).

Suppression of miR24 is thought to contribute to several different cancers including lung, gastric, colorectal, breast, prostate, ovarian and pancreatic cancer, along with leukaemia and squamous cell carcinoma. A range of target genes associated with cancer processes such as proliferation, cell cycle arrest, apoptosis and differentiation, have been validated as targets of miR24. There is

strong evidence for miR24 mediated regulation of the cell cycle, with the nuclear phospho protein MYC and E2F2 (a protein known to be involved in controlling cell cycle) being targets of the miRNA. Other targets involved in the cell cycle that are considered to be down stream of these two proteins are also regulated by miR24 (197). It is interesting to note that even though these targets have been validated using a luciferase assay, conferring direct binding of the miRNA to the mRNA, they did not have a complementary 3'UTR seed region, but were instead recognised by another seedless (non complementary) microRNA recognition site (197). Due to the intricate role this miRNA plays in cell cycle regulation and the clinical evidence supporting a down regulation of miR24 in several cancers including colorectal cancer (196), this miRNA may offer a new therapeutic avenue for the treatment of cancer (196,198).

Other studies have looked at the relationship between miR24 and the TGFß superfamily. TGFß family receptor ALK4 is modulated directly by miR24, preventing erythrocyte proliferation and differentiation. Furthermore, the expression of miR24 is conversely correlated with ALK4 over the process of erythropoiesis (115). Cross talk between the TGFß/BMP signalling pathways and the platelet derived growth factor –BB (PDGF-BB) signalling has also been linked to miR24 with regards to vascular smooth muscle cell (vSMC) phenotype (199). Following vascular injury, the vSMC must change from a contractile to a synthetic phenotype, with TGFß/BMP and subsequent SMAD signalling being associated with the former and PDGF-BB associated with the latter. The switch from contractile to synthetic vSMCs is thought to be mediated through a PDGF-BB induced down regulation of Tribbles-like protein-3 (Trb3), known to facilitate SMAD signalling downstream of TGFß/BMP binding. Therefore, it has been proposed that PDGF-BB up-regulates miR24, which then diminishes Trb3 expression and prevents SMAD signalling. Ultimately, this promotes the switching to PDGF-BB signalling resulting in a synthetic and reparative phenotype of the vSMCs (199).

Investigation into the role of miR24 with regards to OA has also been conducted. Most notably a link between miR24 and tumour suppressor protein, $p16^{INK4a}$ has been established, a relationship that has already been shown in the cancer field (196). Philipot *et al* have reported that miR24 is a negative regulator of $P16^{INK4a}$

which is associated with the senescence-associated secretory phenotype (SASP). The SASP is a collection of catabolic agents including matrix remodelling enzymes MMP1 and MMP-13, along with pro-inflammatory cytokines, IL-8 and IL-6. Data demonstrate that OA associated cytokine IL-1β can increase the expression of $P16^{INK4a}$ along with the other SASP elements via the down-regulation of miR24. Transfection of primary chondrocytes with $P16^{INK4a}$ can also significantly up-regulate MMP-1 and MMP13 and can modulate the expression of IL-8. Furthermore, the OA associated reduction in miR24 expression negatively correlates with $P16^{INK4a}$. Finally, Philipot demonstrates that if miR24 is downregulated *in vitro,* there is a subsequent increase in both $P16^{INK4a}$ and MMP1 expression (195). Therefore, the disease associated drop in miR24 could be contributing to the catabolic insult observed in OA.

Other members of both clusters containing miR24 have also been implicated in OA pathology. MiR27b has been shown to inhibit IL-1β induced MMP-13 expression (200), however, in OA miR27b expression has been shown to be decreased. Transcripts that are known to promote anabolic signalling, such as Insulin-like growth factor binding protein -5 (IGFBP-5), have been shown to be targeted by miR27a (193). MiR27a inhibition in primary chondrocytes, results in increased IGFBP-5 expression after 72 hours, which in the cartilage can lead to decreased ECM breakdown (193).

1.4.6 MicroRNA 342

MiR342 has not been directly linked to OA, but has been studied with regards to obesity which is considered one of the major risk factors for OA. Murine miR342 was shown to be up-regulated during the development of obesity (201). Further work has brought to the forefront miR342's ability to induce adipogenesis in human derived adipose MSCs (203) which have also been linked to OA disease development.

In a similar fashion to miR24, miR342 has also been predominantly studied in the cancer field, with a downregulation of the miRNA being noted in cancer derived tissue compared to normal comparator tissue. Furthermore, miR342 has been recently highlighted as having tumour-suppressing properties, via direct targeting of the 3'UTR of both forkhead box (FOX) superfamily proteins, FOXM1

and FOXQ1. These particular FOX proteins are highly expressed in colorectal cancer inducing invasiveness, migration and proliferation of the colorectal cancer cells. The discovery that miR342 can supress the activities of these proteins could be harnessed therapeutically leading to new treatment strategies (204). There are also implications for miR342 in breast cancer through the modulation of inhibitor of differentiation 4 (ID4) and subsequently the breast cancer gene BRCA, which is known to be implicated in the heritability of breast cancer (205).

1.4.7 miRNA in the Circulation

Biomarker discovery for disease is a large area of intensive research. There are certain criteria that are critical for biomarker identification: disease specificity, ease of access, stability and responsiveness to treatment. Disease specific proteins in the plasma have been the most widely used biomarkers to date, however, they are not always best suited to the task (206). They have been shown to be subject to a variety of post-translational modifications, have complex structural composition in the blood and have relatively low abundance; thus detection can be difficult. There is a need for new and better biomarkers to be identified enabling better diagnosis and prognosis (206) especially for OA. MiRNAs may be the new biomarkers that are being sought. MiRNAs have been shown to be actively transported from the cells in which they are synthesised (207) and can therefore not only be found in circulating cells but also in bodily fluids. In 2010, Weber *et al* looked at the miRNA expression profile of 12 different bodily fluids, including plasma and urine. From this it was concluded that there were both common and fluid specific miRNAs. Therefore, as a potential biomarker they would be easy to measure in blood and/or urine samples. MiRNAs have also been shown to be stable within the plasma as they can secreted in membrane bound particles called exosomes, preventing then from being degraded by ribonucleases (208). Alternatively, the miRNA may be protected via other mechanisms such as association with RNA protein complexes or modification rendering the miRNA non detectable by the ribonucleases. Importantly, miRNAs have been shown to be disease specific, with a large literature detailing the specificity of certain miRNA to disease processes. A recent review has collated all the miRNA that have been proposed as disease

specific biomarkers for a variety of different cancers, including prostate cancer, breast cancer, colon cancer, lung cancer and also for myocardial infarction (206).

In 2015, Beyer *et al* used the Bruneck cohort in order to identify Let7e as a predictor for future need for arthroplasty(209). The cohort contained 816 individuals who had a baseline visit for the study in 1995. At this visit blood samples were taken and these patients were then followed until 2010. A total of 12 miRNA were identified from an initial screen using a subgroup of the cohort, these miRNA were identified as being altered in the plasma of those who had went on to develop OA. These miRNA were then taken forward in the whole cohort and when further analysis was conducted, Let7e was found to be decreased in the individuals who went on to receive one or more joint replacements (209). The Bruneck cohort has now been used to successfully investigate plasma miRNA alterations in diabetes, cardiovascular disease and osteoarthritis.

A miRNA signature has also been determined by Li *et al* between early and late stage OA SF (210). Early OA was defined by the presence of a meniscal tear requiring arthroscopy and late stage disease was defined by the need for arthroplasty. A total of 752 miRNA were screened with 14 being taken forward for validation in a larger cohort. It was found that 7 miRNA were differentially regulated: miR24-3p, miR29c-3p, miR34a-5p, miR23a-3p, miR27b-3p, miR186-5p and miR27a-3p(210). These miRNA remained significantly up-regulated in late stage disease when normalised to gender, age or BMI. Therefore this panel could serve as biomarkers for differentiation between early and late stage OA.

1.5 Aims and Hypothesis

The expression of BMP-7 in OA is negatively correlated with disease severity. Recombinant BMP-7 has been considered as a potential therapy for OA, this is based on its chondroprotective properties which have been robustly established in animal models of OA. A human clinical trial has highlighted the safety and tolerance of BMP-7 and demonstrated improved clinical outcomes as determined by improved WOMAC scores and OARSI responder criteria. However, the expression pattern of BMP-7 in articular chondrocytes is still controversial and the impact of BMP-7 stimulation on chondrocytes and macrophages biology is unknown. Therefore it has been hypothesised that a disease derived loss of BMP-7 and its signalling pathway in OA is mediated by disease-associated miRNA. Further to this, it has been hypothesised that BMP-7 promotes anabolic processes in chondrocytes, via modulation of it's own signalling family, and has an anti-inflammatory impact on macrophages in the joint.

In order to robustly test these hypotheses the following aims were proposed;

- To investigate BMP-7 expression and the expression of associated signalling cascade members, at both the transcript and protein level, in a well-defined cohort of OA patients.

- To deduce if BMP-7 or any of the related transcripts are controlled by OA associated miRNAs.

- To delineate the effect of exogenous BMP-7 on chondrocytes and macrophages.

2 Materials and Methods

2.1 Sample Acquisition

Samples were acquired from patients undergoing Total (TKR) or Partial/Unilateral (UKR) knee replacement at the Glasgow Royal Infirmary. This study was approved by the ethical committee at the Glasgow Royal Infirmary, Scotland. Patients were consented at the clinic prior to elective surgery. On the day of surgery samples were collected in Dulbecco's modified Eagle's medium (DMEM) (Gibco® Life Technologies) with 1% penicillin/streptomycin (P/S) (Sigma Aldrich) and transported back to the laboratory for processing.

For cohort analysis the following samples were acquired from TKR (Figure 2.1).

- Peripheral blood
- Cartilage femoral notch biopsy
- Cartilage from
    - Distal medial condyle (DMC)
    - Distal lateral condyle (DLC)
    - Posterior medial condyle (PMC)
    - Posterior lateral condyle (PLC)
    - Medial tibial plateau (MTP)
    - Lateral tibial plateau (LTP)
- Synovial membrane
- Synovial fluid.

Patients undergoing UKR did not have any of the lateral samples listed or the DMC.

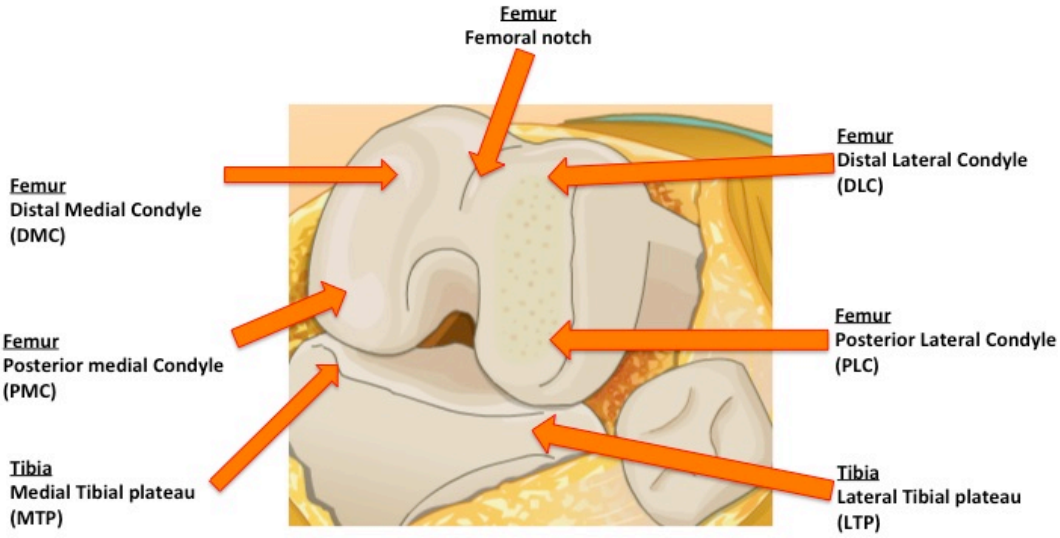


Figure 2.1: Anatomical location of the cartilage samples from total knee replacement.

2.1.1 Peripheral Blood Processing

OA patient peripheral blood or blood from healthy controls was layered over histopaque (Sigma Aldrich) and centrifuged at 300g for 30 minutes (mins), with no break, isolating plasma from red blood cells and peripheral mononuclear cells. Plasma was aliquoted and stored at -80°C.

2.1.2 Synovial Fluid Processing

Synovial fluid was aspirated from the joint at the time of initial incision during surgery. Fluid was stored in an EDTA vacutainer to prevent coagulation. Synovial Fluid was centrifuged at 400g for 5 mins at room temperature (RT) to pellet the cells present in the fluid. The synovial fluid was then aliquoted and stored at -80°C.

2.1.3 Cartilage and Synovium Processing

Cartilage and synovial membrane samples were either processed for RNA extraction (section 2.2/2.6) or placed in formalin for fixation and subsequent Immunohistochemical (IHC) analysis (section 2.8). Femoral notch biopsy was processed for RNA extraction only.

2.1.4 Cartilage and X-Ray Grading

At the time of excision, the cartilage was scored using the Outerbridge grading system for cartilage degradation outlined in Table 2-1. Patient joint X-rays were also scored using the Kellgren and Lawrence outlined in Table 2-2.

Outerbridge Grade	Description
0	Normal
I	Cartilage with softening and swelling.
II	A partial – thickness defect with fissures on the surface that do not reach subchondral bone or exceed 1.5cm in diameter
III	Fissuring to the level of subchondral bone in an area with a diameter more that 1.5cm
IV	Exposed subchondral bone

Table 2-1 Outerbridge classification (87-89)

Kellgren and Lawrence Grade	Description
0	No radiographic features of OA present
1	Unlikely narrowing of the joint space, possible osteophytes
2	Possible joint space narrowing, small osteophytes
3	Joint space narrowing, multiple moderately sized osteophytes, some sclerotic areas and possible deformation of the bone ends.
4	Severe joint space narrowing, multiple large osteophytes, marked sclerosis and definite bony end deformity

Table 2-2 Kellgren and Lawrence Scoring system (84,86).

2.2 Cartilage Processing

2.2.1 Mortar and Pestle

Samples were cut into small sections and placed in a mortar and liquid nitrogen was poured over the samples in order to snap freeze the tissue. Once frozen the samples were crushed into a powder with a pestle. The powder was transferred into an eppendorf and 700µl QIAzol® (QIAGEN®) was added. A 7mm steel bead (QIAGEN®) was placed in the tube and the powder was further homogenised for 30 min using a TissueLyser LT (QIAGEN®).

2.2.2 Liberase™ Digest

Cartilage and synovium samples were cut into small sections and placed into 5ml Roswell park memorial institute medium (RPMI) (Gibco® Life Technologies), supplemented 1% P/S. 150µg/ml Liberase™ (Roche Diagnostics) was added and the tissue was incubated overnight at 37°C with gentle shaking. For femoral notch biopsy samples, the cartilage was removed from the bone and the whole cartilage biopsy was placed in the Liberase™ supplemented media. The Liberase™ solution was passed through a 70µM cell strainer (BD Falcon™) to obtain a single cell suspension. Cells were centrifuged at 400g for 5mins, the pellet was resuspended in Phosphate buffered saline (PBS) (Gibco® Life Technologies) and centrifuged again. Following the centrifugation, the pellet was either lysed in 700µl QIAzol® for subsequent RNA analysis (section 2.6), or cells were plated for in vitro cultures (section 2.3).

2.3 Primary Human Chondrocyte Cell Cultures

Following Liberase™ digestion (section 2.2.2) primary human chondrocytes were plated at 1.5x 10^5/ml in complete DMEM (cDMEM) comprised of DMEM supplemented with 1% P/S, 1% L-Glutamine (Sigma Aldrich), 10% Heat inactived foetal bovine serum (FBS) (Invitrogen) and Fungizone® Antimycotic (Life Technologies). Cell were left for 48hrs at 37°C, 5% CO_2 to settle onto the plastic.

2.3.1 Cytokine Stimulations

Primary chondrocytes were stimulated with a variety of cytokines in order to assess any associated changes in transcript and protein expression. Human recombinant BMP-7, IL-1ß and TGFß (PreproTech) were used at a concentration of 100ng/ml or 10ng/ml. Cells were stimulated for 8 hours before cells were either lysed in Qiazol® for RNA analysis or placed in fresh cDMEM for a further 24 or 48 hours. At each of the subsequent time points the cells were lysed in Qiazol® for RNA analysis (Section 2.6) and the supernatants were harvested for subsequent analysis (section 2.9).

2.3.2 miRNA Mimic/Inhibitor Transfections

In order transfect primary human chondrocytes, the following transfection reagents were tested: Lipofectamine® (Thermo fisher), DharmaFECT 3 (Dharmacon) and TransIT TKO ® (Mirus Bio LLC). Transfection efficiency was used to determine which reagent was optimal for transfecting the miRNA mimic into the cell. Analysis of the transfection efficiency was conducted by evaluating the percentage of cells that were positive for the control inhibitor conjugated to alexa fluor 488 (imaged using the MAQSQuant flow cytometer). The most efficient transfection reagent was determined to be DharmaFECT 3.

2.3.2.1 DharmaFECT 3

DharmaFECT 3 was used according to the manufacturers instructions. MiRNA mimics were all used at a final concentration of 25nM. Briefly, DharmaFECT 3 (2µl) was diluted into 50µl serum free media (DMEM, 1% P/S and 1% L-Glutamine) and incubated at RT for 5mins. MiRNA mimic or control mimic were diluted in serum free media to obtain a concentration of 250nM and incubated for 5 mins at RT. DharmaFECT 3 and the miRNA or control mimic were combined to create a transfection solution and incubated at RT for a further 20 mins. Fresh complete media (400µl) was added to the cells along with 100µl of the transfection solution, creating a final concentration of 25nM mimic or control in each well.

2.4 SW1353 Cell Stimulations

SW1353 cells (human chondrosarcoma cell line) were plated at a variety of seeding densities depending on the passage of the cells. Cells were plated and left O/N at 37°C before being stimulated for either 3, 8 or 24 hours with the following cytokines BMP-7, IL-1β, TGFβ, IL-6 or TNFα (PreproTech) at a variety of concentrations: 5, 10, 50 and 100ng/ml or in combination (see figure legend for specific details for each experiment). Cells were lysed in Qiazol® and stored at -20°C for mRNA analysis (section 2.6).

2.5 Primary Human Macrophage Cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from blood using a histopaque (Sigma Aldrich) density centrifugation (section 2.1.1). The Scottish National Blood Transfusion Service supplied concentrated blood, increasing the numbers of PBMCs available. Monocytes were isolated from the PBMCs using a CD14/CD16 negative selection kit (carried out by Simone Kidger or Lewis Rodgers). The monocytes were plated at 1 x 10^6/ml and in complete RPMI with the addition of 100ng/ml M-CSF (PreproTech). Cells were then left to differentiate into macrophages for 6 days at 37°C, 5% CO_2. On day 6, cells were either primed with IFNγ (PreproTech) 20ng/ml overnight (M1), or placed in fresh M-CSF supplemented media as appropriate. On day 7,cells were stimulated as required with either IL-4 (PreproTech) 100ng/ml (M2), BMP-7 100ng/ml (M0+BMP-7) or M-CSF 100ng/ml (M0/M1). At this point some cells were also challenged with lipopolysaccharides (LPS) from *Salmonella Minnesota* (Sigma Aldrich) 15ng/ml. Stimulations were carried out for 24 hours before supernatants were harvested and stored at -20°C for subsequent analysis (section 2.9). Cells were lysed in Qiazol® and processed for RNA extraction (section 2.6).

2.6 RNA Extraction and cDNA Synthesis

2.6.1 miRNeasy Mini Kit

RNA was extracted using the miRNeasy mini kit (QIAGEN®) following the manufacturers protocol. All centrifugation steps were at RT unless otherwise stated. In short, 140µl chloroform (Sigma Aldrich) was added to the sample which had previously been lysed in QIAzol®. This was then shaken at RT for 5 min. Samples were then spun at 12,000g for 15mins at 4°C and the resulting upper aqueous phase was transferred to a new eppendorff. Ethanol was added at approximately 1.5X the volume of the aqueous phase and mixed thoroughly by pipetting. The sample was transferred to an RNeasy® mini column and spun at >8000g for 30sec. Flow through was then discarded. RWT Buffer was added to the spin column and the spin step was repeated. Flow through was again discarded. RPE buffer was then added to the spin column and spun as before. A further 500µl RPE buffer was added to the column and this was spun at >8000g for 2 min, flow through was then discarded. The column was transferred to a new 2ml collection tube and spun for 1min at full speed to dry the column. The RNeasy® Mini column was placed in a new 1.5ml collection tube and 30µl RNase free water was added to the center of the RNeasy® Mini column membrane. This was spun for 1 min at full speed to elute the purified RNA. RNA concentration was determined (section 2.6.4) and was then stored at -20°C.

2.6.2 DNase Digest

For all primary chondrocyte RNA extractions carried out for MRNA analysis a DNA digest step, using DNase Set – RNase Free kit (QIAGEN®), was included. RNA was processed as previously described up to the addition of the RWT buffer. At this point 350µl RWT was added to the column and centrifuged at >8000g for 30 secs at RT. Flow through was then discarded. 10µl DNase1 and 80µl RDD buffer were then added to the column and left at RT for 15 mins. A further 350µl RWT buffer was added to the column before it was spun >8000g for 30 secs at RT. The flow through was then discarded. The RNA extraction then proceeded as outlined above with the addition of RPE buffer.

2.6.3 Serum and Plasma Extraction with *C.Elegans* miR39 Spike

Plasma and synovial fluid from OA patients (sections 2.1.1/2.1.2) was taken and subject to RNA extraction. The columns from the miRNeasy® kit (detailed previously) were used with the QIAGEN® protocol for their serum and plasma kit. In short, 200µl plasma or synovial fluid was added to 1ml Qiazol®. To this, $1.6x10^8$ copies/µl of miR 39 (miRNeasy® Serum/Plasma Spike-In Control, QIAGEN®), were also added. Choloroform, 200 µl, was added and tubes were shaken and incubated for 3mins at RT before being spun at 12,000g for 15min at 4°C. The upper aqueous layer was then transferred to a fresh tube and mixed with 1.5X 100% ethanol. The resulting mixture was then added to a spin column. The column was spun at >8000g for 30 secs at RT and the flow through was discarded. RWT buffer was added to the column and spun as before. RPE buffer was added and the column spun as before. Flow through was again discarded. 500µl 80% ethanol was added to the column and spun at 8000g for 2 mins at RT. The spin column was then transferred to a fresh 2ml collection tube and centrifuged at full speed for 1min at RT to dry the membrane. The column was then placed in a 1.5ml eppendorf and 14µl RNase free water was added to the membrane to elute the RNA. RNA was stored at -20°C.

2.6.4 RNA Quantification

RNA concentration was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and the NanoDrop™ software (Thermo Scientific). The 260/230 values were used to assess RNA purity and 260/280 to assess contamination.

2.6.5 cDNA Synthesis

2.6.5.1 AffinityScript™

RNA was converted to cDNA using AffinityScript™ cDNA synthesis kit (Agilent) following the manufacturers instructions. Unless otherwise stated cDNA was synthesised at 5ng cDNA/µl. A volume of 10µl RNA at a concentration of 10ng/µl was added to 3µl random primers (0.1µg/ml) and 2.7µl RNase® free water. This was incubated at 65°C for 5mins then cooled to 20°C for 10mins. To

this, 0.5µl RNase block ribonuclease inhibitor (40U/µl), 0.8µl dNTP's (100mM), 2µl AffinityScript RT Buffer (X10) and 1µl AffinityScript multiple temperature reverse transcriptase RT were added, bringing the total volume to 20µl. This was then incubated at 25°C for 10 mins, 42°C for 5mins, 55°C for 60mins, 70°C for 15mins and finally cooled to 4°C. All temperature adjustments were made using a 2720 thermal cycler (Applied Biosystems).

2.6.5.2 Miscript ®

cDNA synthesis from the cartilage for miRNA analysis was carried out using MiScript® reverse transcription kit (QIAGEN®). In short, 10µl RNA at a concentration of 10µg/µl was added to 4µl miScript® HiFlex buffer (5X), 2µl miScript® nucleics mix (10X), 2µl RNase™ free water and 2µl MiScript® reverse transcriptase. This was incubated at 37°C for 60mins followed by 95°C for 5mins. Incubations were carried out using a Veriti 96 well thermal cycler (Applied Biosystems). After the incubation 200µl RNase® free water was added to the RNA, resulting in a final cDNA concentration of 0.45ng/µl.

For plasma 10µl of the RNA at an unknown concentration was used to make the cDNA using the same protocol. This was carried out in order to keep the concentration of the spike in control, miR 39, consistent over all the samples.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Endpoint Polymerase Chain Reaction (PCR)

2.7.1.1 Nested PCR

PCR for endpoint analysis was carried out using MyTaq Red™ (Bioline) following the manufacturers instructions. In short, 10ng cDNA was added to 25µl MyTaq Red™ master mix along with 1nM final concentration of 'outside' BMP-7 forward (3'GACGCTGGTCCACTTCATC5') and reverse primers (5'GAGCAATGGAGGATCCAGAAA3'), and 21µl of RNase® free water. A non template control (NTC) which included all the components of the reaction excluding the cDNA was also set up. In the NTC, cDNA was substituted by 2µl RNase® free water. A PCR reaction was carried as follows; samples were

incubated at 94 °C for 5 mins, followed by 35 cycles of 94°C for 15sec, 55°C for 30secs and 72°C for 30sec and a final incubation at 72°C for 10mins before cooling to 4°C. All temperature-controlled incubations were carried out in a 2720 thermal cycler (Applied biosystems). Samples were run on a 1.8% agrose gel containing 0.015% ethidium bromide with 10µl of sample loaded per well. The gel was run for approx. 45 mins with a current of 110V in Tris-base, acetic acid and EDTA (TAE) buffer. The product from the 1st PCR (2µl) was added to fresh myTaq red™ master mix and RNase® free water as before. However 'inner' BMP-7 forward (3'CCACTTCATCAACCCGGAAA5') and reverse primers (5'GAGCAATGGAGGATCCAGAAA3') were added to the samples. Another PCR reaction was run using the same conditions as previously stated. This resulted in a total of 70 PCR cycles. A second NTC was also set up using 2µl of the NTC reaction from the first PCR. The product for the second PCR was then taken and run on a 1.8% gel containing 0.015% ethidium bromide. The gel was run for 1hour at 110 volts in TAE buffer and visualised as before using the AlphaImager ™ (Alpha Innotech).

2.7.2 Quantitative Polymerase Chain Reaction (qPCR)

2.7.2.1 TaqMan® Analysis

Transcript analysis with TaqMan® primer probes was carried out as follows. 5µl TaqMan® master mix, without uracil N-glycosylase (UNG) was combined with 0.5 µl TaqMan® primer probe and 3.5 µl RNase® free water. cDNA at a concentration of 5ng/ul was added to the 9µl of the above combined reagents, and the total 10µl solution was added to a 384 well plate for each sample in triplicate. A NTC was also set up for each gene analysed. The following thermal cycle was applied to the plate using a 7900 HT fast real-time PCR machine (Applied Biosystems); 10 minute incubation at 95°C followed by 40 cycles of 95°C for 15 secs and 60°C 1 min, using SDS software (version 2.4). Analysis was conducted using RQ manager (version 1.2.1) (Applied Biosystems).

2.7.2.2 Power SYBR® Analysis

Power SYBR® green was used for analysis of mRNA transcripts. A total of 3ng cDNA (equivalent of 0.6µl cDNA) was added to 5µl power SYBR® green, and 1nM of forward and reverse primers (0.1µl of each primer) see table 2.3 for primer sequences. This mix was added to each well in a 384 well plate and was made up to 10µl with RNase free water. Each sample was run in technical triplicate, and each gene run had an associated NTC. Plates were run on a 7900 HT fast real-time PCR machine (Applied Biosystems), using SDS (version 2.4) software and analysed using RQ manager (version 1.2.1) both from Applied Biosystems. The following thermal profile was applied to all samples, 50°C 2mins, 95°C 10mins then 40 cycles of 95°C 15secs and 60°C 1min.

2.7.2.3 MiScript SYBR Green Analysis

MiScript SYBR® green allows the quantification of miRNAs following MiScript® reverse transcription cDNA synthesis. cDNA at a final concentration of 0.025ng/µl was added to 5µl MiScript SYBR® green, 1µl miRNA specific primer (QIAGEN®), 1µl universal primer (QIAGEN®). This was added to each well of a 384 well plate and brought to a final volume of 10µl with RNase® free water. Each sample was analysed as a technical triplicate and a NTC was also set up for each gene analysed. Plates were run on a 7900 HT fast real-time PCR machine (Applied Biosystems), using SDS (version 2.4) software from Applied Biosystems. The plate was incubated for 15 mins at 95°C before being subject to 40 rounds of PCR with the following conditions, 94°C for 15secs, 55°C for 30secs and 70°C for 30secs. All data collection was carried out at the final step in each cycle. Analysis was carried out using RQ manager (version 1.2.1) from Applied Biosystems.

Gene	Size	Forward Primer	Reverse Primer
BMP-7	216	GACGCTGGTCCACTTCATC	GAGCAATGGAGGATCCAGAAA
BMPR2	120	GACAGGAGACCGTAAACAAGG	CCATATCGACCTCGGCCAATC
COL1A1	140	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
COL2A1	244	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
FOLLISTATIN	128	GAGCAATGCAAACCTCACAAG	CAGTGTCCATCGTAATCAACCTG
AGGRECAN	81	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
CD44	109	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT
ACVR1	77	GTGAAGGTCTCTCCTGCGGTA	GCCATCGTTGATGCTCAGTGA
GREMLIN 1	151	TCATCAACCGCTTCTGTTACG	GCTGTAGTTCAGGGCAGTT
BMPR1B	130	TTTTGCGAAGTGCAGGAAAAT	TGTTGACTGAGTCTTCTGGACAA
Furin	187	CCTGGTTGCTATGGGTGGTAG	AAGTGGTAATAGTCCCCGAAGA

Table 2-3 Primers sequences as found using the online resource Primer Bank (https://pga.mgh.harvard.edu/primerbank/).

2.7.3 Digital ddPCR™ (Bio-Rad)

Digital PCR was carried out on the cartilage and synovium. Bio-Rad supplied all machines for this protocol. cDNA (2µl) from cartilage or synovium at a concentration of 2ng/µl, was added to 10µl ddPCR™ master mix with Taqman® specific probe and 8µl RNase® free water. This solution was then added into the droplet generator cartridge along with 70µl ddPCR™ droplet reader oil. This was then placed into the QX200 droplet generator, creating 20,000 droplets for each sample, with a random distribution of target positive and negative cDNA in the droplets. The droplets were then transferred to a 96 well plate where they were subject to PCR using a thermal cycler (C100 touch, Bio-Rad). The droplets now with the amplified cDNA were then read by the QX200 droplet reader. Positive droplets contain target cDNA are detected based on the incorporation of the fluorophore from the TaqMan® primer. Quantasoft software then measured the number of positive and negative samples and the Poisson algorithm was used to calculate the number of copies per µl of cDNA.

2.7.4 qPCR Data Quantification

Analysis for qPCR data was conducted as follows. CT values for the gene of interest were subtracted form the CT values from the housekeeping gene giving the ΔCT. This was then either converted into $2^{-\Delta CT}$ or was used to calculate fold change $2^{-\Delta\Delta CT}$ ($2^{-(}$$^{(\Delta CT)\ (\text{gene of interest})}$$_{-\ \Delta CT\ (\text{control})}$$^{))}$.

2.8 Immunohistochemistry

2.8.1 Sample Fixation and Processing

Cartilage and synovium samples were fixed in 10% neutral buffered formalin (Leica Biosystems) for approximately 2 weeks before either being stored in 70% ethanol (synovium) or being decalcified (samples containing bone). Decalcification was carried out via rotation in EDTA buffer for approximately 2 – 4 weeks. EDTA was refreshed every 2-3 days for the first week then every week thereafter. Following decalcification, samples were stored in 70% ethanol. All samples were then penetrated with wax using a Shandon CITADEL 1000 (Thermo

Scientific). The samples are placed into cassettes and subject to the following: 30mins 70% ethanol, 1hour 70% ethanol, 1 hour 90% ethanol, 1hour 95% ethanol, 2 hours 100% ethanol, 2.5 hours 100% ethanol, 3 hours 100% ethanol, 2 hours xylene, 2 hours xylene, 2.5 hours xylene, 5 hours wax, 6 hours wax (When the same reagent is used in subsequent stages, fresh solution is used unless otherwise stated). After wax penetration, the samples were embedded into wax blocks and cooled to 4°C , allowing the wax to set. The blocks were then stored at room temperature until needed. Blocks were pre-cooled to 4°C before 2-4µm sections were cut using a stainless steel feather microtome blade, N35HR (PFM Medical). In some cases Mollifex® (VWR) was used to soften the cutting edge of the tissue.

2.8.2 IMMPRESS Method

Sections were placed in a 60°C oven for 1 hour to allow the wax to soften. Sections were then hydrated as follows: xylene 5mins, xylene (fresh) 5mins, 100% ethanol 3mins, 95% ethanol, 90% ethanol 3mins, 70% ethanol 3mins, running water 5mins. The sections were then washed in Tris-buffered saline, 0.05% Tween20 (TBST) for 5mins. For all cartilage sections, antigen retrieval was carried out with an overnight incubation in Unitrieve (Gentaur) at 42°C . Synovial sections were exposed to heat induced antigen retrieval method. Slides were immersed in boiling 0.01M citrate buffer and boiled for a further 8mins. Peroxidase activity was then quenched using a 30min incubation in 0.5% hydrogen peroxide in methanol (both Sigma Aldrich). Sections were blocked with 2.5% horse serum (Vector) and 2.5% human serum for 30mins followed by a 30mins FC block (Innovex Biosciences). The BMP-7 primary antibody either raised against the whole protein (ab54904 Abcam) or against the pro region of BMP-7 (ab56023 Abcam) was added at 6µg/ml along with the appropriate isotype control either mouse monoclonal IgG1 or rabbit polyclonal DA1E mAB IgG (Cell Signalling) respectively. These were then incubated in a humidity chamber at 4°C O/N. Slides were brought back to RT and washed for 5mins in TBST then 5mins in distilled water. A species specific ImmPRESS™ (Vector) kit was then added to the slides for 1hr: total BMP-7 – mouse ImmPRESS™ or pro BMP-7 – rabbit IMMPRESS. Sections were then washed as before then incubated with ImmPACT™ DAB peroxidase substrate kit (Vector) until the desired stain intensity

developed. The sections were then rinsed in running water. Nuclear staining was carried out with haematoxylin (Cell Path) before the sections were dehydrated through alcohol back to xylene as follows. 70% ethanol (2 dips), 90% ethanol (20secs), 100% ethanol (2X 3mins) and finally xylene (2X 5min). Finally slides were mounted with a cover slip in DPX mountant (Raymond A Lamb) and left to dry before images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

2.9.1 IL-6, IL-10, and TNF α ELISA

IL-6, IL-10 and TNF α secreted into the supernatants from cultured macrophages under a variety of differentiation conditions were analysed using Cytoset assays (Life Technologies), following the manufacturers protocol. In short, plates were incubated with specific capture antibody at 4 °C overnight, followed by a PBST (Phosphate Buffered Saline, 0.05% Tween) wash. Plates were then blocked in assay buffer (PBS + Bovine serum Albumin 0.5%) for 1hr at RT followed by PBST wash (X5). Samples and standards were added to the plates, along with detection antibody and incubated on a shaker for 2hrs at RT. Plates were then washed as before. The top standards were diluted to 1000pg/ml for both IL-6 and IL-10 and to a concentration of 2000pg/ml for TNF α. Strepavidin-horse radish peroxidase (HRP) was then added to the plates for 30mins and washed as before. Tetramethylbenzidine (TMB) (E-Biosciences) substrate was added to the plate. Once the assay had developed to the desired intensity, the reaction was terminated with the addition of 4N Sulfuric acid. The plates were then read on a Tecan Sunrise™ absorbance reader and analysed with Magellan™ data analysis software.

2.9.2 BMP-7 ELISA

Human BMP-7 Duoset ELISA development kit (DY354) (R&D Systems) was used to assess BMP-7 levels in plasma and synovial fluid samples, manufacturers protocol was followed. In brief, plates were coated with capture antibody overnight at room temperature. Plates were then washed (X3) in PBST (Phosphate Buffered Saline, 0.05% Tween) in order to remove the excess capture antibody. The wells were blocked in reagent diluent (1% BSA in PBS) for 1 hour. The plates were then washed (X3) before the addition of the patient samples and recombinant BMP-7 standards. The top standard was diluted to a concentration of 4000pg/ml and all patient samples were incubated with heteroBlock (Omega Biologics) to eliminate non-specific binding before being added to the plate. Following standard and sample addition the plate was placed on a shaker at RT for 2 hours. The wash step was again repeated (X3), and the detection antibody at a concentration of 0.5µg/ml was added (reagent diluent supplemented with 2% heat inactivated normal goat serum was used to dilute the detection antibody). Plates were left to shake for 2 hours at RT, before being washed as previously stated. Streptavidin-HRP was then added into each well and incubated for 20mins with shaking at RT. This was followed by a final wash and the addition of TMB substrate to the plate. Once the assay had developed to the desired intensity, the reaction was terminated with 4N Sulfuric acid. The plates were then read on a Tecan Sunrise™ absorbance reader and analysed with Magellan™ data analysis software.

2.10 Western Blot

Protein lysates were created using RIPA buffer (Thermo Scientific) containing 1X HALT™ protease and phosphatase inhibitor cocktail (Thermo Scientific). The buffer was added on ice and the samples were shaken for 10mins before being spun at 20,000g for 10min at 4°C. Supernatant was then removed and stored at -20°C. Samples were loaded according to volume, with 40µl protein lysate being added to 1X NuPAGE sample reducing agent (Novex Life Technologies) and 1X NuPAGE sample buffer (Novex Life Technologies). The solution was then boiled at 95°C for 5mins, denaturing the protein content of the sample. A NuPAGE 4 -12 % Bis Tris gel (Novex Life Technologies) was loaded with 30µl of sample per well,

NOVEX® Sharp pre-stained protein ladder (10µl) (Novex Life Technologies) was also loaded onto the gel. Samples were run in 1X NuPAGE MOPS SDS running buffer for 1hour using a 200V current. Following this, gels were transferred onto a nitrocellulose membrane using the iBlot system (Thermofisher). Efficiency of transfer was assessed using Ponceau Red (Sigma Aldrich) staining.

For specific protein detection the membrane was blocked in 5% milk (Marvel) in TBST for 1 hour at RT with shaking. Membranes were then incubated with BMP-7 antibody raised against the pro region of BMP-7, 1µg/ml (ab56023 rabbit polyclonal, Abcam) at 4°C O/N with shaking. Membranes were then washed (X3) in PBST before the appropriate secondary antibody conjugated to HRP was diluted at 1:1000 in TBST and added to the membrane shaking for 1 hour at RT. Secondary antibody was a polyclonal goat anti rabbit immunoglobulin/HRP (Dako). The membrane was washed as previously stated, the HRP substrate WestPico (Thermofisher scientific) was added to the membrane and the chemiluminescence was visulaised using the C500 gel imager (Azure Biosystem) and the C series software program (Azure Biosystem).

Membranes were stripped using Restore™ western blot stripping buffer (Thermo Scientific). Stripping buffer was incubated with the membrane for 15 mins at 37°C then for 15 mins at RT shaking. Housekeeping analysis was then carried out on the membrane. The membrane was blocked in 5% milk, followed by the addition of the HRP conjugated GAPDH (D16H11) XP (R) Rabbit antibody (Cell Signalling) for approximately 2 hours shaking at RT. WestPico addition and analysis was conducted as previously stated.

2.11 Luminex Assay

Analysis of a panel of inflammatory cytokines in the supernatants from primary chondrocyte cultures was carried out using a human cytokine 14 plex assay (Life Technologies). Analytes included IL-1ß, IFNα, IL-6, IL-12, RANTES, MIP-1α, MIP-1ß, GM-CSF, MCP-1, IL-Rα, IL-17, IP-10 and IL-2R, IL-8. The assay was carried out as per manufacturers instructions. Analysis was conducted using the Bioplex systems platform (Biorad).

2.12 In Silico Analysis

In Silico analysis was carried out on ATTA binding sites for the 3'UTR of the BMP-7 transcript using AREsite2 (http://nibiru.tbi.univie.ac.at/AREsite2/welcome).

2.13 Statistics

All statistical analysis was carried out using Graphpad Prism version 6e. The specific tests that have been applied are outlined in each figure legend. Where appropriate, D'Agostino-Pearson normality test was applied in order to determine if the data displayed a Gaussian distribution. In this study a P value of 0.05 was considered significant, with * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3 Optimisation of cartilage analysis techniques for *ex vivo* and *in vitro* studies

3.1 Introduction

Osteoarthritis (OA) is a common degenerative disease of the joint, whereby all elements of the joint can display pathology. However, one of the overarching features of this disease is cartilage erosion. Cartilage is a fundamental component of the joint and is mainly comprised of ECM. The ECM constitutes 90% of the total cartilage volume (211), with the remaining 10% being comprised of chondrocytes, the only cell present in the cartilage. As cartilage is one of the main tissues involved in OA pathogenesis it is imperative to understand what is happening to this tissue in a disease setting. To this end, acquisition of tissue from OA patients undergoing total or partial knee replacement offers great insight into the end stage of OA. There are, however, challenges within the field regarding the acquisition of high quality genetic information from this tissue due to its sparse acellular nature.

Previously, other groups have tried several methods of RNA extraction from cartilage with all reporting varying levels of success. As stated above, one of the main issues facing extraction of good quality RNA is the scarcity of cells (212). However, this is not the only problem, the high abundance of proteoglycans present in the tissue also poses difficulties as these are often co-purified along with the RNA (212). Historically this has hindered the study of transcripts in these cells in a direct *ex vivo* setting, with many studies resorting to culturing the cells before molecular analysis (212). As this study aims to look at the cartilage in an *ex vivo* capacity it is essential to determine and optimise the most efficient method for RNA extraction. Over recent years, groups have worked on new protocols for the extraction of RNA from cartilage. A popular technique is to freeze the tissue in liquid nitrogen and grind it into a powder before the addition of lysis buffer and subsequent RNA analysis (212-214). Alternatively, the ECM can be enzymatically digested releasing the chondrocytes, which can then be lysed.

Grinding the tissue before homogenisation is a popular method of *ex vivo* analysis, with a various versions of this method that that have been optimised. It has been suggested that all equipment should be kept at a steady temperature via incubation in liquid nitrogen (212,213), thus making the tissue more brittle. This works well if a tissue mill is available, however, if a mortar and pestle are the only available tools for grinding the tissue, this is not always practical for the user, as only the tissue can be snap frozen. Further to this, many protocols suggest that once the tissue had been ground and the lysis reagent had been added, the sample should be spun at a high speed for a prolonged period of time ranging from 10mins – 60mins (212,213). Most of the optimisation for grinding the tissue has been done in order to facilitate the extraction of RNA from small tissue samples, thus the time taken to carry out the homogenisation is not a limiting factor. However for the current study, large tissue samples are to be obtained, which must be taken into consideration, as time to homogenise the tissue may become a limiting factor. Due to impracticalities the incorporation of all of these factors into a protocol for RNA extraction is not always feasible.

Enzymatic digestion has been used as a method of chondrocyte release from the ECM before RNA extraction. Manning and Bonner detailed a protocol for this method in 1967. The use of a short trypsin incubation followed by a longer collagenase (6-18hr) incubation has been widely reported for the release of the cells from the tissue (214). Further work has been carried out in the field to define the optimal method of digestion with one group comparing combinations of collagenase II, trypsin/EDTA and tosyl-lysyl-chloromethane. They reported that when used in combination the yield of chondrocytes from cartilage samples was not improved, but the time taken to digest the cells was reduced. One of the drawbacks of digesting the cells in a collagenase mix is that the most of the traditional commercially available collagenase is distilled as a by-product of bacterial fermentation (Sigma-Aldrich). This may cause issues if the cells that are being digested can be activated or altered by bacterial stimuli. Liberase™ is an alternative enzyme blend of both collagenase I and collagenase II that has not been purified from bacteria (Roche). Therefore does not run the risk of activating the cells. Previous data generated in the McInnes lab have confirmed that digestion with Liberase™ is as effective as digestion with conventional digestion reagents.

For the study of *ex vivo* chondrocytes it is essential to determine what processing method is optimal with the practical restraints in place, whether mortar and pestle processing or Liberase™ digestion will give the optimal RNA quality. By ensuring the best possible isolation of the RNA, better analysis of both the mRNA and miRNA of interest from the chondrocytes can be conducted. Isolating high quality genetic information from the cartilage for *ex vivo* analysis is not the only hurdle that must be overcome. It is known that during the prolonged culture of primary chondrocytes dedifferentiation of the cells can occur. Here, primary chondrocytes will be not only be analysed *ex vivo*, but they will also be cultured *in vitro* in order to perform functional studies to ascertain the role of exogenous BMP-7. Hence, it is important to ensure to that the primary chondrocyte cultures phenotypically resemble *in vivo* chondrocytes as much as possible. Current literature suggests that after isolation from the cartilage, chondrocytes can lose their phenotype over time. This is most notably observed in changes to ECM components, with type II collagen and aggrecan decreasing as passage number increases and type I collagen expression increasing. This suggests an alteration in phenotype in proliferative chondrocytes (215). Many other studies have looked at the loss of the differentiated phenotype in chondrocytes in long term culture. It has been shown in both rabbit and human epiphyseal chondrocytes that long term culture again results in loss of phenotypic type II collagen and proteoglycans, as well as up-regulation of type I pro collagen (216,217). Previous studies tended to look at phenotypic drift in chondrocytes that are encouraged to proliferate over increasing passages, however looking at the phenotypic profile of the cells in a confluent environment over short term cultures has not been well defined.

3.2 Aim

There are several methodological challenges to be addressed in order to obtain good quality RNA for molecular analysis of human articular cartilage *ex vivo,* as well as issues with phenotypic drift with primary chondrocyte cultures for functional *in vitro* studies. Therefore the overall aim of this chapter was to identify and optimise the most appropriate techniques for working with the cartilage over the duration of the study. The aims were further broken down as follows,

1) Determining optimal technique for extraction of high quality RNA from cartilage.

2) Deducing the most appropriate method of cDNA synthesis and determining the optimal housekeeping genes for qPCR analysis.

3) Defining the chondrocytic phenotype in short term cultures and identifying the optimum window for use for *in vitro* assays before dedifferentiation occurs.

3.3 Results

3.3.1 Optimisation of Cartilage Processing for Molecular Analysis.

In order to analyse cartilage *ex vivo* from OA patients, it first had to be excised from individuals under going total or partial knee replacement. From here samples were either processed via mortar and pestle homogenisation or by Liberase™ digestion (Figure 3.1). Mortar and pestle homogenisation was carried out after the tissue was snap frozen in liquid nitrogen. This process involves the lysis of the whole tissue, including ECM. The tissue was ground into a powder and dissolved in Qiazol (phenol based lysis solution). When the tissue was processed using Liberase™ digestion, the extracellular matrix was dissolved releasing the chondrocytes. Digestion resulted in removal of the ECM and thus only cells of interest were lysed in Qiazol. The caveat to this method is that it took a considerable period of time for adequate digestion to occur. In order to evaluate which method produced purer RNA, determination of the most stable housekeeping genes was first carried out using qPCR analysis (Figure 3.2A). The resulting data suggested that individual patient CT values for the housekeeping gene β -actin were more consistent and displayed less variation from the median CT value when Liberase™ digestion was applied. This was also reflected in the $^{260}/_{280}$ values for RNA (Figure 3.2B) as Liberase™ gave significantly purer RNA, with less variation from the desired value of 2.

From the data shown above, Liberase™ digestion was taken forward as the system of choice for tissue processing. Next, housekeeping genes were investigated to determine the optimal normalisation control for mRNA analysis with regards to chondrocytes. In the first instance, the housekeeping genes β -actin, GAPDH and ribosomal RNA (rRNA)18s were investigated (Figure 3.3A). The data show that the CT values for rRNA 18s were detected between cycles 5-9, β -actin between 15-18 and GAPDH between 31-35. Variation in housekeeping CT values across the patients was minimal (Figure 3.3Bi). The effect of chondrocyte origin, e.g. from eroded versus non-eroded regions of cartilage, was also investigated in order to determine if this would impact housekeeping expression (Figure 3.3 Bii). Tissue was classified as follows, eroded cartilage had an Outerbridge score between II-IV and non-eroded had a score between 0-I (Table

2.1). Regardless of whether or not chondrocytes were harvested from non-eroded or eroded tissue, extraction of RNA from the isolated cells resulted in good quality RNA that again displayed minimal disparity in the CT values for each gene. As all genes were consistent across the different patients and in eroded and non eroded cartilage, ß-actin was taken forward as the candidate housekeeping gene for all further studies, as it had the most optimal CT value range compared with 18S (which was too low) and GAPDH (which was too high).

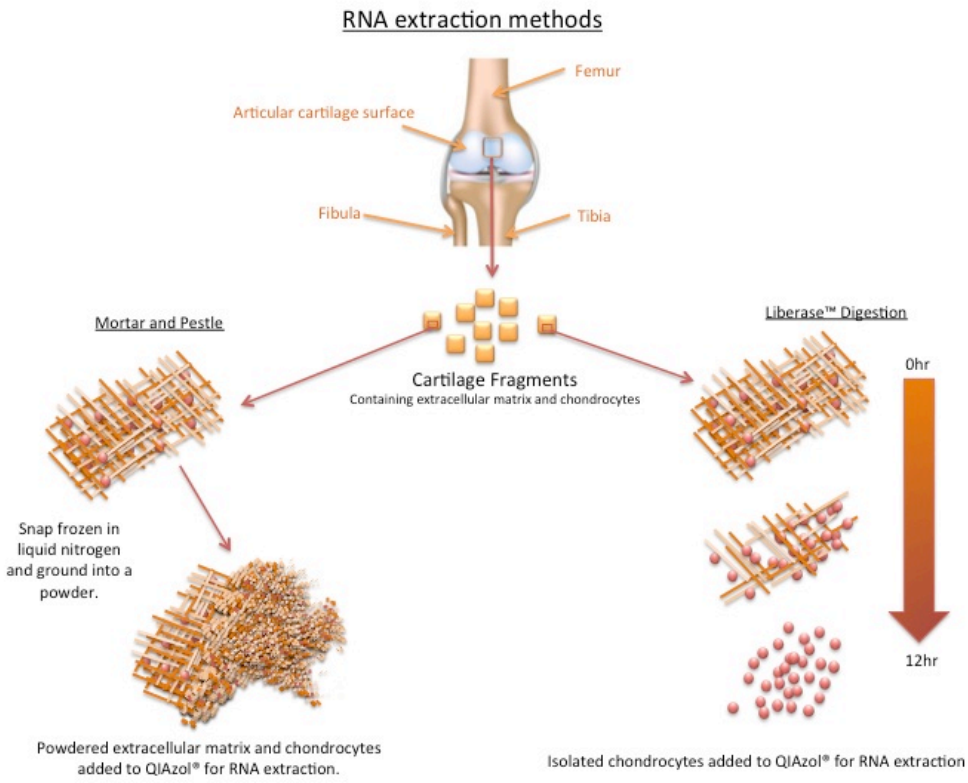


Figure 3.1 RNA Extraction Methods from Human Articular Chondrocytes.
Diagrammatic representation of two RNA extraction methods used for human articular cartilage: mortar and pestle homogenisation and Liberase™ digestion. A 12-hour digestion was carried out with the Liberase™ as shorter incubations resulted in insufficient breakdown of the ECM.

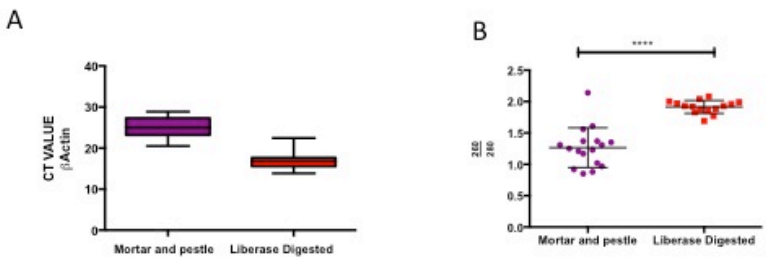


Figure 3.2 Liberase Digestion Offers Optimal Cartilage Processing Compared to Mortar and Pestle Method.
A) House keeping gene β-actin expressed as a CT value from cDNA generated either using a mortar and pestle (N=7) or Liberase™ digestion (N=10). B) RNA purity determined by $^{260}/_{280}$ ratio for RNA extraction via mortar and pestle, N=3 (Average of 5 cartilage sections per patient) or Liberase™ digestion, N=4 (Average of 6 cartilage sections per patient).
Mann Whitney test P< 0.0001 ****.

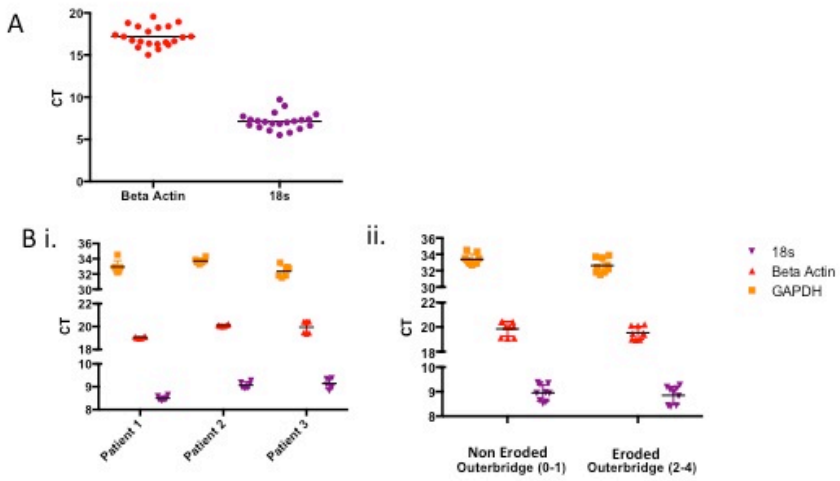


Figure 3.3 mRNA Housekeeping Comparison β-Actin, 18s and GAPDH.
A) Housekeeping analysis of βactin and rRNA 18s, values shown as CT, N=4. B) i. Housekeeping comparison for β-actin, GAPDH and rRNA 18s N=3. B) ii. Comparison of housekeeping genes β-Actin, GAPDH and rRNA 18s across non eroded (cartilage intact with no noticeable fissuring – Outerbridge score 0-1), and eroded (cartilage with fissuring and erosion typically down to the bone – Outerbridge score 2-4) tissue. Values shown as CT, N=3.

As miRNA analysis was also desirable for this study, a normalization gene for miRNA work was also considered. The current gold standard for miRNA housekeeping is the small non coding RNA U6. Therefore U6 expression was investigated and it was found to be detectable with a mean CT value of 15 (Figure 3.4A). When the cartilage was defined as either eroded and non-eroded, minimal variation was again observed between the CT values (Figure 3.4B). Finally, the expression of U6 was interrogated across all samples taken from the joint: femoral notch biopsy, DMC, DLC, PMC, PLC, MTP, LTP and synovial membrane (Figure 3.4C). Variation was evaluated using standard deviation (SD). The average SD across all samples was 1.1, thus indicating relatively small differences in each compartment across 5 patients. Evaluation of an alternative housekeeping gene, U1, was also carried out (data not shown). Again, minimal variation was observed between and within the patients. Therefore, as U6 is the standard housekeeping for miRNA analysis in the lab and there were no additional benefits of using U1, it was decided that U6 would be taken forward as the miRNA normalization gene for the remainder of the study.

Having established the most appropriate housekeeping genes for transcript and miRNA analysis, the next step was to determine the most optimal kit for cDNA synthesis with regards to mRNA detection. Ideally, the use of one cDNA synthesis kit that would allow for the detection of both miRNA and mRNA would be most practical. To this end, investigation of cDNA synthesised from affinity script (2 phase reactions optimised for mRNA) or MiScript (one phase reaction optimised for both miRNA and mRNA) was carried out. Two chondrocyte specific genes, type II collagen (COL2A1) and MMP-13, and one BMP associated gene, BMP receptor ACVR1 (ALK2), were used to screen for variation between the two cDNA synthesis methods (Figure 3.5). cDNA synthesised from the one phase MiScript kit gave consistently higher CT values (CT value >30) than the affinity script kit (Figure 3.5A). COL2A1, β - actin and ALK2 all showed substantial variance in CT values when comparing AffinityScript to MiScript.

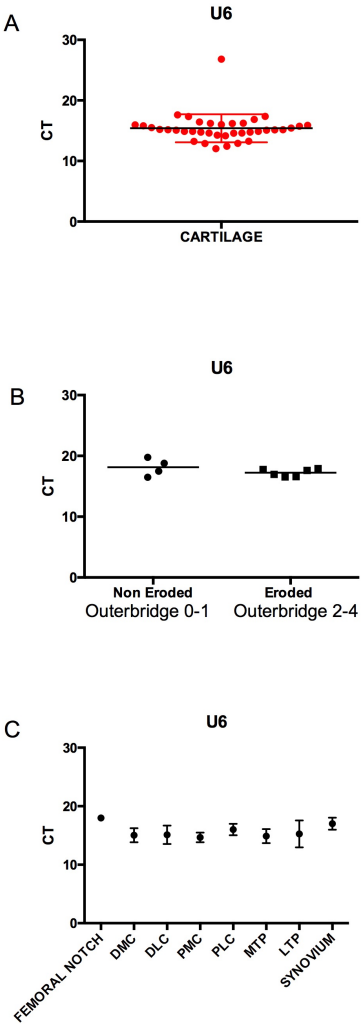


Figure 3.4 U6 Housekeeping for miRNA Analysis
A) U6 expression in cartilage shown as CT, N=10. B) U6 comparison between non eroded (cartilage intact with no noticeable fissuring – Outerbridge score 0-1), and eroded (cartilage with fissuring and erosion typically down to the bone – Outerbridge score 2-4) tissue. Cartilage taken from a total of 7 areas of the joint over 2 patients. C) U6 analysis in cartilage taken from 7 different articular areas of the knee along with synovium from the corresponding joints, N=5. Error bar represents standard deviation (SD).

To determine whether the method of cDNA synthesis skewed the data, it was normalised to a housekeeping gene. The CT value for the housekeeping gene was subtracted from the CT value of the genes of interest before it was transformed to $2^{-\Delta CT}$ ($2^{\wedge-}$ ($CT_{\text{gene of interest}} - CT_{\text{house keeping}}$)). If the cDNA synthesis method had no bearing on the results, it would be expected that the data would have a similar trend in expression despite any differences in the CT values. The data show that COL2A1 expression was similar in all chondrocytes regardless the cDNA kit used (Figure 3.5Bi), whereas ALK2 (Figure 3.5Bii) displayed clear disparity in transcript expression between kits. The MiScript kit showed significantly increased expression of ALK2 in the DMC and MTP compared to the PMC, whereas the AffinityScript kit showed consistent expression of this gene. Similarly disparity was observed in MMP-13 expression between the kits (Figure 3.5Biii). As there were noticeable inconsistencies in gene expression between the two different kits, it was not possible to rely on one kit for the analysis of both the miRNA and the mRNA. It was therefore decided that miScript would be used for all miRNA analysis, however affinity script would be used for all subsequent cDNA synthesis for mRNA analysis. The CT values obtained using AffinityScript were all consistently below 30 for the majority of the genes investigated, whereas the miScript cDNA reported CT values of up to 36 in this analysis. A CT value this high would normally indicate a gene that was not expressed. However, from the AffinityScript data, this is clearly not the case.

3.3.2 Chondrocyte Cultures for *In Vitro* Analysis.

As previously stated, part of this study will also focus on the effect of exogenous BMP-7 on chondrocytes in an *in vitro* setting. With this in mind, it must be considered that it has been widely reported that chondrocytes have a high tendency to lose their phenotype in culture (218). Therefore determining the most suitable time range for using cultured primary chondrocytes was a fundamental parameter in setting up this study. Three different culturing platforms were investigated: plastic alone, bovine collagen coated plastic or nanofibre plates. The latter was chosen as the literature suggests that mimicking the ECM in culture can help the chondrocytes maintain their phenotype (219), (Figure 3.6).

A

Gene	Sample	AffinityScript CT	MiScript CT	CT Differences
ALK2	p20 DMC	31	32	1
	P20 PMC	29	32	3
	P20 MTP	29	33	4
	P20 SYN	27	30	3
MMP13	p20 DMC	33	34	1
	P20 PMC	29	33	4
	P20 MTP	32	33	1
	P20 SYN	32	33	1
COL2A1	p20 DMC	26	35	9
	P20 PMC	25	33	8
	P20 MTP	24	33	9
	P20 SYN	32	36	4
β ACTIN	p20 DMC	26	33	7
	P20 PMC	25	31	6
	P20 MTP	24	33	9
	P20 SYN	20	25	5

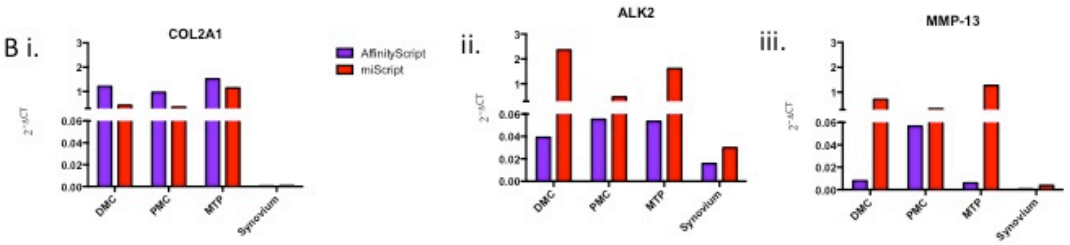


Figure 3.5 AffinityScript and miScript cDNA Synthesis Yield Different CT Values for mRNAs. cDNA was synthesised from cartilage (DMC, PMC, and MTP) and synovium with both AffinityScript and Miscript kits to enable direct comparisons of CT values (N=1). A) Table comparing the CT values for AffinityScript and MiScript cDNA over 4 different genes, ALK2, MMP-13, COL2A1 and housekeeping gene β-Actin. CT values for each cDNA synthesis kit are given along with the difference in CT values (miScript CT – AffinityScript CT). B) Expression of the three genes of interest shown as $2^{-\Delta CT}$ for both AffinityScript and Miscript cDNA. i) COL2A1 ii) ALK2 iii) MMP-13.

Previously, other groups have looked at the expression of COL2A1 (type II collagen) and aggrecan as positive markers of chondrocyte phenotype. COL1A1 has also been studied, as up-regulation of this collagen is associated with dedifferentiation. Cells were plated for a maximum of 7 days, with daily analysis of the cells. The chondrocytes cultured on plastic plates appears to shown the least variation across time. The expression of COLA1 (type I collagen) in the plastic plates was stable for up the duration of the culture, whereas on the bovine coated plates it was altered by day 3 and on the nanofibre plates the expression was altered by day 5. The expression of COL2A1 (type II collagen) was stable with the plastic culture plates for 4 days, after this the transcript expression dropped dramatically. The other platforms again showed slightly more variance in terms of the COL2A1 expression. Finally aggrecan expression was assessed, it was observed that in both the plastic and bovine collagen plates there was a sharp increase in expression at day 4, which by day 5 had dropped back down below baseline, this was less pronounced in the nanofibre plate.

In order to confirm cell viability and rule out cell death as a factor responsible to the change in transcript levels, cells were cultured for 96 hours and analysed using Flow Cytometry using the live dead marker DRAQ7. This demonstrated that chondrocytes kept in culture for 4 days displayed 80% cell viability at this time point (Figure 3.63B). It was therefore likely that the increased CT values were due to an altered transcript profile due to dedifferentiation rather than cell death due to prolonged culture. Analysis of the different platforms used for chondrocyte culture showed that there was very little variation in gene expression across the board and the cell behaved in a fairly similar fashion irrespective of the culture platform. Based on these finding chondrocytes were only kept in culture on plastic for a maximum of 4 days, as between 96 and 120 hours, the transcript profile of the cells appeared to change, with a notable decrease in both COL2A1 and aggrecan.

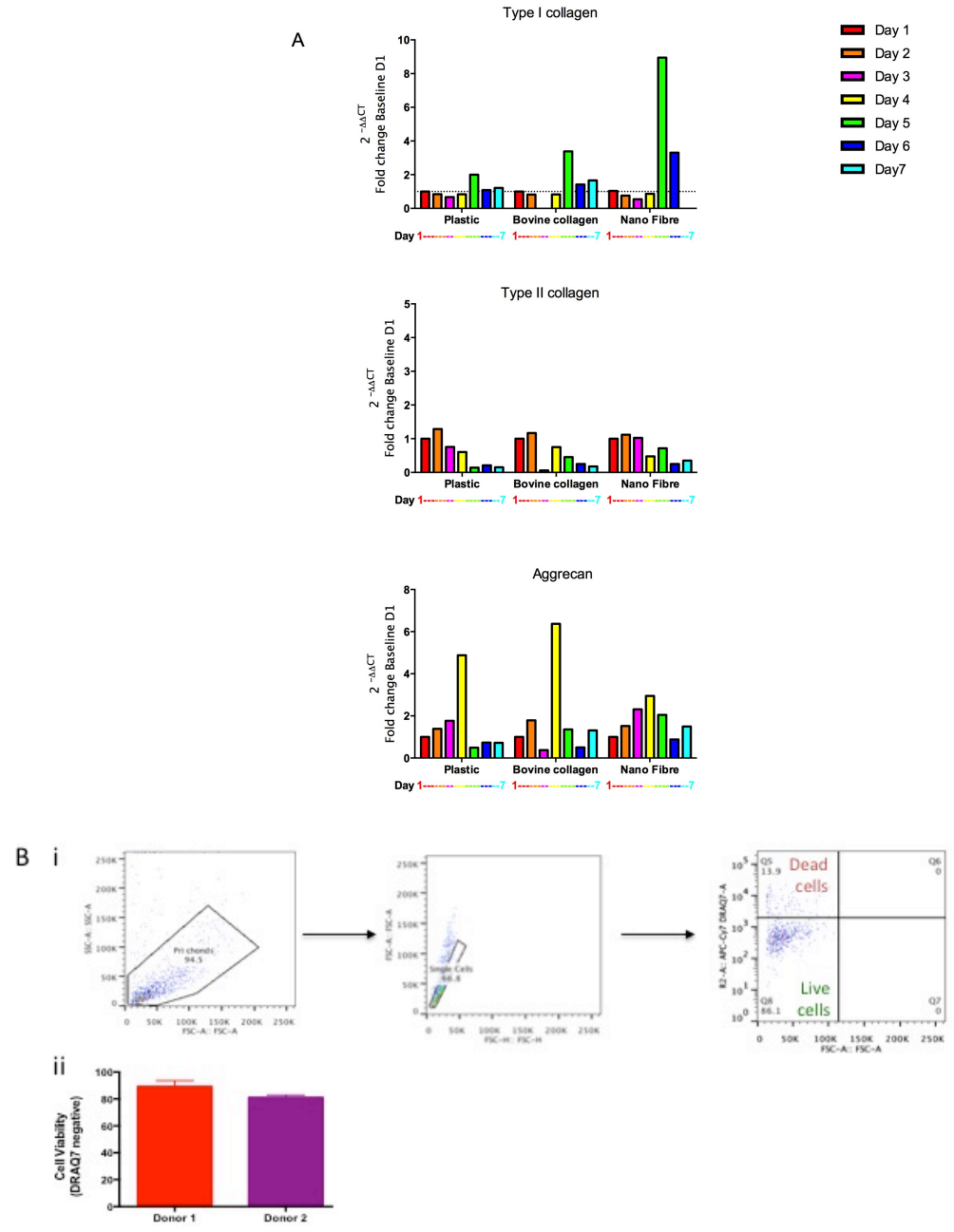


Figure 3.6 Dedifferentiation of Primary Articular Chondrocytes in Culture After 5 Days. Chondrocytes cultured for 7 days in plastic, bovine collagen coated plastic or nanofibre plates. (N=1) A) Fold change, ($2^{-\Delta\Delta CT,}$) was calculated for each gene of interest using the values at Day 1 on each plate as a baseline, House keeping gene beta actin was used to calculate all ΔCT values . i) Type I collagen ii) Type II collagen iii) Aggrecan . B) i) Gating strategy to assess cell viability with DRAQ7 (APC-Cy7) data acquired using a MACSQuant Flow Cytometer and analysed using FlowJo Software. ii) Percentage cell viability for 2 donors following a 4 day culture (donors are not the same as used in A).

3.4 Discussion

From the data presented above, it was deemed that Liberase™ digestion was the optimal method for processing cartilage samples. Liberase™ gave the most consistent CT values for the housekeeping gene ß-actin, and a significantly higher purity of the RNA compared to mortar and pestle processing (Figure 3.2). The most probable explanation for the increased purity of RNA from the Liberase™ digestion is the removal of the ECM matrix before lysis and spin column purification. With the mortar and pestle, both the chondrocytes and ECM components were ground into a powder in liquid nitrogen before being added to the column. Some literature states that spinning the samples for a prolonged period of time before ethanol is added increases RNA purity. The extra spinning step is used in order to try to remove some of the debris from the samples (213). As the samples here were not spun after, it is possible that the high salt solution added to facilitate RNA binding on the silica membrane is unable to efficiently distribute through the mixture and bind to the desired RNA due to the excess ECM debris present, thus giving rise to contaminated RNA.

Assessment of housekeeping genes revealed ß-actin, GAPDH and rRNA 18s were all detectable in the chondrocytes (Figure 3.3). Expression of all three genes was consistent across three different donors. Classification of the cartilage via Outerbridge score as either non eroded (0-I) or eroded (II-IV) also had no impact on the expression of all three housekeeping genes. From these initial experiments, it was decided that ß-actin would be the ideal house keeping gene to take forward. GAPDH was detected at a very late CT of around 34 and rRNA 18s was detected at a very early CT of around 9, whereas ß-actin was detected in range with the CT values for other genes of interest that had been interrogated. MiRNA housekeeping genes U6 and U1 were also investigated. No difference in expression was noted between the eroded and non-eroded cartilage. All samples from the joint (femoral notch, DMC, DLC, PMC, PLC, MTP, LTP and synovial membrane) were analysed to check the consistency of U6 between samples (Figure 3.4). Expression was found to be consistent across all samples. As no differences were brought to light between consistency of U6 and U1 housekeeping gene expression, it was decided that U6 would be taken

forward as the normalisation gene for all miRNA analysis. This would also keep the study consistent with other miRNA work being carried out in the group.

cDNA synthesis was carried out using either a two step reaction (AffinityScript) which is optimised for mRNA cDNA synthesis, or a one step reaction (MiScript) optimised for miRNAs and mRNAs. The transcript expression levels of ALK2, MMP-13, COL2A1 and β-actin were investigated with both kits (Figure 3.5). It was hypothesised that any trends in the data would be present regardless of the manner in which cDNA was synthesised. This would be independent of any possible variation in CT values observed between the two types of cDNA (due to normalisation with a housekeeping gene). However, this was not the case, with obvious differences in the qPCR data generated. MiScript one step reaction incorporates a poly A tail onto miRNAs, enabling oligo(dT) binding which initiates cDNA synthesis. At the same time the kit also uses random primers to create cDNA from mRNA transcripts. In this way it should be optimised for the detection of both miRNA and mRNA. AffinityScript uses a 2 step process with the first step denaturing the RNA and facilitating random primers annealing to the RNA at multiple points along the sequence. During the second phase of the reaction, primer extension occurs. The dNTPs and the reverse transcriptase enzyme are able to extend from the end of the primers sequence to the end to the RNA thus creating cDNA. Finally, termination of the reaction is carried out by a 70°C incubation. The AffinityScript method uses a variety of temperature controlled steps to achieve optimal conditions for each stage of cDNA synthesis, whereas MiScript generates cDNA in a one temperature reaction. The use of a single temperature may not be as effective in creating cDNA from the mRNA present in the reaction. It is also possible the concentration of cDNA added to the qPCR has an impact. The miScript cDNA reaction is diluted to a final concentration of 0.025ng/µl whilst the AffinityScript cDNA is diluted to 5ng/µl, which would account for the lower CT values (indicating higher levels of transcript expression) seen with AffinityScript. Without a clear answer for the differences obtained between the kits it was decided that for all analysis carried out on mRNA transcripts the AffinityScript kit would be used and for miRNA analysis the MiScript kit would be used.

It has been reported that chondrocytes in culture can dedifferentiate over time and lose their phenotype (215-217). Previous reports have shown that if chondrocytes are expanded in culture, expression of collagens and aggrecans are altered after the first passage (215). The main focus in the field thus far has been on phenotypic drift during expansion of the chondrocyte population in culture over several passages of the cells. This has been of interest therapeutically as these cells could potentially be grown *in vitro,* then be implanted into patients to promote extracellular matrix growth in areas where cartilage lesions have occurred. For the purpose of this study the dedifferentiation of chondrocytes was investigated over a shorter time period than in previous studies (7 days) and the cells were not encouraged to proliferate. The chondrocytes were cultured on plastic, bovine collagen coated plastic or nanofibre plates to determine if this had any effect on transcript expression. On all platforms the chondrocytes appeared to lose their transcriptional phenotype with regards to type II collagen expression after 96 hours in culture (Figure 3.6). This is largely in agreement with the work carried out by Hamanda (2013), they also reported an increase aggrecan over time which is also see here most dramatically after 4 days in culture. From the data obtained showing alterations in gene expression after 96 hours, it was decided that the cells would only be kept in culture for 4 days. The viability of the chondrocytes after 4 days was also investigated (Figure 3.6) using a fluorescent live dead marker, which indicated that approximately 80% of the cells were viable after culture. This data further shows that keeping the cells in culture for 4 days is optimal to enable *in vitro* functional assays to be carried out.

In summary, the work carried out in this chapter has lead to the optimisation of RNA extraction methods from human articular cartilage for *ex vivo* analysis. Additionally, the best methods for molecular analysis of these chondrocytes has also been defined, including the most appropriate cDNA synthesis strategy for mRNA and miRNA analysis and the most suitable housekeeping genes for their normalisation. Furthermore, a chondrocyte culture system has been established in order to carry out *in vitro* analysis of these cells.

4 Expression and Regulation of Endogenous BMP-7 and the BMP-7 Signalling Cascade in an Osteoarthritic Cohort.

4.1 Introduction

Bone morphogenetic protein 7 (BMP-7) is a member of the TGF-ß superfamily and plays a number of key functions within the body; these range from kidney formation to osteoinduction in bone. Further to this, exogenous BMP-7 has been shown to be chondroprotective in several animal models of OA (1.2.4.1) (160,220,221). However, little is known about the expression and role of endogenous BMP-7 and its signaling family in human OA tissue.

In the early 2000's, an initial study reported an increase in transcriptional expression of BMP-7 in OA cartilage compared to both healthy adult and neonatal tissue (147). This was later refuted in a subsequent study that suggested that BMP-7 transcript is only detected in foetal cartilage and not in OA or healthy adult articular cartilage (149). In addition to the transcriptional discrepancies, there is also controversy surrounding the expression of the BMP-7 protein within the joint. Depending on the studies, the level of soluble BMP-7 in synovial fluid is either reported to be elevated in OA (153,228), or unaltered when compared to healthy controls (151). Protein within the chondrocytes has also been reported to be decreased in OA (150). It should also be appreciated that studies have looked at the level of BMP-7 in plasma, suggesting that it is elevated in OA, however, this is still open to interpretation due to the data presented being below the detectable range of the assay (153). These discrepancies currently seen in the literature may be due to a modification or regulatory mechanism that render the transcript undetectable with current techniques or preventing maturation into bioactive protein. Methylation is one of these potential mechanisms (section 1.3.1.2), Loeser *et al* have shown that BMP-7 promoter methylation links to an age related decrease in BMP-7, (222). Another epigenetic regulatory mechanism that can target transcript for rapid degradation, thus making detection difficult, are AU rich elements (ARE) in the

3' UTR (section 1.3.1.1). The ARE can be bound by the zinc finger protein tristetraproline (TTP) inducing instability of the mRNA and ultimately degradation (223). An alternative explanation for the disease-associated changes in the BMP-7 transcript are alterations in the epigenetic environment. Several studies have highlighted altered miRNA expression profiles in OA (194,224,232)(section 1.4.4). MicroRNAs (MiRNAs) are small non-coding RNA molecules that over the past decade have been widely investigated and have led to a greater understanding of the epigenetic control in many disease associated pathways (section 1.4). MiRNAs are approximately 22 nucleotides in length and function to maintain homeostasis in cells by controlling protein expression (225). MiRNAs can work to inhibit protein synthesis by targeting the 3'UTR of mRNA, causing either translational repression or mRNA degradation.

A previous link between miR24 and OA has been established by work carried out by Philipot *et al,* showing a decrease in the expression of the miRNA in OA compared to healthy cartilage (195). Philipot *et al* went on to further elucidate a role for this miRNA with regards to OA, suggesting it is involved in the regulation of cell senescence marker $p16^{INK4a}$ (section 1.4.1.1). The most recent data confirming the link between miR24 3p and OA reports that this miRNA was increased in the synovial fluid in end stage disease compared to early stage (210). Other miRNA, such as let7e, were also brought to the forefront as predictive markers for the need for arthoplasty in OA by Beyer *et al* (209). They also identified miR342 3p as an OA associated miRNA, however, they found no evidence for a correlation between this miRNA in circulation and the need for arthoplasty.

Despite the uncertainty regarding the presence of BMP7 in the joint, the signalling cascade for this protein has been relatively well defined to date (Figure 4.1). Firstly, BMP-7 is cleaved from an inactive pro-form to its functional conformation by the enzyme furin at the furin type consensus cleavage site (112). Upon cleavage, the mature form of BMP-7 can fold and, via displuphide linking, either homodimerise or heterodimerise with other mature BMPs (112). These mature ligands can then bind to receptors initiating the subsequent signalling cascade. There are two types of BMP serine-threonine kinase receptors, type I and type II . BMP-7 has affinity for several type I receptors

including ALK6 (BMPR1B), ALK2 and ALK3, (226,227). It also has affinity for more than one type II receptor, however preferentially binds (BMPR2) over ActRII or ActRIIB (227,235), (Figure 4.1). Ligand activation of these tyrosine kinase receptors leads to the recruitment and phosphorylation of Smads 1/5/8, which interact with Smad 4 before translocating to the nucleus where gene specific promoter activation can occur (Figure 4.1). This BMP-7 mediated promoter activation has been linked to the extracellular matrix homeostasis (234) via the up-regulation of hyaluronic acid and its receptor CD44, both of which have been implicated in matrix synthesis (Figure 4.1), (156), however little more is known about its anabolic role in the cartilage (115).

It is well characterised that BMP-7 can be regulated by antagonistic proteins (Section 1.2.1.4) such as follistatin and gremlin (162). A disease-associated increase in both gremlin and follistatin has been observed in an experimental model of OA in dogs (236).

Despite the considerable work in elucidating the expression of BMP-7 in disease, the expression of the BMP-7 signalling family as a whole, including BMP receptors, inhibitors and accessory signalling components such as CD44, remains ill defined in human OA cartilage.

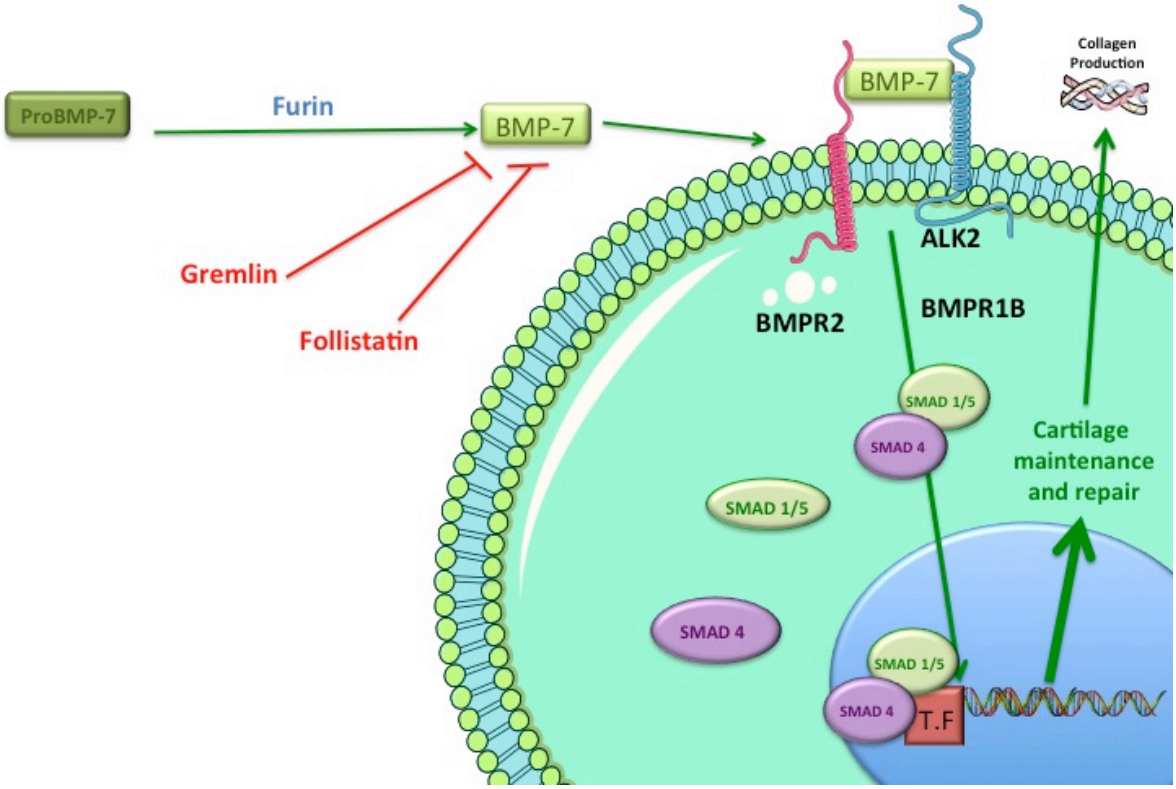


Figure 4.1 BMP-7 signalling cascade
Basic depiction of the BMP-7 signalling cascade: pro BMP-7 is cleaved by furin releasing the mature BMP-7 protein. BMP-7 signals via type I (BMPR1B/ALK2) and type II (BMPR2) BMP receptors. Phosphorylation of the receptors then leads to subsequent SMAD phosphorylation and translocation to the nucleus. Here, gene activation can occur resulting in ECM production.

4.2 Aims

The aims of this chapter were three fold, in the first instance, the aim was to resolve discrepancies within the literature regarding BMP-7 expression in articular chondrocytes. Secondly, given the protective role of exogenous BMP-7 in animal models of OA, here it has been hypothesised that altered expression of members of the BMP-7 signalling family cascade, along with other BMP-7 associated genes, may have an impact on how individuals with OA would respond to BMP-7 therapy. Thus, the second aim of the work in this chapter was, for the first time, to evaluate the expression of the BMP-7 signalling family cascade, along with other BMP-7 associated genes, in the same cohort of OA patients. Finally, it has been hypothesised that miRNA predicted to target both BMP-7 and its signalling family, may be altered in disease. Therefore, the studies in this chapter will aim to identify whether these are altered and have a role in the regulation of this anabolic pathway.

To this end, the aims of this chapter are to:

1. Determine the expression and localisation of BMP-7 within OA cartilage and synovium.

2. Deduce if the expression of certain BMP-7 signalling family members varies between different grades of OA cartilage.

3. Delineate the expression of BMP-7 regulating miRNA in the OA cohort and determine if any of the signalling components are direct targets of these miRNA.

4.3 Results

4.3.1 BMP-7 Transcript Analysis in Primary Human Cartilage.

In an attempt to settle the current disparity in the literature with regards to the expression of BMP-7 transcript in human articular cartilage, a variety of techniques were implemented to locate and quantify BMP-7 mRNA. All primary cells used within this study were isolated from patients who had given informed consent at the time of TKR surgery for end stage OA. Cartilage erosion was scored *ex vivo* using the Outerbridge scoring system (section 2.1). In the first set of studies, self-designed primers were used in standard qPCR analysis. Primers were designed using the publically available mRNA FASTA sequence (National Centre for Biotechnology Information (NCBI) website). The primers were subjected to BLAST analysis (NCBI) (section 2.7.2.4), assessing their specificity to the mRNA they were designed to identify. Synthesis of cDNA from chondrocytes (section 2.6.5) residing in either non-eroded (Outerbridge score 0 - I) or eroded (Outerbridge score II-IV) cartilage was carried out and the product was subject to PCR with the self-designed primers. The data was presented as a cycle threshold (CT) value. In an initial study, samples from both the eroded and non-eroded sections of tissue show that BMP-7 was detected in the cartilage; CT>30 with the NTC ranging from a CT of 36 to undetermined (Figure 4.2). These initial results suggest that only very low levels of transcript are present within the cartilage; CT values were close to that of the NTC in some cases. Importantly, expression of the housekeeping gene ß-actin was consistent across the eroded and non-eroded cartilage confirming that the PCR was successful (Figure 4.2A) and that technical error did not account for the high CT values. As the published data could not robustly be replicated in this instance it was decided to evaluate the expression of BMP-7 in a positive control. To this end, human embryonic kidney (HEK) 293 cells were evaluated along with human articular cartilage, using manufacturer validated TaqMan™ primers for BMP-7. Thus testing if the previous data were simply a product of poorly designed primers. HEK 293 cells were expected to produce the transcript due to its essential role on kidney development and maintenance (237).

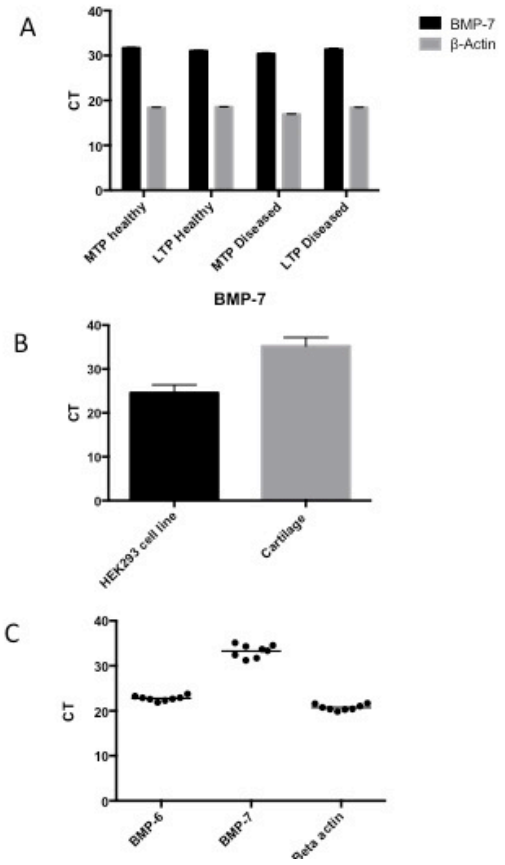


Figure 4.2 BMP-7 Transcript Not Detected in Human Primary Chondrocytes Using qPCR. All cartilage was obtained from patients with end stage disease. A) Sections from non-eroded (Outerbridge score 0-I) or eroded cartilage (Outerbridge score II-IV) taken from either the MTP or the LTP. N=1 for each section (analysed in technical triplicate). Self-designed primers were used to probe for BMP-7 with SYBR green master mix. B) HEK293 cell and cartilage (both N=1), were probed for BMP-7 using TaqMan™ primers along with TaqMan™ universal master mix, (no UNG) C) Cartilage from 2 patients, 1 total knee and 1 unilateral knee replacement. Six sections from the total knee were investigated, DMC, DLC, PMC, PLC, MTP and LTP and two sections from the unilateral knee, PMC and MTP (N=2). TaqMan™ primers were used to probe from both BMP-7 and BMP-6, with TaqMan™ universal master mix, (no UNG). All values shown as CT (cycle threshold) and β-actin was used as a housekeeping gene for each with self-designed primer (A) and TaqMan™ Primers (B/C). Self designed primer NTC was a CT value of 36 and undetermined, TaqMan NTC was undetermined.

The cell line, as predicted, produced a CT value below 20 (Figure 4.2B) using the Taqman™ primers in this particular assay. The CT values obtained for BMP-7 transcript within the cartilage were consistent with the self designed primers, giving rise to CT values ranging from 33 -37, indicating extremely low levels of expression. BMP-6 expression was also evaluated in the samples as a another positive control, as it has previously been reported to be highly expressed in human adult cartilage (149). BMP-6 transcript was present in the same two patients that had previously been interrogated with the TaqMan™ primers for BMP-7 expression (Figure 4.2C), confirming that previously published data could be replicated using patient samples from this study (149). In order to rule out any patient variation, a further two patients were interrogated for BMP-7 expression (Table 4.1), however, the CT values were again in excess of 30. Cartilage from other donors also displayed CT values similar to that of the NTC in separate analysis (data not shown). Therefore, both the self-designed and the validated TaqMan™ primers were only able to detect very low levels of BMP-7 in the chondrocytes in some of the cartilage samples examined suggesting that low level BMP-7 expression may be patient specific.

The data above suggests that, contrary to some of the literature, cartilage may not be a ready source of the BMP-7 transcript within the joint, therefore, other possible sources were also investigated. To this end, the synovium, bone and bone marrow of OA patients were interrogated. The CT values for BMP-7 in synovium of the three patients investigated were lower, by 3 CT's, (Table 4.1) than those observed in the cartilage and were below the CT values detected in the NTC, indicating that the synovium can produce low levels of transcript within the joint. Whereas the CT values for transcript expression in both the bone and bone marrow demonstrate lower expression than that of the cartilage suggesting that these were not likely sources for the transcript (Table 4.1).

All of the methods utilised up this point in the study had been for relative quantification of transcripts and were all unsuccessful in robustly reproducing the previously published findings. To address this, absolute quantification by digital PCR was implemented as an alternative, more sensitive, detection method. Digital PCR offers an ultrasensitive platform determining the number of copies of a transcript present per oil emulsion droplet generated (Figure 4.3A).

Cartilage (PMC)		Synovium		Bone		Bone Marrow	
P44	35	**P32**	37	**P52**	34	**P53**	UND
	34		31	**(PMC)**	33		UND
	35		31		33		UND
P43	35	**P47**	33	**P52**	34		
	33		32	**(MTP)**	34		
	33		32		34		
		P48	29				
			30				
			30				

Table 4.1 BMP-7 Expression in Cartilage, Synovium, Bone and Bone marrow.
PMC shown as a representative section of the cartilage, N=2, where P number## represents different patients. Synovium values N=3; Bone samples, N=2; bone marrow, N=1. Technical triplicate values shown for all samples run with Taqman primers. All NTC were undetermined with the exception of the synovium, which varied between 36 and undetermined. All data shown as CT value. UND = Undetermined

The cDNA is equally distributed between the 20,000 oil droplets, with only a proportion of the droplets being positive for the genes of interest, thus creating two populations, one positive and one negative. Housekeeping gene ß-actin could be detected in a robust manner with a good separation between the positive and negative populations (Figure 4.3B). Detection of positive control gene BMP-6 along with BMP signalling receptor, BMPR2, was successful using the digital PCR (Figure 4.3C/D). When BMP-7 was interrogated with the system, it could not reliably be detected across cartilage from 5 patients and synovium from 2 patients (Figure 4.3E). There could be very low levels of transcript present in the T2 synovium, however it is hard to determine, as the number of samples examined were not significant. Thus, the digital qPCR suggests that BMP-7 mRNA maybe present at very low level in some of the synovium samples, but not the cartilage, in the small number of patients investigated.

As a final attempt to replicate the data that had been published by Chubinskaya *et al* in 2002, demonstrating that articular chondrocytes have detectable BMP-7 transcript, their published nested PCR approach was adopted. Primers for BMP-7 mRNA were designed against the FASTA sequence, the product of this PCR was then used in order to create a second inner pair of primers, which would be run with the resultant product from the initial RT PCR reaction. No bands were observed at the expected weight of 216bp in the initial PCR with the outer primers (Figure 4.4). However, after the nested reaction, bands could be visualised in all of the samples. The housekeeping gene ß-actin could also be clearly observed in all of the samples after one round of RT PCR. NTCs were run for the outer primers and were also carried over to be subject to the nested PCR, in order to check that DNA contamination had not occurred: the control was negative at each stage of the reaction. These data suggest that along with the initial qPCR data there may be very low levels of BMP-7 transcript produced from the primary chondrocytes, however the data from the digital PCR analysis did not support this and it may be patient specific.

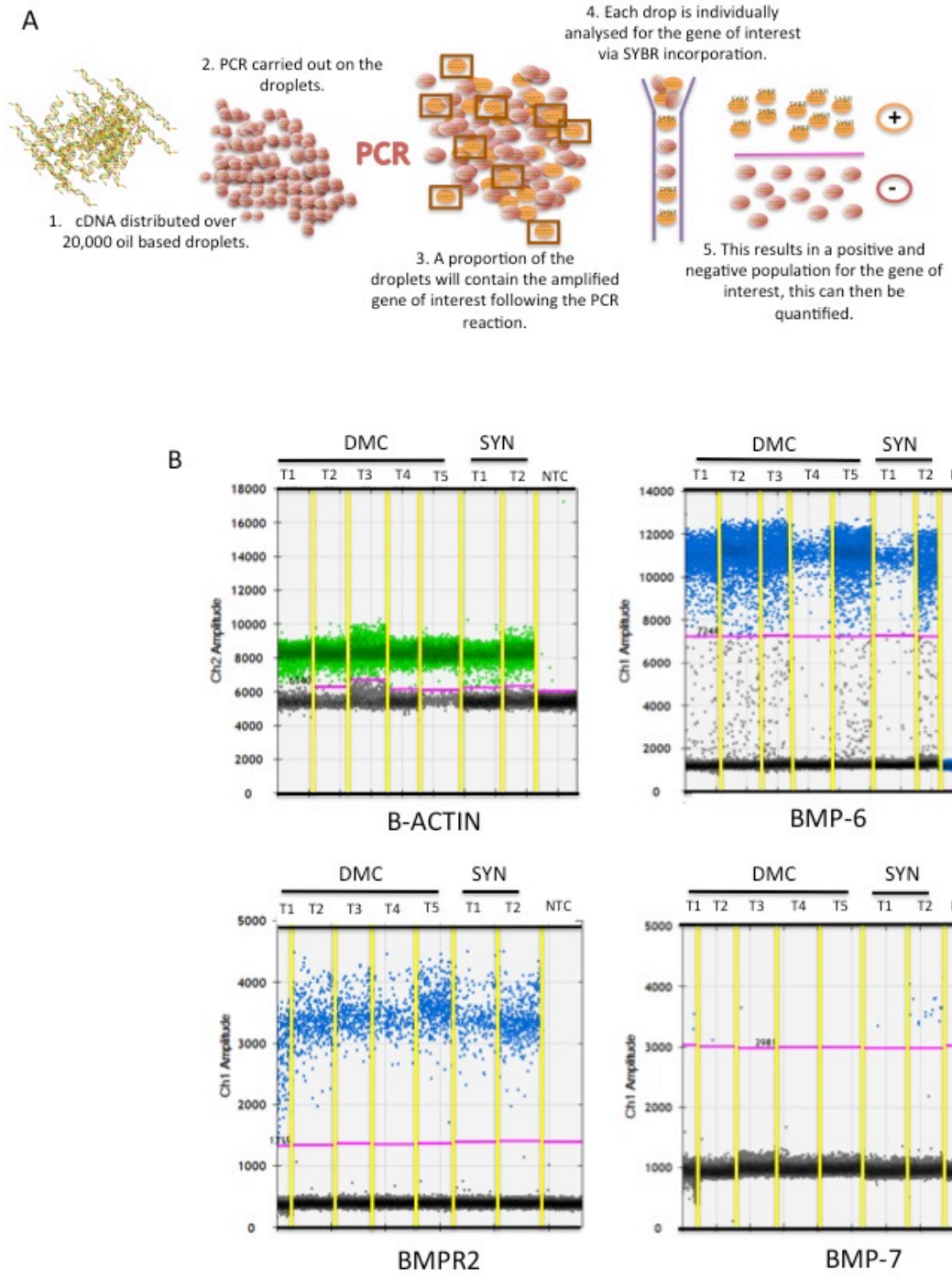


Figure 4.3 BMP-7 mRNA Was Not Detected Using Digital PCR.
A) Schematic representation of the digital qPCR assay system. The distal medial condyle (DMC) from 5 patients along with synovium from 2 patients were analysed for B) β-actin, BMP-6, BMPR2 and BMP-7. The pink line in each of the plots represents the separation between the positive (green/blue) and negative (black) droplets. A negative control lacking the cDNA (NTC) was also run for each transcript. All primers used in the analysis were TaqMan™.

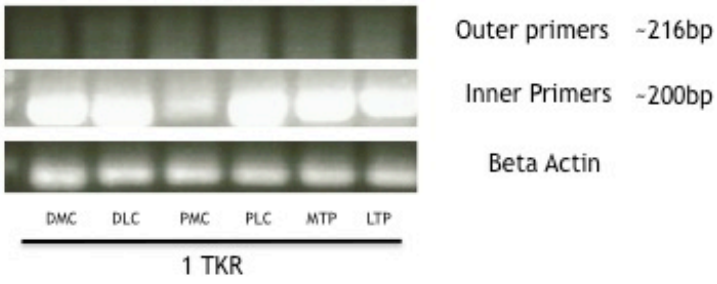


Figure 4.4 BMP-7 Can be Found in Human Primary Chondrocytes Using Nested Primers. Cartilage from each section of joint from a single patient was subject to nested PCR. Firstly 5 µg cDNA was subject to 35 rounds of PCR with outer BMP-7 primers, expected product size 216 bp. A second round of PCR (again 35 cycles) was carried out on the product from the 1st PCR resulting in a 200 bp product. Products from both the 1st and 2nd rounds of PCR with the outer and inner primers respectively were run on a 1.8% agarose gel for 45 mins. β-actin was used a loading control and was run on the gel after an 1st round of PCR.

4.3.2 BMP-7 Protein Quantification and Localisation in OA Cartilage

As only low levels of BMP-7 transcript expression were observed in a patient specific manner in the cartilage, an investigation was carried out to determine if the protein could be either quantified or qualified. Firstly, an antibody raised against the pro-peptide of BMP-7 was used to investigate protein levels (Figure 4.5A). Chondrocytes from OA patients were lysed in RIPA buffer before being subject to gel electrophoresis, transferred to nitrocellulose membrane and probed with BMP-7 specific antibody (section 2.10). In total 4 patients were investigated. Cartilage taken from several areas within the joint were all digested together from 3 patients and chondrocytes lysed in RIPA buffer. For the 4th patient each specific area harvested from the joint was digested and lysed individually (U8: PMC and MTP). The transcript data (section 4.2.1) would suggest that only low levels of BMP-7 protein should be present within the cartilage. Accordingly in order to maximize the detection of any expressed protein, the maximum concentration of protein was loaded into the gel (this was based on sample volume). Incubation with the BMP-7 antibody, raised against the pro-domain of the BMP-7 peptide (Figure 4.5A), revealed faint bands in all samples at the expected molecular weight of approximately 40 kDa. Housekeeping protein GAPDH, which was used as a positive control, was also detected in all the samples. (Figure 4.5B). Human recombinant BMP-7 was run as a negative control for the antibody, as this does not contain the pro-domain of the protein (Figure 4.5A); as expected no band was detected in the negative control, (Figure 4.5B).

After confirming that the BMP-7 antibody binds to the pro-domain of the BMP-7 protein and detects the full-length peptide in the OA chondrocytes (as shown by the weight at which it detected BMP-7), the antibody was used to determine the cellular and anatomical location of the protein, via immunohistochemistry (IHC). The evaluation of the BMP-7 protein in the cartilage required optimisation of the IHC technique. This was due to the inherent properties of the tissue that resulted in the cartilage becoming non-adhered from the slide during the staining process. This mainly occurred during antigen retrieval, a process critical to the IHC analysis. Antigen retrieval is carried out in order to unmask epitopes

that have become cross-linked during the fixation process, thus facilitating antibody binding. Heat induced antigen retrieval with citrate buffer is the standard antigen retrieval method, however, in the case of cartilage mounted sections, this resulted in almost all of the sections falling off the slides. Any sections that did remain on the slides after this process were only ever partially adherent, which gave rise to reagents accumulating under the section which in turn caused issues in the analysis due to unspecific staining. Therefore, it was essential to find an alternative form of antigen retrieval for this tissue before BMP-7 analysis could begin. To this end, UNI-TRIEVE, a solution that does not require high temperature or extreme pH, was assessed as an alternative. This resulted in a vast improvement in the number of sections remaining adhered to the slides post antigen retrieval. Some of the sections were however still only loosely adhered meaning that the section could freely move around the slide whilst others still fell off the slides and were lost in the antigen retrieval buffer. The same antibody raised against the pro-region of the BMP-7 peptide, that had been used in the WB analysis, was also employed with IHC to investigate the expression and localisation of the protein in cartilage. As BMP-7 is essential in kidney development and maintenance, the antibodies were initially optimised in rat kidney (Figure 4.6). The BMP-7 antibody was evaluated at several concentrations - 4, 6 or 8 µg/ml, in order to identify the optimal concentration for use in IHC (Figure 4.6A). From the images obtained with the various concentration it was decided that 6 µg/ml was the optimal concentration to take forward as the 4 µg/ml staining was too faint and the 8 µg/ml gave rise to artificial edge staining. Next, BMP-7 protein expression (Figure 4.7) was investigated in human OA articular cartilage. Following incubation with the BMP-7 antibody, clear staining (brown deposits) was observed in the PMC from three patients. The staining was abundant in areas close to cartilage lesions where characteristic stress clustering of the chondrocytes (229) was apparent (Figure 4.7A). Furthermore, cartilage sections from the patients were also positive for intracellular BMP-7 staining (Figure 4.7A), which was confirmed by using digested chondrocytes cytospun onto slides and stained for BMP-7 (Figure 4.7B). This staining suggests that these cells can produce the protein in times of cellular stress and as only a small proportion of these cells appear to be in this OA typical cluster formation, this may account for the low transcript abundance previously observed (section 4.2.1). A second antibody, raised against the whole

BMP-7 peptide, was used in order to confirm if the initial staining observed in the cartilage would remain consistent (Figure 4.8). BMP-7 was most abundantly observed again in areas of chondrocytic stress (Figure 4.8) and intracellular staining could also be observed (Figure 4.8) thus confirming the initial staining patterns observed. Taken together, the data obtained for BMP-7 staining in the cartilage suggests that BMP-7 protein is discretely detected in the intracellular regions of the chondrocytes and is also prominent in areas of chondrocytic stress determined by chondrocyte cluster formations and cartilage lesions. Thus, the low level of transcript expression in the cartilage may account for the moderate levels of protein observed.

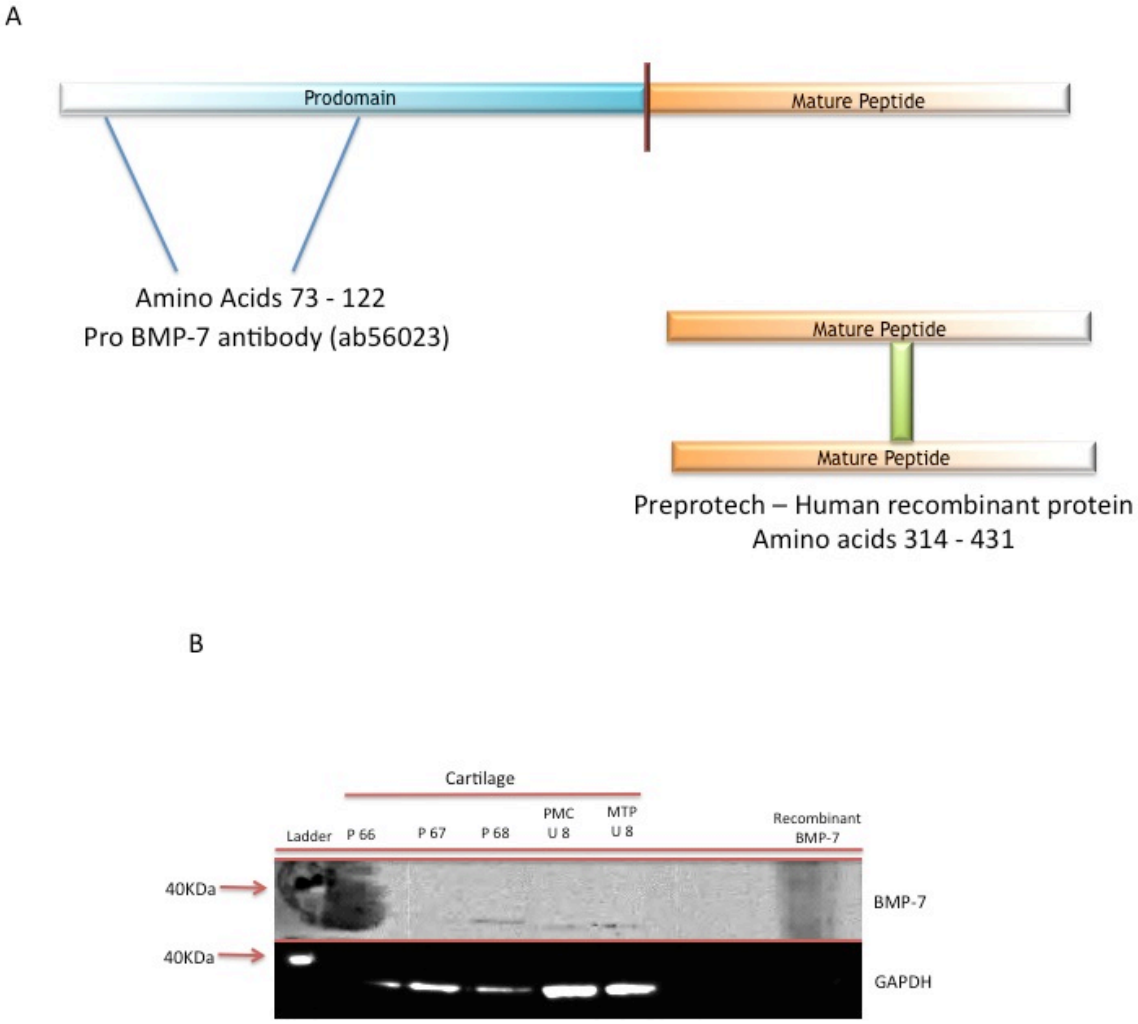


Figure 4.5 BMP-7 Protein can be Visualised Using Western Blotting.
A) Shematic showing the region on the BMP-7 protein that the antibody is raised against.
B) Cartilage from 3 patients (all cartilage from each patient digested together) was lysed in RIPA buffer producing patient specific protein lysates. From a 4th patient lysates were creating by digested the cartilage according to the sections of the joint the cartilage was excised from (i.e. a separate lysate for both PMC and MTP). Samples were loaded by volume onto a 4 -12% bis tris polyacrylamide gel. Following electrophoresis, the protein was transferred onto a nitrocellulose membrane using iBlot system. BMP-7 antibody (ab56023 rabbit polyclonal) was used at 1 µg/ml. Recombinant BMP-7 was used as a negative control for the antibody and GAPDH was probed as a loading control.

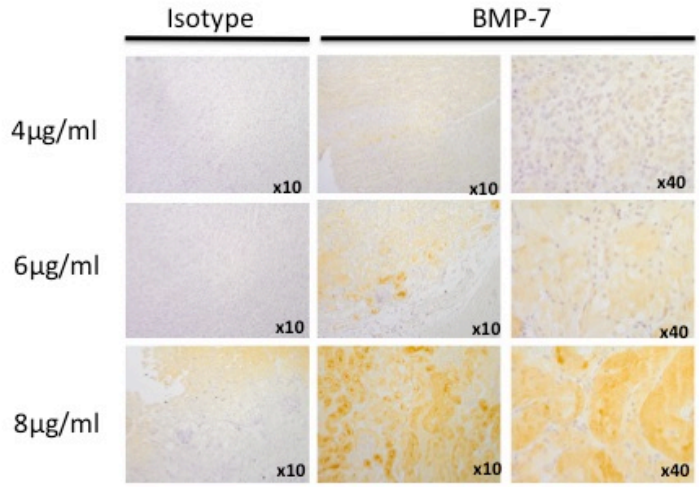


Figure 4.6 Validation of BMP-7 Antibody on Rat Kidney.
Sections were prepared for staining using the HEAT induced antigen retrieval technique. A) Three concentrations of the BMP-7 antibody (raised against the pro domain of the peptide) were added to the sections (ab56023 rabbit polyclonal) - 4 µg/ml, 6 µg/ml and 8 µg/ml. Isotype used was rabbit polyclonal DA1E mAB IgG. Isotype is shown at a 10X magnification, with all BMP-7 staining being shown at 10X and 40X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

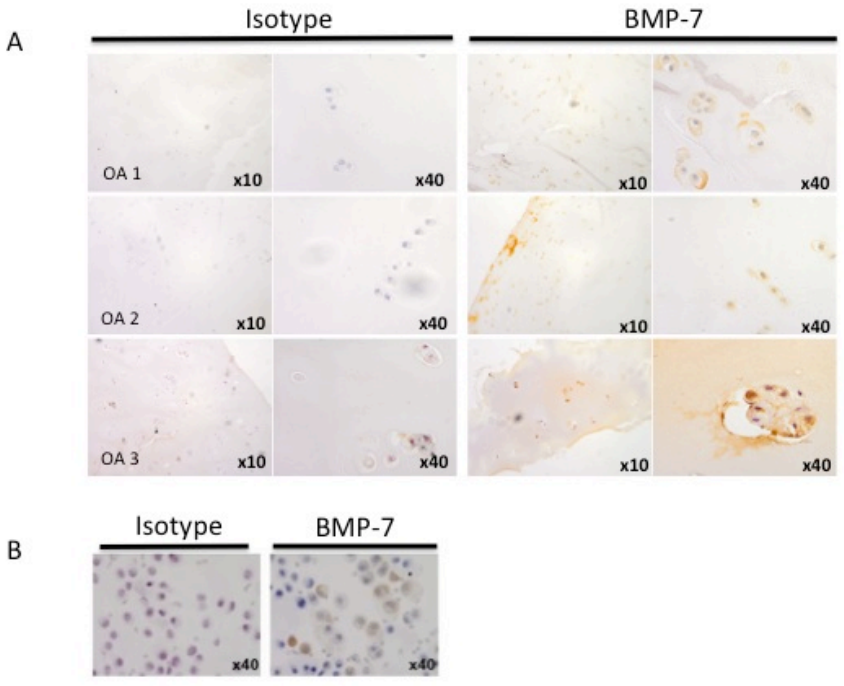


Figure 4.7 BMP-7 Positive Staining in OA Cartilage
A) Cartilage from three patients (OA1, OA2 and OA3) was subject to UNITRIEVE antigen retrieval before being stained with BMP-7 antibody, raised against the pro-domain of the protein (ab56023 rabbit polyclonal) at 6 µg/ml. Isotype shown at 10X magnification, BMP-7 staining shown at both 10X and 40X magnification. Isotype used was rabbit polyclonal DA1E mAB IgG. B) Isolated chondrocytes were cytospun onto slides before being subject to HEAT induced antigen retrieval and BMP-7 staining with the BMP-7 antibody raised against the pro-domain of the protein (ab56023 rabbit polyclonal) at 6 µg/ml. Isotype and BMP-7 shown at 10X and 40X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

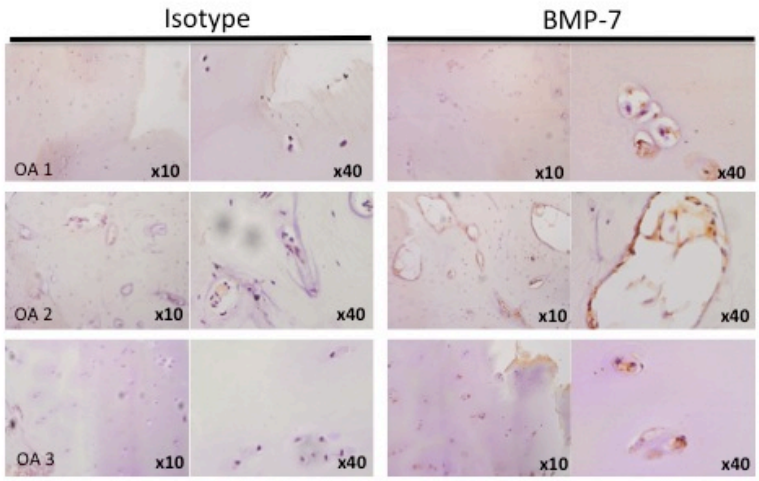


Figure 4.8 Validation of BMP-7 Staining in the Cartilage with an Alternative Antibody. Cartilage sections from 3 patients were subject to UNITRIEVE antigen retrieval before being stained with BMP-7, raised against the whole peptide for BMP-7 (ab54904, mouse monoclonal antibody). Isotype - Mouse IgG1 negative control. Representative sections from three different patients (OA1, OA2 and OA3) stained with 6 µg/ml total BMP-7 antibody at either 10X or 40X magnification. Isotype shown at 10X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

4.3.3 BMP-7 Localisation in Synovium

From the transcript analysis (section 4.3.1), it was shown that BMP-7 mRNA was detectable in the synovium (Table 4.1). Therefore, it was decided to further investigate this finding by determining the specific location of BMP-7 within the synovial tissue. Initial staining was carried out on the synovium from 4 OA patients with end stage disease. The results show intense BMP-7 staining around the endothelial cells of the blood vessels (Figure 4.9). This staining pattern was again confirmed with a second antibody, which has been raised against the whole peptide as opposed to the pro-region of the protein (Figure 4.10) in a further 6 OA synovial tissues. BMP-7 staining was also observed in areas of higher cellular density (Figure 4.10), thus both the transcript and protein can be detected within the synovium of the OA patients.

In order to confirm if the BMP-7 staining in the synovium was specific to OA, synovial sections from RA patients were also probed with BMP-7 antibody. OA tissue was used as a positive control for all RA tissue staining (Figure 4.11). In the RA sections investigated, no staining was observed around the blood vessels in the synovium (Figure 4.11). However, the control OA staining was consistent with the previous findings (Figure 4.9 and Figure 4.10). These data potentially suggest that the expression of BMP-7 protein is not limited to the cartilage but may have other roles within the synovium; however, further investigation is required to fully elucidate any of these roles.

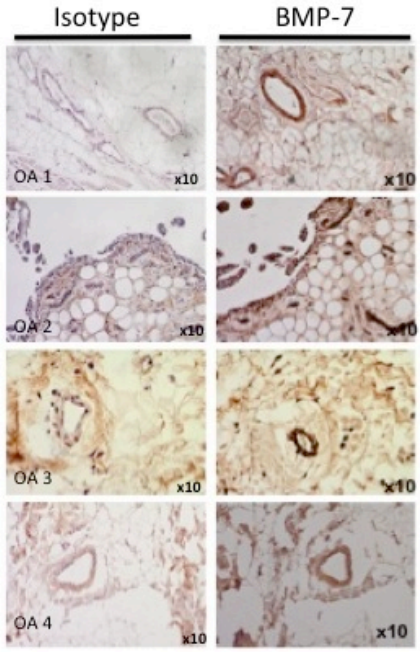


Figure 4.9 BMP-7 Staining in the Synovium of OA Patients
A) Synovial sections from 4 OA (OA1, OA2, OA3 and OA4) patients prepared from staining using HEAT induced antigen retrieval. Sections were stained with BMP-7 antibody (ab56023 rabbit polyclonal), raised against the pro-domain of BMP-7, at 6 µg/ml. Isotype used was rabbit polyclonal DA1E mAB IgG. Both the isotype and BMP-7 antibody shown at 10X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

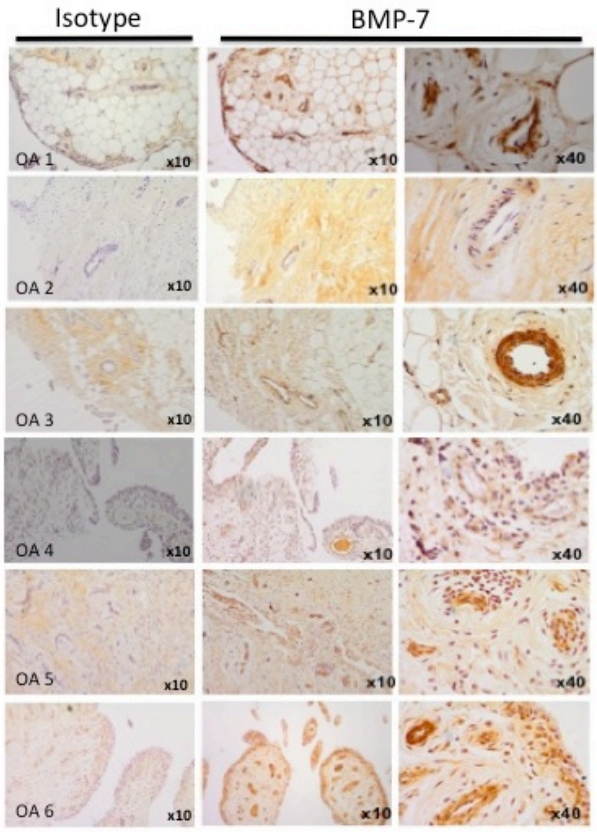


Figure 4.10 Validation of BMP-7 Staining in the Synovium with an Alternative Antibody. Synovial sections were prepared for IHC using HEAT induced antigen retrieval. A) Representative sections from 6 OA patients (OA1, OA2, OA3, OA4, OA5 and OA6) stained with total BMP-7 antibody (ab54904, mouse monoclonal antibody) at 6 µg/ml. Isotype Mouse IgG1 negative control. Images shown at both 10X and 40X magnification. Isotype shown at 10X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

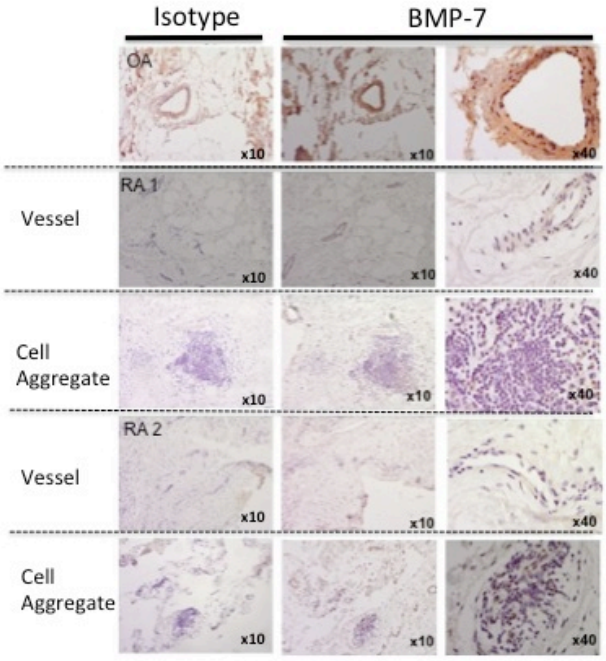


Figure 4.11 BMP-7 is Not Present in the RA Synovium.
BMP-7 staining (ab56023 rabbit polyclonal) in 1 OA patient and 2 RA patients (RA1/RA2). All isotype images shown at 10X magnification, whilst all other sections are shown at both 10X and 40X magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

4.3.4 BMP-7 Protein in OA Plasma and Synovial Fluid

As BMP-7 protein could be found in the synovial membrane and cartilage of the OA patients, it was of interest to investigate the levels of soluble BMP-7 in both the plasma and synovial fluid of the OA patients. There are again discrepancies in the literature around the concentration of BMP-7 present in the plasma, as one group reported concentrations in the region of ng/ml and another reported concentrations on the region of pg/ml. In this study an ELISA based approach was used in order to determine the concentration of the BMP-7 in OA synovial fluid along with comparator synovial fluids from RA, psoriatic arthritis (PsA) and ankylosing spondilytis (AS). OA plasma was also investigated along with comparator RA plasma and healthy (aged-matched) plasma. The samples were all subject to a heteroblock® incubation in order to prevent non-specific binding of rheumatoid factor, from the RA samples. The lower limit of detection of the ELISA was 62.5 pg/ml (Figure 4.12). In all the samples measured, the levels of BMP-7 were markedly below the detection limit of the assay. Thus no conclusion could be made regarding the soluble concentrations of BMP-7 within the plasma or synovial fluid.

4.3.5 Disconnect Between BMP-7 Transcript and Protein Expression.

This study has so far demonstrated that BMP-7 protein can be observed in human articular cartilage from OA patients, however transcript expression is very low. To provide some insight into the regulation of BMP-7, *in silico* analysis of the 3'UTR of BMP-7 was carried out identifying any regions, such as AU rich elements, likely to be targeted for modification that may account for these inconsistencies (Figure 4.13). AU rich regions are often bound by ARE binding proteins that are able to affect transcript stability and translational capacity (230). The expression of the AU binding regions is not the sole factor for attachment of the ARE-binding proteins. Recent advances (both experimentally and computationally) have confirmed that the presence of AU motifs along with structural accessibility of the mRNA are vital to this mechanism (230). The internet resource AREsite is the first predictive algorithm that combines multiple factors in order to determine AU elements that may be bound, allowing

modification of transcript expression. Here AREsite has been utilised to identify 5 predicted ATTA regions on the 3' UTR of the BMP-7 transcript which could potentially be targeted by AU binding proteins (Figure 4.13). As previously alluded to (section 1.3.1.1), the binding of these proteins targets the transcripts for rapid degradation. Interestingly, it is currently thought that tristetraproline (TTP) may act as a bridge between the AU rich element on the mRNA and the elements used for decay of the transcript by the recruitment of decay enzymes (231). Given the predicted sites for binding (Figure 4.13), these regions could potentially be responsible for rapid degradation of the mRNA by factors such as the zinc finger protein, TTP, hence rendering the transcript undetectable. Due to time constraints the *in silico* analysis could not be taken forward for verification using *in vitro* techniques.

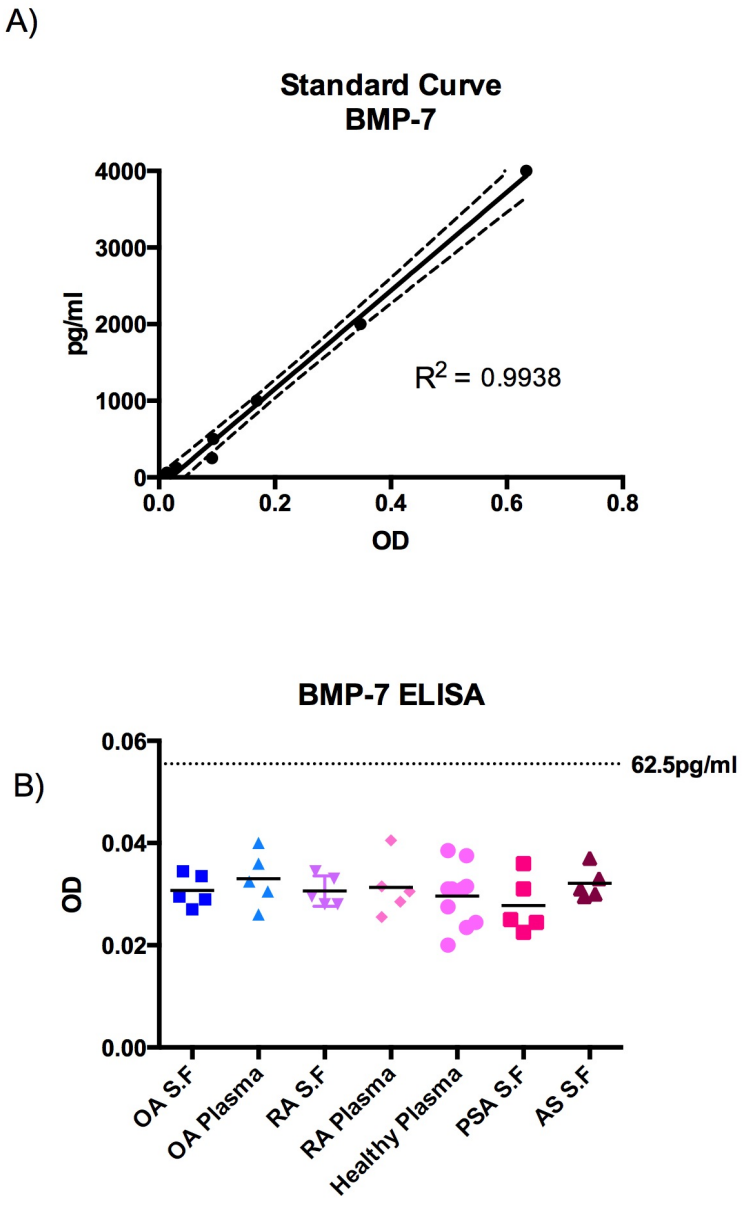


Figure 4.12 BMP-7 Protein Cannot be Detected in SF or Plasma from OA, RA, PSA or AS Patients or Healthy Controls.
A) Standard curve was generated using recombinant human BMP-7 from the commercially available ELISA B) Samples from OA synovial fluid (N=5), OA plasma (N=5), RA synovial fluid (N=5), RA plasma (N=5), Healthy plasma (N=10), PsA synovial fluid (N=5) and AS synovial fluid (N=5), were screened for BMP-7 protein expression. The sensitivity of the assay as defined by the standard curve was 62.5 pg/ml, represented by the broken line.

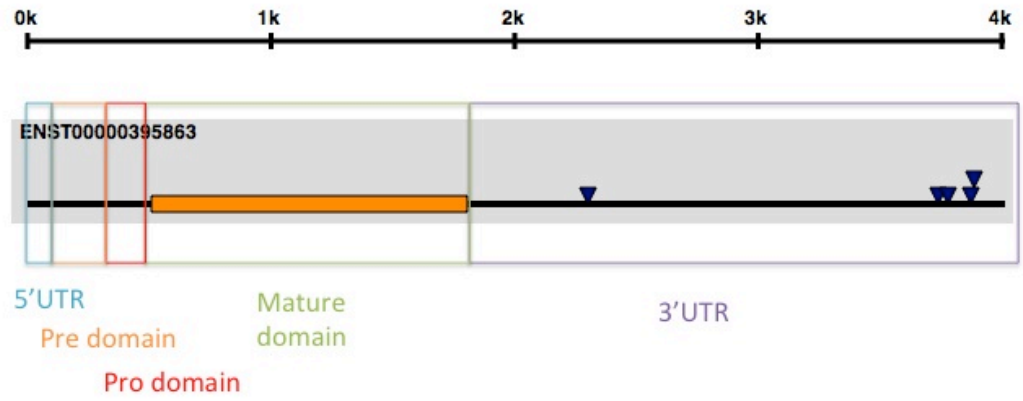


Figure 4.13 *In Silico* Analysis of BMP-7 3'UTR: Identificaton of Predicted TTP Binding Sites. Analysis carried out and images taken using the AREsite software courtesy of the University of Vienna and E!Ensemble (ENST00000395863). AREsite have predicted 5 ARE's in the 3'UTR of BMP-7, shown by the blue triangles,

4.3.6 Patient Cohort Analysis: Expression of the Endogenous BMP-7 Signalling Cascade in OA Cartilage.

4.3.6.1 OA Patient Demographics

For BMP-7 to elicit a signalling response as a therapeutic agent, components of the signalling family would need to be expressed at the sites where the therapy would be administered i.e. the joint environment; specifically the cartilage. A strong case has already been provided in the literature for the chondroprotective properties of exogenous BMP-7 in the treatment of OA (23), however, the expression of the receptors and accessory molecules involved in the BMP-7 signalling cascade have not previously been evaluated all together in OA. To this end the following were assessed in the cartilage of a small cohort of OA patients: anabolic components (BMP receptors BMPR1B, ALK2 and BMPR2; cleavage enzyme furin; and accessory receptor CD44), ECM components (type II collagen; type I collagen; and aggrecan) and catabolic/BMP inhibitory components (MMP-13; BMP-7 inhibitors follistatin and gremlin). Tissue was obtained from individuals with end stage OA, who had given informed consent at the time of TKR at the Glasgow Royal Infirmary. The characteristics of the full cohort are detailed in (Table 4.2).

The whole cohort contained 6 males and 5 females, however due to prolonged timing of the sample collection, the cohort was analysed in two batches. This was broken down as follows; T1, T2, T3, T4 and T5 (red) and T10, T11, T12 and T13 (green). The first group contained 4 male patients and 1 female. The mean age of the group was 79.5 years. The second cohort consisted of 2 males and 2 females and had a mean age of 60.75 years. Patients T6 and T7 (black) were collected for cohort analysis, however the only viable samples obtained were peripheral blood and synovial fluid these therefore could not be included in either cohort for cartilage analysis. The majority of the patients suffered from some degree of synovial inflammation, as assessed by the surgeon at the time of joint replacement. Most of the patients also suffering from multi-compartmental disease as defined by the modified Kellgren and Lawrence X-ray scoring system (1.1.10.2) (84,238). Cartilage damage was also scored *ex vivo* using the modified Outerbridge scoring system (1.1.10.3) (88) and was either defined as non-eroded (Outerbridge 0-1), or (eroded, Outerbridge 2-4). All the cartilage

acquired from the 1st cohort had an Outerbridge score of ≥ 2 and was therefore all classed as eroded. The second cohort had a wider range of Outerbridge scores, thus giving a range of samples in both the eroded and non-eroded subgroups. Whether the patients had any former arthroplasty or not was also noted from the patient records.

Table 4.2 OA Patient Cohort: Clinical Data

Patient Identifier (T#)	Gender	Age	Synovial Inflammation	Kellgren and Lawrence score			Outerbridge score						Other Joints Replaced
				M	L	P/F	DMC	DLC	PMC	PLC	MTP	LTP	
T1	M	71	Y	4	3	3	4	3	4	2	4	3	N
T2	F	79	Y	0	2	4	3	3	2	3	3	3	N
T3	M	85	N/A	4	1	0	4	3	4	3	4	3	N
T4	M	67	Y	4	0	0	4	4	3	2	4	3	Y
T5	M	65	N	4	1	0	4	3	4	2	4	3	Y
*T6	F	55	N	4	2	4	4	2	3	3	4	2	N
*T7	F	59	Y	4	2	2	4	2	3	1	4	-	N
T10	F	56	Y	4	0	1	4	2	4	2	4	2	Y
T11	F	69	Y	N/A	N/A	N/A	0	4	1	4	0	4	-
T12	M	63	N	2	4	2	4	4	0	2	2	4	-
T13	M	55	Y	4	2	3	4	3	0	1	2	1	-

*T6 and T7 were only included in plasma analysis. N/A = not available

4.3.7 OA Cohort: Cartilage Analysis of the BMP-7 Signalling Family

Cartilage was taken from 6 areas of the joint that are routinely removed during knee replacement surgery. These include: four sections from the femur DMC, DLC, PMC and PLC and two sections from the tibia, MTP and LTP (Figure 2.1). Each sample of cartilage was graded according to the Outerbridge scoring system, as previously described (Table 2.1). With each patient having 6 samples, some of these had equivalent grading's and thus in these instances the results for the same grade were averaged and represented as one data point for that patient. The overall transcript expression of the main components of the BMP-7 signalling pathway were initially determined in the OA cartilage using qPCR. It is thought that there may be decreased BMP-7 expression within the OA joint (150), thus there is no ligand to bind to the receptor. Therefore, if replenishment of BMP-7 is beneficial in treating OA, the expression of the BMP receptors will be of interest as administration of exogenous BMP-7 would first lead to a receptor- ligand interaction with subsequent activation of relevant associated signalling cascades. To this end, BMP-7 receptors ALK2, BMPR2 and BMPR1B were analysed in the cartilage (Figure 4.14A). Results revealed that the receptors were readily detectable within the cartilage: ALK2 (CT range 19-22), BMPR2 (CT range 21-28) and BMPR1B (ALK6) (CT range 24-28). In addition to BMP-7 receptors, the expression of ECM fibres, type I/II collagens and aggrecan were interrogated as both type II collagen and aggrecan are essential ECM components that have been shown to be induced by BMP-7 (160,233,234) (Figure 4.14B). The expression of the ECM associated type II collagen (CT range 15-18), aggrecan (CT range 16-18) (CT range 15-24) were found to be abundant within the cartilage. Variation in the abundance of Type I collagen was noted with over a 3 log order difference in expression between the samples analysed, which may reflect the heterogeneity of the disease. Finally, the BMP-7 antagonists gremlin and follistatin were analysed (Figure 4.14C) showing that follistatin (CT range 16-19) and gremlin (CT range 18-22) could be detected in articular cartilage. After confirming that the transcripts of interest were present within the cartilage, further analysis was carried out by subgrouping the patients according to the Outerbridge score (Figure 4.15). Due to the Outerbridge scores of all the cartilage collected in this subgroup being greater than 2, all of the cartilage

analysed here is considered eroded. The expression of the BMP-7 receptors were not significantly different across the Outerbridge scores (Figure 4.15A). Furthermore, the accessory components, follistatin and gremlin, along with hyaluronic acid receptor CD44, were also not significantly different across the Outerbridge scores (Figure 4.15B). Both follistatin and CD44 potentially decrease as cartilage erosion becomes more severe, and thus it is possible that based on sample size there is a type II error, resulting in a false negative result in the data. Finally, expression of type I and type II collagen along with aggrecan was analysed (Figure 4.15C), with only aggrecan potentially decreasing with increased severity in erosion, however there was no statistical significance in this observation.

As there was no significant difference between the different grades of eroded cartilage it was decided that an investigation with another cohort with a larger range of Outerbridge scores would be conducted. In the second cohort, the cartilage was analysed in 2 groups - non-eroded (Outerbridge score 0-1) or eroded (Outerbridge score 2-4) as opposed to looking at each grade individually due to the small size of the cohort.

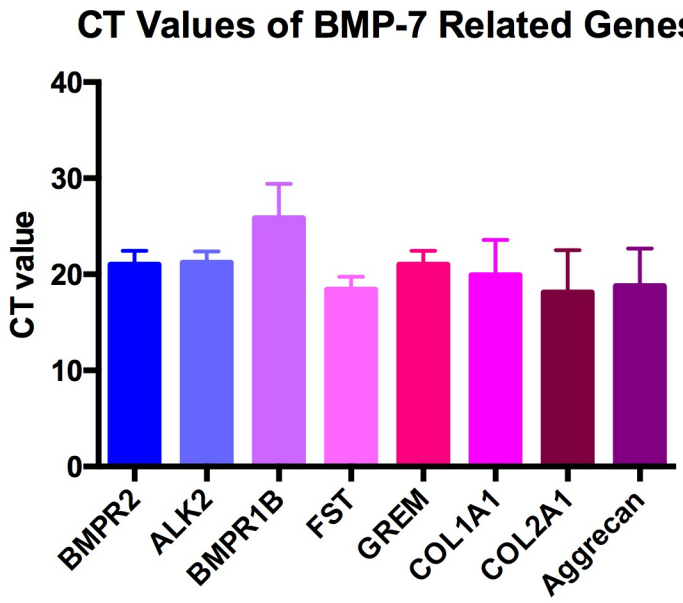


Figure 4.14 Transcript Expression Validation in Human Articular Cartilage.
Cartilage taken from 5 OA patients with a total of 6 sections were taken from each joint, DMC, DLC, PMC, PLC, MTP and LTP (one patient missing the DLC and another missing MTP). A variety of transcripts were investigated to assess if they were detectable within the cartilage. All data shown as CT values.

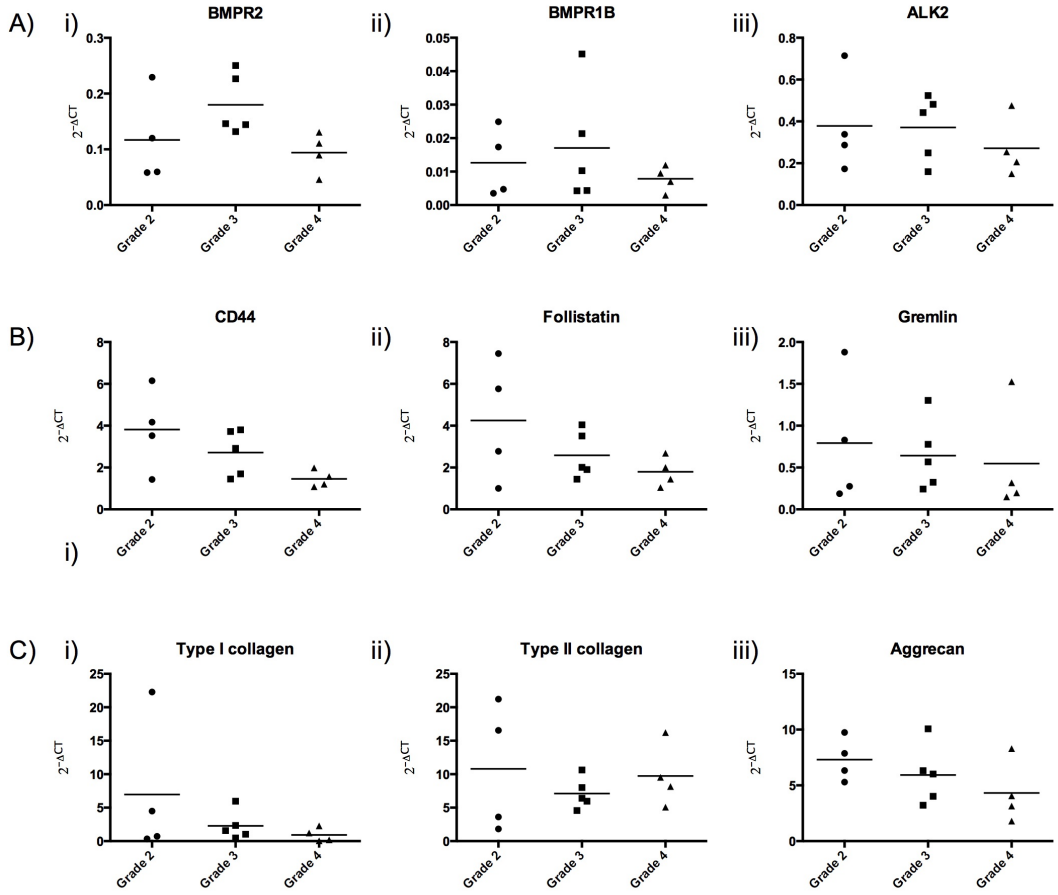


Figure 4.15 Initial Cohort Analysis Reveals no Changes in Transcript Expression in Eroded Cartilage.

Cartilage taken from 5 OA patients with a total of 6 sections were taken from each joint, DMC, DLC, PMC, PLC, MTP and LTP (with one patient missing the DLC and another missing MTP). Each section was given an Outerbridge score; all data from sections obtained from one patient with the same score were averaged. A) Expression of BMP receptors i) BMPR2, ii) BMPR1B and iii) ALK2. B) Expression of components associated with BMP-7 signalling i) CD44, ii) Follistatin and iii) Gremlin. C) Expression of extracellular matrix constituents, i) Type I collagen, ii) Type II collagen and iii) aggrecan. All normalised to β-actin and shown as $2^{-\Delta CT}$. Statistical analysis carried out using a Kruskal Wallace test.

All transcripts were displayed as fold change using the average value of the non-eroded cartilage samples, grades 0-I, for all patients as a baseline, (Figure 4.16). Expression of both type I BMP receptor, ALK2 and type II receptor, BMPR2 were analysed (Figure 4.16A). Some samples had over a 2-fold increase in expression of both BMPR2 and ALK2 in the eroded cartilage, however, in other samples there was no change in expression (Figure 4.16A). Hyaluronic acid receptor, CD44, also showed modest increases in expression when calculated as fold change over the non-eroded cartilage (Figure 4.16B), however, there was no increase in the expression of cleavage enzyme furin. All other transcripts investigated showed no alteration between the eroded and non-eroded cartilage, with the exception of Type II collagen which was increased at least 2 fold in over half of the samples interrogated. None of these observation were statistically significant, most likely due to the small size of the cohort. The observed increase in type II collagen and the receptors suggests that these chondrocytes may be up-regulating anabolic pathways in the eroded cartilage. However, the expression of the BMP-7 signalling family remains statistically unaltered between the eroded and non eroded cartilage.

In order to further investigate the increase in type II collagen, ALK2 and BMPR2 as the spread of the data in these transcripts displayed similar patterns in expression (Figure 4.16), linear regression analysis was carried. Thus allowing any correlation between these transcripts in the patient cohort to be determined. It was found that a strong positive correlation existed between the BMPR2 and ALK2 within the patient cohort, R^2 0.62, $P<0.0001$ (Figure 4.17). A significant correlation also exists between type II collagen and both BMPR2, R^2 0.47, $p=0.0004$ and ALK2, R^2 0.33, $p=0.0051$ (Figure 4.17). Thus this potentially suggests intact anabolic signalling in some of the patients as those who have an up regulation in the expression of the receptors also displayed increased ECM component type II collagen. No significant correlation was found with furin, despite its similar expression profile.

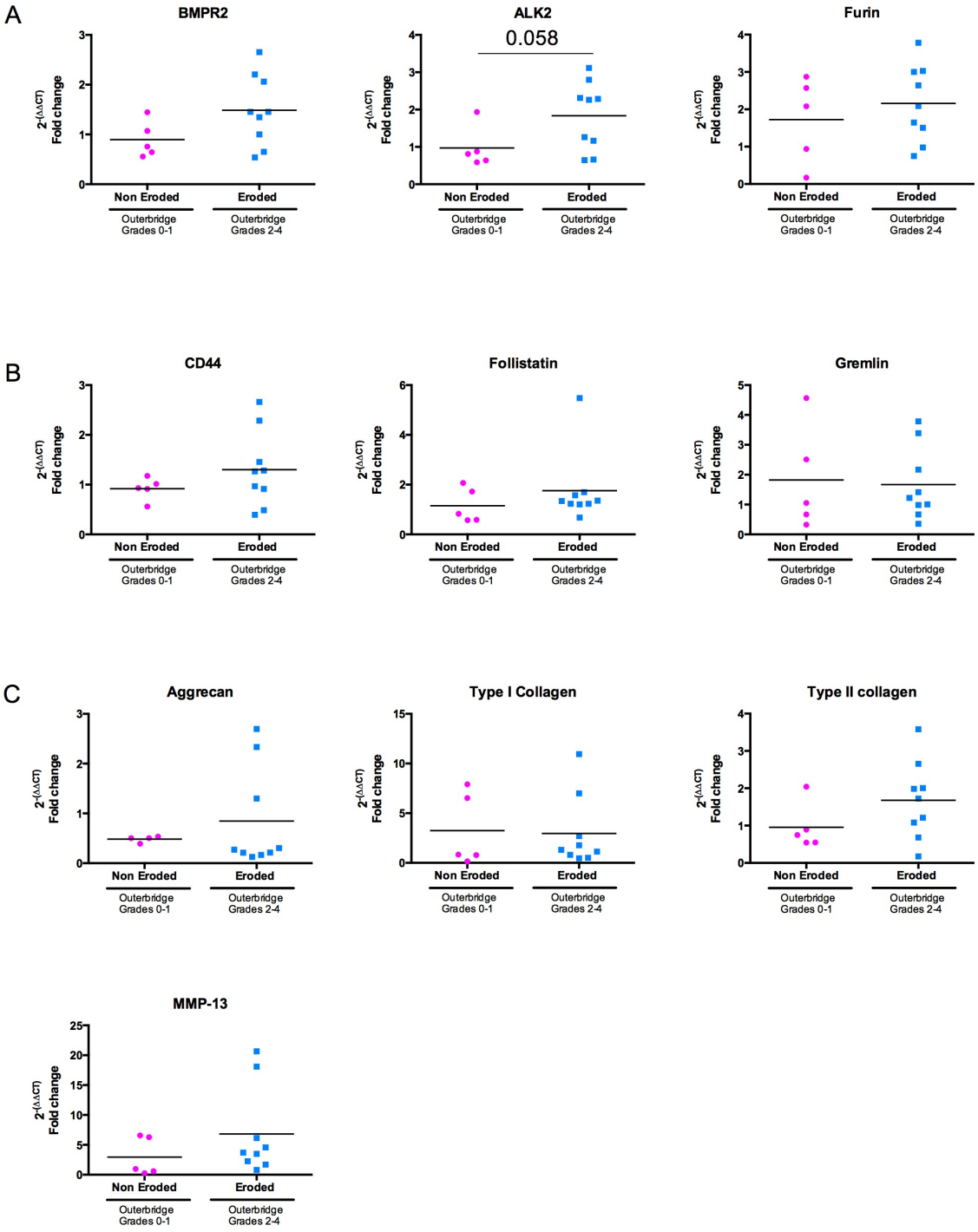


Figure 4.16 Expression of BMP-7 Signalling Components in Non-Eroded and Eroded Cartilage.

Cartilage taken from 4 OA patients (T10, T11, T12 and T13). With a total of 6 sections were taken from each joint, DMC, DLC, PMC, PLC, MTP and LTP (with one patient missing the PMC and another missing LTP). Each section was given an Outerbridge score; all data from sections obtained from one patient with the same score were averaged. Fold change, $2^{-\Delta\Delta CT,}$ was calculated using the mean of all of the Healthy tissue (Outerbridge 0-1) as a baseline. The ΔCT normalisation was calculated using β-actin. A) Expression of direct BMP-7 signalling components i) BMPR2, ii) ALK2 and iii) Furin. B) Expression of non-direct BMP-7 signalling components i) CD44, ii) follistatin and iii) Gremlin. C) Expression of extracellular matrix components i) Aggrecan, ii) Type II collagen, iii) Type I collagen and iv) matrix degradation enzyme, MMP-13. All analysis carried out using SYBR green. Statistical analysis carried out using Mann Whitney T-test.

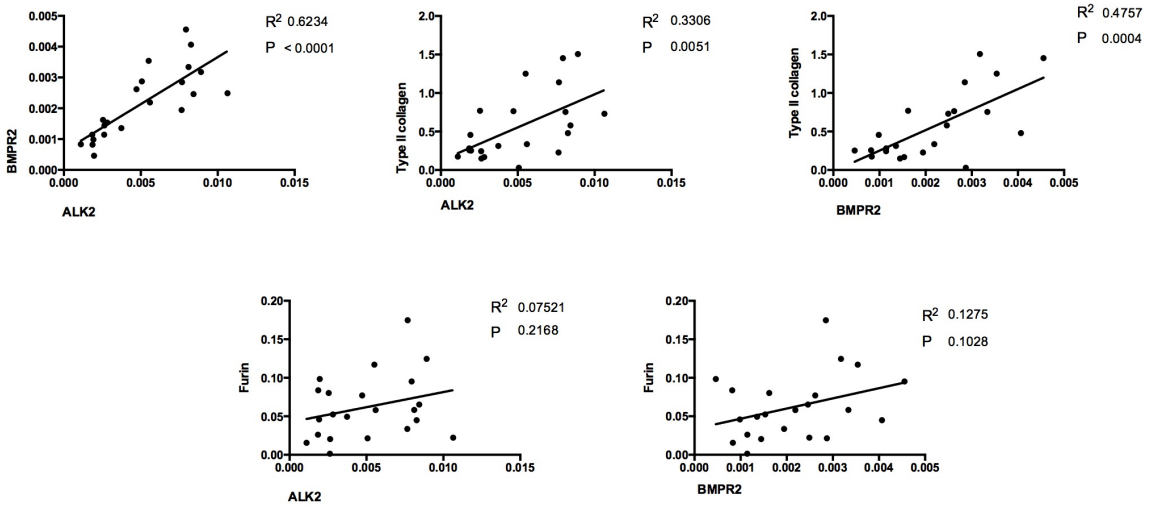


Figure 4.17 Positive Correlations Exist Between the Expression of BMPR2, ALK2 and Type II Collagen Within the OA Patient Cohort.
Linear regression analysis of all samples cartilage taken from T10, T11, T12 and T13.

4.3.8 MiRNA Expression in OA Cartilage

In this study, patient variation within the transcript expression has been shown across the BMP-7 signalling cascade in a small cohort of OA patients. Therefore, to begin to address why these patients have differential expression of these transcripts, studies to investigate the epigenetic regulation of BMP-7 and the BMP-7 signalling family members were conducted. This was based on the growing literature which provides evidence that dysregulation of certain miRNA's are associated with OA (194,202,232). Human TargetScan is an internet based mathematical algorithm that is used to predict miRNA targeting of mRNA based on the seed region complementarity to the 3'UTR. Using the BMP-7 signalling cascade as a starting point, miRNA that were predicted to regulate more than one key member of the BMP-7 signalling pathway were identified. To this end, miR24 3p, miR342 3p and miR342 5p were highlighted as potential miRNA's of interest (Figure 4.18). It is predicted that miR24 3p will target BMP-7, the cleavage enzyme furin, BMP receptor ALK2, BMP inhibitor follistatin and intracellular proteins SMAD1/5/4. While miR342 3p is predicted to target BMP-7, BMP receptor BMPR2 and SMAD1/5. Finally, miR342 5p is predicted to target cleavage enzyme furin and BMP inhibitor gremlin. In this instance, a biopsy that had been taken from the non-weight bearing femoral notch of each OA knee (Figure 2.1) was simultaneously analysed along with the other cartilage from the OA cohort (Table 4.2), thus providing an internal control for each patient. It is understood that this internal control is still exposed to the same disease environment as the rest of the cartilage, however, this area is not exposed to any form of mechanical loading, thus it can control for any mechanical related changes in the disease process. All three miRNA's were detected in cartilage (Figure 4.19). MiR24 3p was detected within a CT range of 14-20, miR342 3p within a CT range of CT 17-25, and miR342 5p within a CT range of CT 21-28. Interestingly, OA associated Let7e, a miRNA whose plasma levels negatively correlate with risk of arthroplasty (209), could not be detected within chondrocytes (data not shown). No differences in miRNA levels were observed between the weight bearing cartilage and the non-weight bearing femoral notch internal control (Figure 4.19).

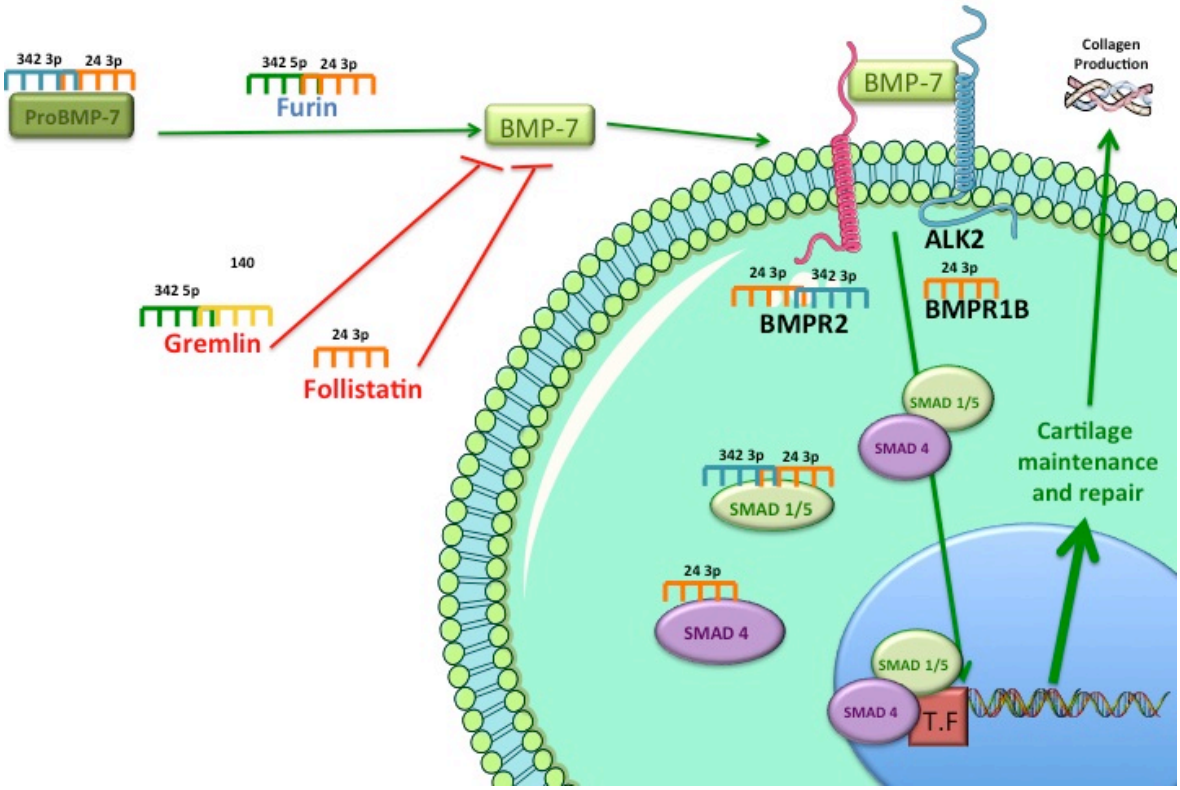


Figure 4.18 Schematic of miRNA Targeting of the BMP-7 Signalling Family as Predicted by Human TargetScan.
Basic depiction of the BMP-7 signalling cascade highlighting the miRNAs that are predicted via human TargetScan to target members of the BMP-7 signalling family. MiR24-3p is predicted to target; BMP-7, furin, BMPR2, BMPR1B, follistatin, SMAD1/5 and SMAD4. MiR-342-3p is predicted to target: BMP-7, BMPR2 and SMAD1/5. Mir342-5p is predicted to target; furin and gremlin and miR140 is predicted to target gremlin.

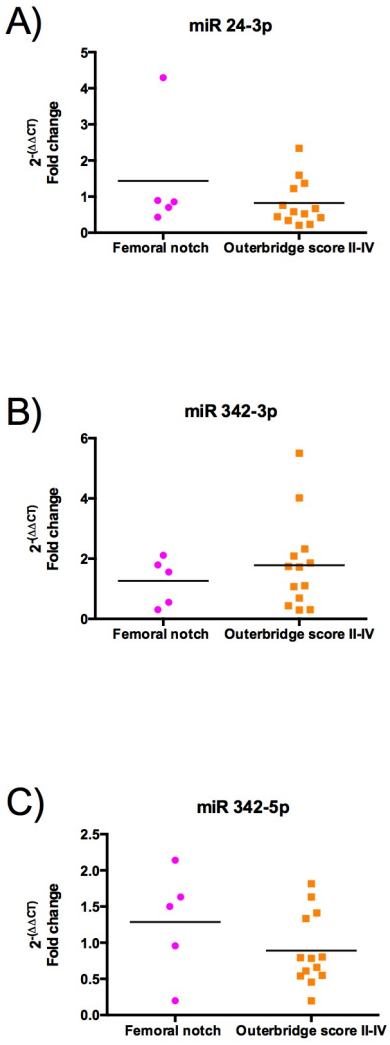


Figure 4.19 Unaltered miRNA Expression in OA Patient Cartilage using an Internal Non-Weight Bearing Control.
Cartilage taken from 5 OA patients with a total of 6 sections were taken from each joint, DMC, DLC, PMC, PLC, MTP and LTP, with one patient missing the DLC and another missing MTP along with a punch biopsy from the femoral notch. Each section was given an Outerbridge score, all data from sections obtained from one patient with the same score were averaged. Data is shown as fold change ($2^{-\Delta\Delta CT}$) with an average value of the femoral notch samples from all the patients used as baseline. MiRNA analysis was carried out with validated primers from Qiagen, the following miRNA were analysed within the cohort, A) miR24 3p B) miR342 3p and C) miR342 5p. ΔCT calculated using U6 as a normalisation gene. All analysis carried out with MiScript SYBR green from Qiagen. Statistical analysis; Mann-Whitney test.

4.3.9 MiRNA Targeting of BMP-7 Signalling Transcripts

As the miRNA's that were predicted to target BMP-7 signalling family members were detected in the cartilage of the OA patients, it was decided that investigation into the potential of these miRNA's to directly modulate the expression of the BMP-7 signalling family would be of interest. Despite the fact that OA related changes in their expression could not be confirmed due to a lack of healthy controls. Primary chondrocytes were excised from cartilage with an Outerbridge score of ≤1 from three individuals suffering from end stage OA (Section 2.3). Cells were plated in plastic culture plates and given time to acclimatise and recover from the stress caused when removed from the ECM; timings determined from the optimisation experiments carried out in Chapter 3 (Figure 3.6). Following the rest period, chondrocytes were transfected with either miR24 3p mimic or miR342 3p mimic for 24 hours. To ensure that the transfections were specific to the 3p isoform of the miRNA that was chosen for this investigation, qPCR was conducted. (Figure 4.20). It is clearly demonstrated that the cells transfected with either miR24 3p or miR342 3p mimic (Figure 4.20 A/B) display increased expression of the miRNA for that isoform only. Further to this, they also show that the control mimic (CM) has no effect on the expression of the miRNA compared to the untransfected cells. The effect of the transfection protocol with regards to the endogenous expression profiles of the mRNA of interest (BMP-7, IL-1β, BMPR1B, ALK2, BMPR2, furin, follistatin and gremlin) was also evaluated (Figure 4.21). IL-1β, follistatin and BMPR2 displayed modest alterations following transfection; however these were not significant. This confirmed that CM transfection had a negligible impact on the expression of the genes of interest and could subsequently be used as the baseline for future analysis. Leading on from the initial optimisation, cells were transfected with either miR342 3p mimic or miR 24 3p mimic (Figure 4.22) and (Figure 4.23) respectively. The observed data show that the miR342 3p mimic had no repressive impact on any of the genes of interest (Figure 4.22) suggesting no direct interactions between the miRNA and the transcript. However, there was an increase in the expression of gremlin following miR342 3p mimic transfection, possibly suggestsing some form of indirect interplay between miR342 3p and this mRNA (Figure 4.18). MiR24 3p mimic analysis was also carried out for the transcripts of interest, (Figure 4.23). Here some direct

interactions between miRNA and mRNA were observed (Figure 4.18). With one of these interactions having been predicted by human target scan, BMPR1B repression, and one having not, ALK2 repression. The data show a 60 – 90% inhibition in BMPR1B receptor expression and between 40% - 50% inhibition of ALK2 following treatment with the miR24 CM. IL-1β and BMP-7 were also substantially down-regulated in two out of the three chondrocyte donors, suggesting heterogeneity in the chondrocyte ability to respond to this miRNA. Given that the data suggests a direct relationship between the miRNA and the mRNA of both BMPR1B and ALK2, it was decided to go back to the OA cohort to conduct correlation analysis of the miRNA and mRNA in the patients (Figure 4.23J). A negative correlation was observed between the expression of the miRNA and ALK2 in the OA patients, surprisingly a correlation was also found between miR24 and BMPR2 even though it did not seem to have a profound effect on the transcript in the transfection assay, 30% inhibition in 2 of the chondrocyte donors. No correlation was observed between miR24 and BMPR1B. For the first time, a role for miR24 3p in the regulation of BMP receptor expression has been demonstrated, implicating this miRNA in some form of regulatory network for the BMP-7 signalling pathway. In the future a luciferase assay would be needed in order to confirm direct targeting of receptors by miR24 3p.

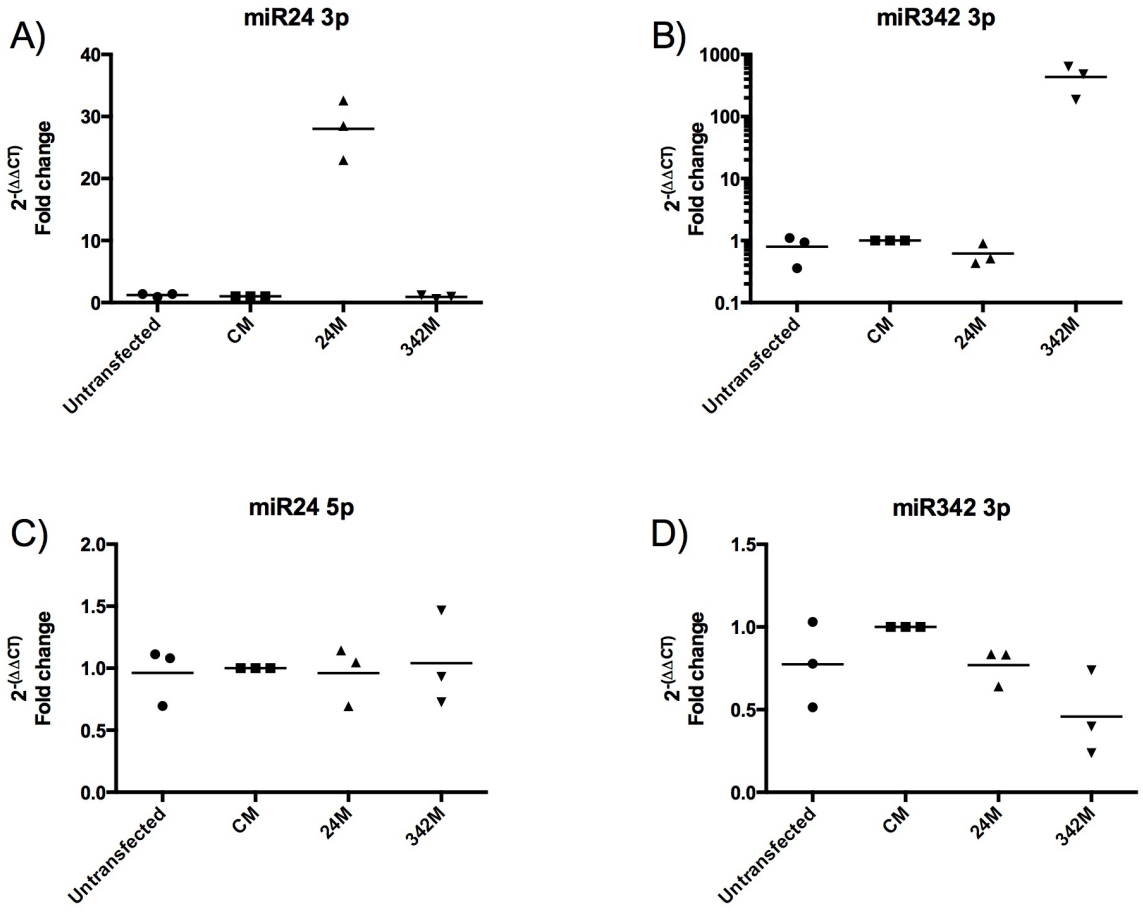


Figure 4.20 Validation of Isoform 3p Specificity in Mimic Transfections.
Primary chondrocytes from three cartilage donors (N=3) undergoing TKR surgery, Outerbridge score ≤1 were plated at $1X10^5$/ml. Following 48 hours acclimatisation, the cells were transfected using Dharmafect transfection reagent with 10ng of either control mimic (CM) miR24 3p mimic (24M) or miR342 3p mimic (342M). Cells were transfected for 24 hours before media was changed and the cells were harvested 48-hour post transfection. qPCR analysis was carried out to assess the uptake of the mimic into the cells. All fold change ($2^{-\Delta\Delta CT}$) was calculated using the CM as a baseline. A) MiR24 3p expression B) miR342 3p expression C) miR24 5p expression D) miR342 5p expression.

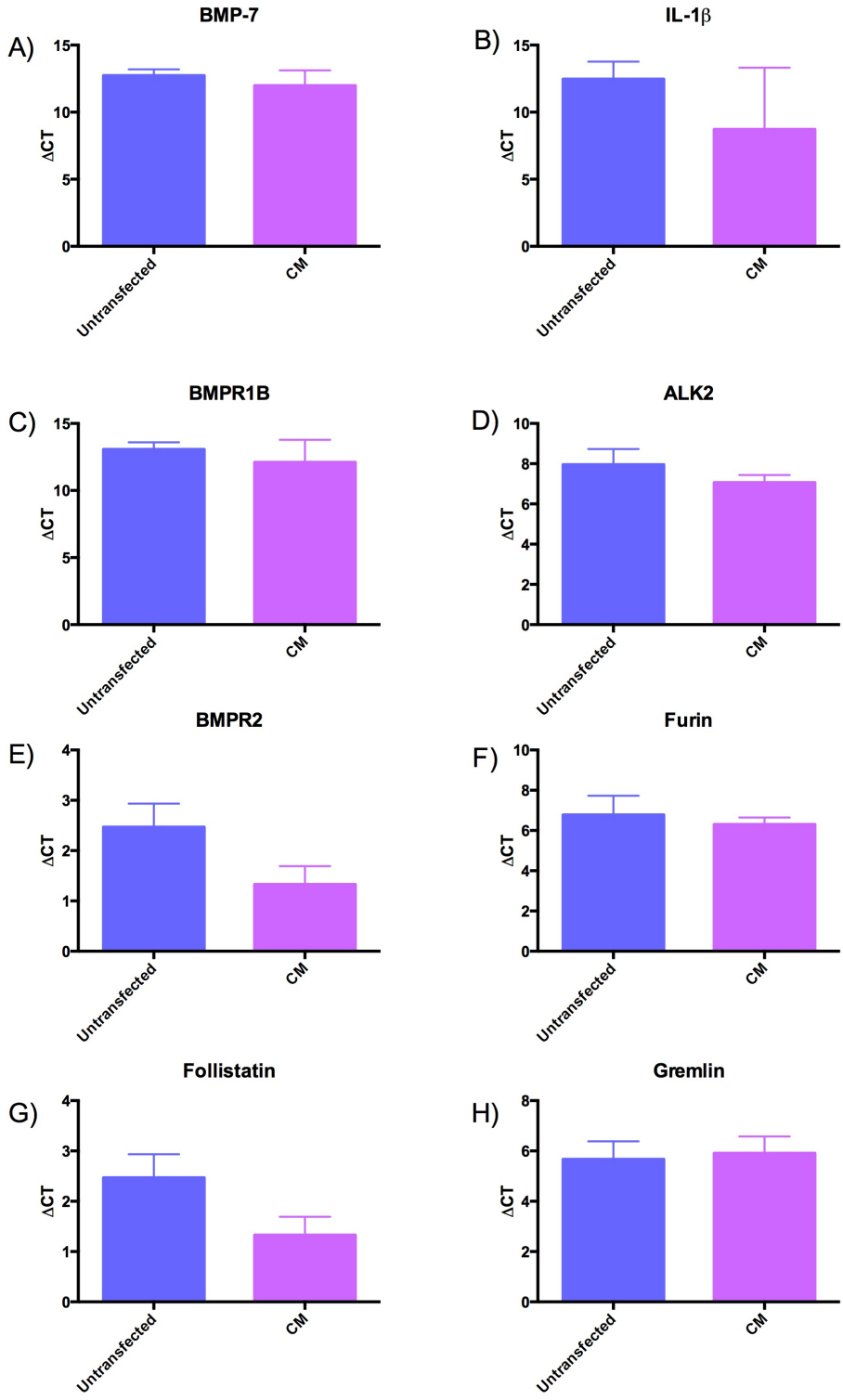


Figure 4.21 Minimal Differences Observed in the Gene Expression of the Control Mimic Cells Compared to the Untranfected Cells

Primary chondrocytes from three cartilage donors (N=3) with an Outerbridge score ≤1 were plated at $1X10^5$/ml. Following 48 hours acclimatisation, the cells were either transfected using Dharmafect transfection reagent with 10ng control mimic (CM) or placed in control media (no transfection reagents). Cell were transfected for 24 hours before media was changed and the cells were harvested 48-hour post transfection. qPCR analysis was conducted comparing the ΔCT value (CT value gene of interest – CT value housekeeping (β-actin)) for the CM transfected and untransfected cells. All relevant genes of interest were analysed A) BMP-7, B) IL-1β, C) BMPR1B, D) ALK2, E) BMPR2, F) Furin, G) Follistatin and G) Gremlin. Statistical analysis; Wilcoxon test

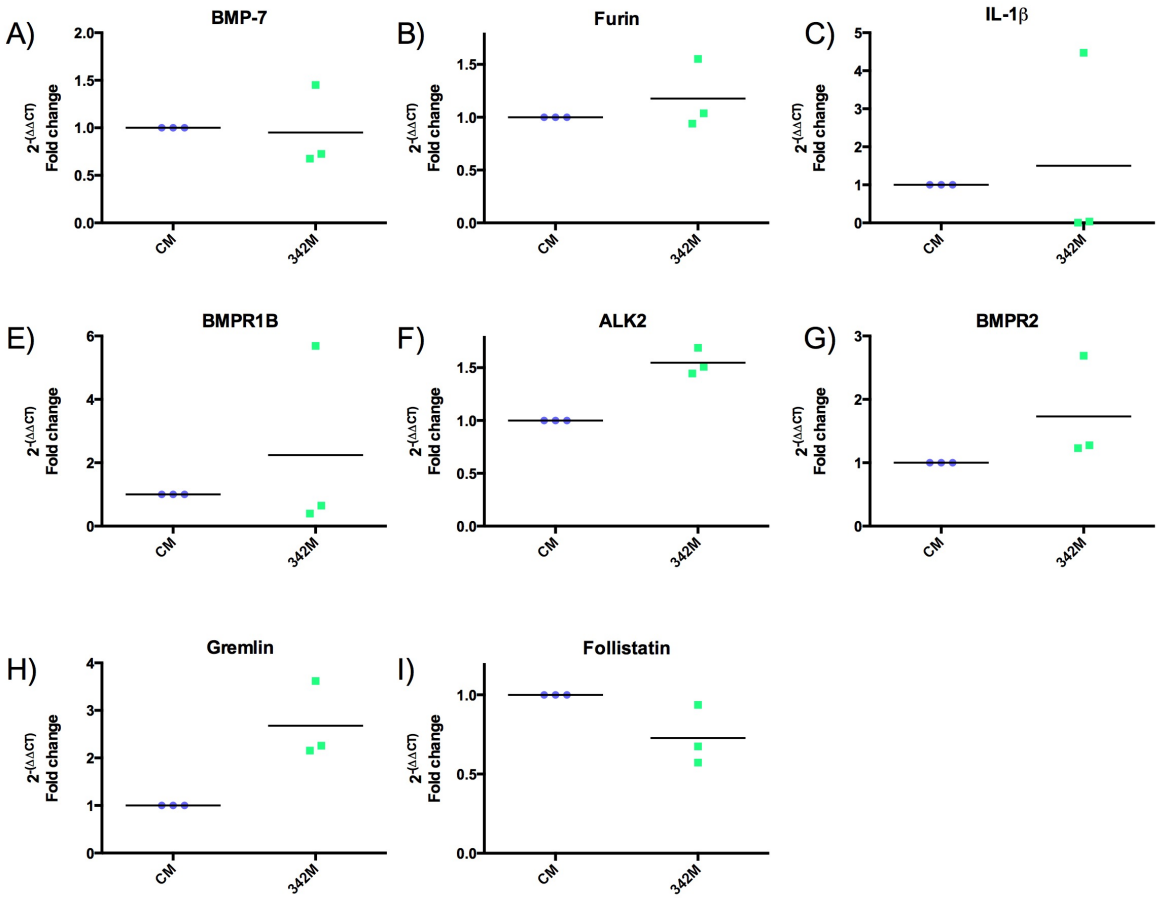


Figure 4.22 miR342 3p has an Indirect impact on the Expression of BMP-7 Signalling Family Member Gremlin

Primary chondrocytes from three cartilage donors (N=3) with Outerbridge score ≤1 were plated at $1X10^5$/ml. Following 48 hours acclimatisation, the cells were transfected using Dharmafect transfection reagent with 10ng of either control mimic (CM) or miR342 3p mimic (342M). Cell were transfected for 24 hours before media was changed and the cells were harvested 48-hour post transfection. All fold change ($2^{-\Delta\Delta CT}$) was calculated using the CM as a baseline. Genes interrogated were A) BMP-7, B) Furin, C) IL-1β, D) BMPR1B, E) AK2, F) BMPR2, G) Gremlin H) Follistatin. The housekeeping gene used for ΔCT was β-actin. Statistical analysis; Wilcox test.

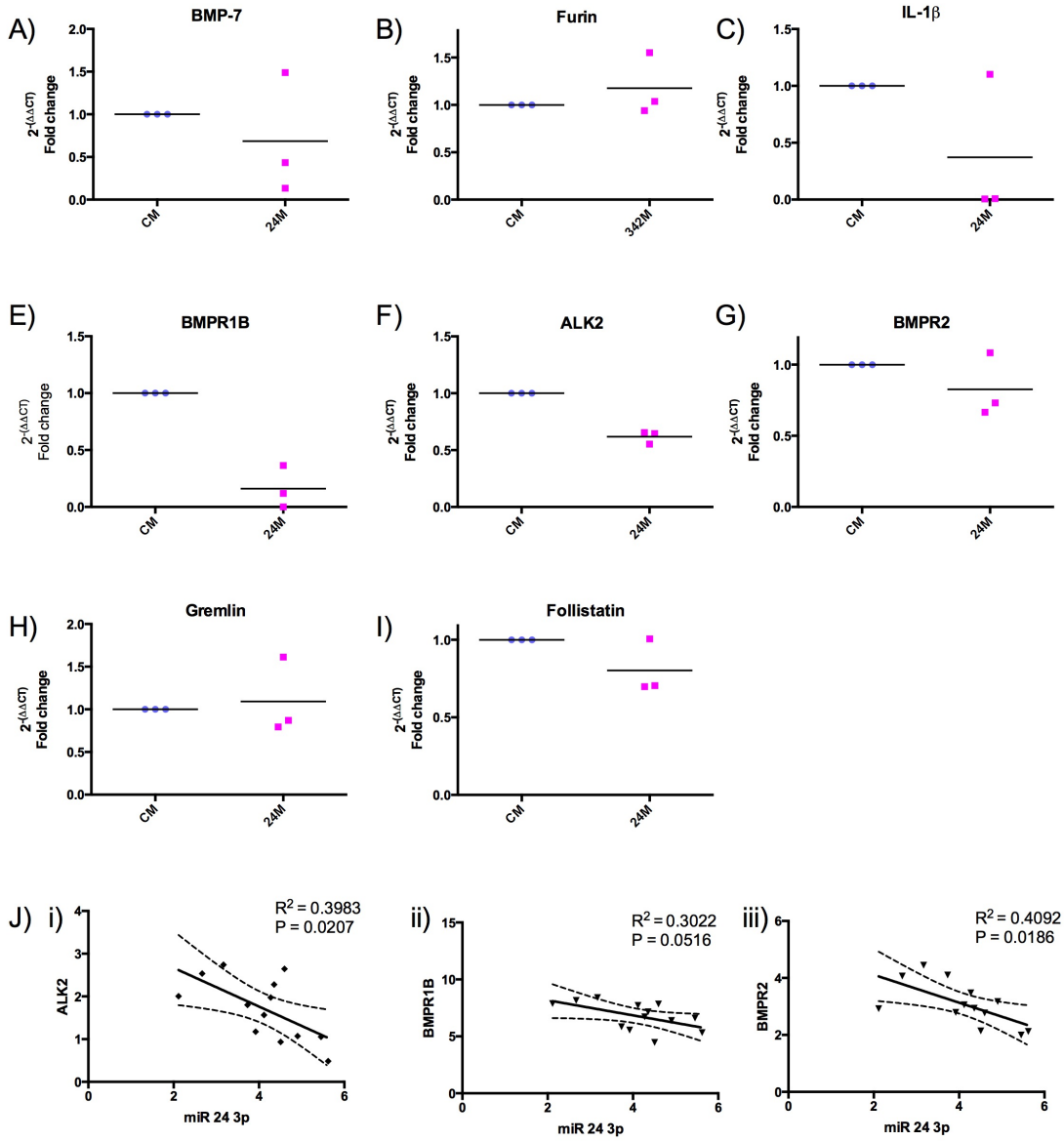


Figure 4.23 miR24 3p has a Direct Effect on Gene Expression Across the BMP-7 Signalling Family.

Primary chondrocytes from three cartilage donors (N=3) with Outerbridge score ≤1 were plated at 1X10^5/ml. Following 48 hours acclimatisation, the cells were transfected using Dharmafect transfection reagent with 10ng of either control mimic (CM) or miR342 3p mimic (342M). Cell were transfected for 24 hours before media was changed and the cells were harvested 48-hour post transfection. All fold change ($2^{-\Delta\Delta CT}$) was calculated using the CM as a baseline. Genes interrogated were A) BMP-7, B) Furin, C) IL-1β, D) BMPR1B, E) AK2, F) BMPR2, G) Gremlin H) Follistatin. The housekeeping gene used for ΔCT was β-actin. Statistical analysis; Wilcoxon test. J) Correlation analysis of miR24 3p with i) ALK2, ii) BMPR1B and iii) BMPR2, was conducted using ΔCT values for the mRNA (ΔCT = mRNA Ct value - β-actin CT value) and the miRNA (ΔCT = miRNA CT value – U6 CT value). Cartilage was taken from 5 OA patients at the stage of TKR, (total of 6 sections were taken from each joint, DMC, DLC, PMC, PLC, MTP and LTP, with one patients missing the DLC and another missing MTP) each section was given an Outerbridge score. All sections obtained from one patient with the same score were averaged. Linear regression analysis with 95% confidence was applied using the line of best fit to determine the R^2 value.

4.3.10 miRNA Expression in Plasma

Recent studies have shown differences in the plasma expression of miRNA in OA (32). Based on this published work it was decided that the miRNA of interest in this study would also be investigated in the plasma of the OA patients compared to healthy aged matched controls. For the purpose of this investigation blood was taken from all patients on the morning of their surgery. Beyer *et al* have previously suggested that Let7e may be a predictive marker for arthroplasty (209): negatively correlating miRNA plasma levels and the number of joint replacements. Levels of Let7e in our OA cohort demonstrated that compared to healthy controls (Figure 4.24A) there is less circulating Let7e. This is consistent with the findings reported in the Beyer study; where Let7e levels in the plasma were decreased in those who went on to develop OA. The expression of miR24 3p and miR342 3p that are predicated to modulate the BMP-7 signalling cascade were also interrogated in the plasma, (Figure 4.24C/D). There was a significant decrease in both miRNA in the OA cohort compared to the controls. MiR140 was of also of interest as it has been predicted to target the BMP inhibitor gremlin (Figure 4.18). Furthermore, it has also has been linked to OA with decreased expression being associated with disease and overexpression being shown to be protective in animal models of disease (191,239). The cartilage specific miR140 is also decreased in OA compared to the healthy control plasma (190) (Figure 4.24B). Despite the fact that a statistical difference was observed in all of the miRNA's of interest, there is still a lack of validation of the OA specificity of these changes. As OA shares several common features with other musculoskeletal diseases such as RA, other disease controls would be needed in order to verify if these miRNA alterations are OA specific.

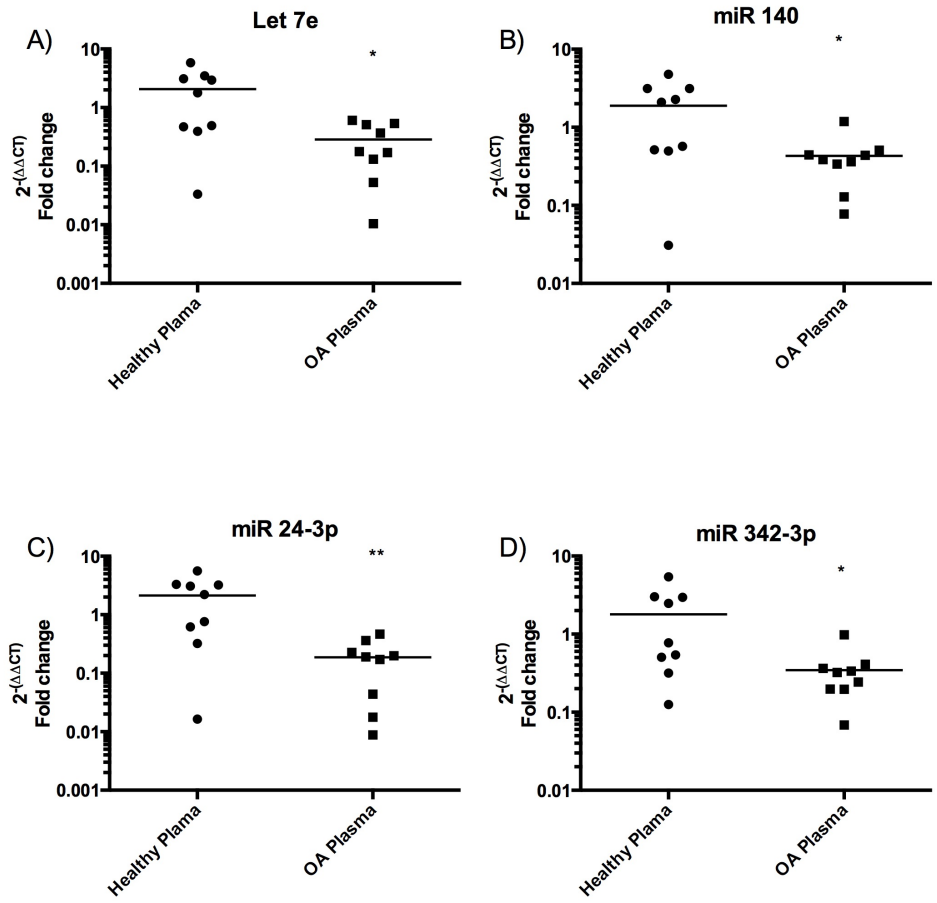


Figure 4.24 Decreased Levels of miRNA in the Plasma of OA Patients Versus Healthy Controls.

Plasma was collected in EDTA collection tubes from OA patients on the day of TKR surgery, N=9, mean age 69.6 years or from healthy, age and sex matched controls, N=9, mean age 69.5 years. *C. Elegans* specific miRNA, miR39, was used as a spike in control for normalisation at 1.6 X 10^8 copies/µl. Fold change ($2^{-\Delta\Delta CT}$) was calculated using the average of the healthy plasma samples as a baseline value. Analysis was carried out for the following miRNA A) Let7e B) miR140 C) miR24 3p and D) miR342 3p. Statistical analysis; Unpaired T-Test *P < 0.05, **P< 0.01.

4.4 Discussion

4.4.1 Summary of Results

This chapter was designed to address three key aims, with the first being the determination of BMP-7 and its signalling components expression in an OA cohort. In addressing this it was found that BMP-7 mRNA could not be robustly detected using conventional methods for molecular analysis. Only very low levels of mRNA appear to be expressed raising the question of the physiological relevance of this transcript. Additionally, these low levels of expression were only detected in some of the samples analysed suggesting donor specific expression of this transcript. Protein was qualified using IHC within the cartilage, confirming that the protein is present. Furthermore, it was found that BMP-7 was expressed at high levels in the vascular endothelial cells in the synovium of OA patients. This was not observed in the RA synovium, thus highlighting the molecular differences between these two disease states. The second aim addressed the expression of BMP-7 signalling cascade members and other BMP-7 associated genes in a small cohort of OA patients. No significant changes were observed between eroded and non eroded cartilage, possibly due to the cohort being underpowered. It was found that there were strong significant correlations between the expression of the BMP receptors, BMPR2 and ALK2, along with type II collagen within the cohort. This suggests that intact anabolic capacity in an OA setting may be patient specific, displaying heterogeneity in the small cohort with respect to this. Finally, the work conducted in this chapter aimed to delineate the expression of BMP associated miRNA in OA and furthermore validate if any of the signalling components were direct targets of these miRNA's. It appears that 4 miRNA's are differentially regulated in the plasma of OA patients compared to age matched controls; Let7e, miR140, miR24 3p and miR342 3p. Furthermore, it was also shown that miR24 3p may have a direct effect on the expression of BMP receptors; with a strong negative correlation in the OA cohort between miR24 3p and the expression of ALK2 being shown, potentially highlighting a regulatory role for miR24 in OA pathogenesis.

4.4.2 The On-Going Debate Over BMP-7 Expression

Expression of BMP-7 transcript has been reported in primary human chondrocytes (147,148). However, validation of this expression by other groups using qPCR has not been successful (149), thus leaving discrepancy in the published literature over the expression of this transcript in articular cartilage. This study confirms that BMP-7 transcript is difficult to detect in primary human chondrocytes using conventional molecular analysis such as qPCR, thus supporting the work of Chen *et al* (2006). Previous studies that report BMP-7 expression in articular chondrocytes used very sensitive methods such as *in situ* hybridisation or nested PCR. Thus it is not surprising that the conventional methods of mRNA analysis were unable to recapitulate these findings and the different techniques deployed offer an explanation for the discrepancies between the findings of Chubinskaya (2000) and Chen (2006). Data generated in this chapter did, to some extent, verify the expression of BMP-7 transcript using nested PCR methodology. However, validation of the nested PCR was not successful using other hypersensitive readouts such as digital PCR. The negative results obtained only give rise to more doubt as to the expression of the transcript in chondrocytes and thus leave the unanswered question; can chondrocytes robustly express BMP-7 transcript?

BMP-7 protein can be visualised within the joint in both the articular cartilage and synovium. It would appear to be most abundant in areas of chondrocytic clustering typical in OA/stress (240)(241). It is plausible that certain mediators that are released in response to stress may be able to induce BMP-7. Data obtained here regarding the expression of BMP signalling components such as receptors, cleavage enzyme furin and type II collagen may further support this. All three of these mediators appear to increase modestly at the transcript level in the eroded cartilage, which would be regarded as more stressed, when compared non-eroded OA cartilage. It was also found that when patients were investigated individually, those with higher levels of the receptors also had higher expression of Type II collagen suggesting that this signalling pathway may still be intact in some of the patients. It is feasible that these stressed cells attempt to increase BMP-7 signalling in order to enhance anabolic repair. Furthermore, as these stressed cells are sparsely divided throughout the

cartilage it may be very hard to pick up the small levels of BMP-7 transcript produced, hence why only nested primers are able to be successful in detecting the mRNA. With this in mind, it still does not account for the lack of validation from the digital PCR.

4.4.3 Variance Between the Expression of BMP-7 Transcript and Protein.

Computational data from this study may suggest that the BMP-7 transcript has a short half-life, potentially due to degradation by regulators such the zinc finger protein TTP. Again making detection at the level of the transcript very difficult. TTP has been shown to be modified by miRNA in the other forms of arthritis such as RA, where Bruton's tyrosine kinase (Btk), a negative regulator of TTP, is negatively regulated by miRNA 346 (242). This suggests that miRNA 346 may have an anti-inflammatory role via indirect up-regulation of TTP, which has further been shown to down regulate TNFα. Futhermore, miR 346 has been reported to be elevated in RA synovial fibroblast (243). Based on these studies, it is not inconceivable for TTP expression to be altered in OA as it is in RA, causing changes in effector transcripts, such as BMP-7. Alternatively, it may be influenced indirectly by miRNA's that are altered as a result of the disease, as has been shown with miRNA 346 in RA. Many groups have now identified a collection of miRNA's that are deregulated in OA (186,194,244), therefore one of these may lead to a similar situation with regards to TTP as to the one observed in RA. MiR140 has been reported to be down-regulated in OA (202) and is predicted to target TTP (prediction via human target scan), therefore a disease associated decrease in these miRNA may result in an subsequent increase in TTP. It may be beneficial to compare TTP expression in the OA and normal cartilage, thus providing evidence as to whether it is altered in disease. From here work could be carried out to identify miRNA that may be involved in disease regulation of TTP. Furthermore, as various splice variants of BMP-7 exist, it would be of interest to investigate which of these are more commonly expressed in OA, as some will have more TTP binding sites than others, thus impacting their potential for TTP targeted regulation.

Another explanation for the presence of the BMP-7 protein, despite the lack of transcript, could be that the observed protein may be latent and the transcript may have been produced at an earlier point in the disease process. It must be considered that one of the major caveats of this study was that only diseased tissue could be accessed. It has been reported in the literature that if BMP-7 is not processed to its mature form then the prodomain is able to anchor the protein complex to the N terminal of the ECM component fibrillin 1 (112) thus creating a store of inactive protein. Further to this it has also been suggested that the mature protein could be stored in a similar fashion, as it has been reported that after cleavage the pro-region can remain non-covalently associated with the mature protein; again facilitating binding to the ECM (111). Therefore, it is possible that when the protein is required the cleavage enzyme furin may be up-regulated, activating the latent 'pro' protein and initiating signalling. The data presented above (Figure 4.16) would offer support to this, as furin was increased in some of the donors in the eroded cartilage compared to the non-eroded cartilage suggesting that these cells may be trying to activate the anabolic capacity of the BMP signalling pathway at this time of stress. If the study had been adequately powered (power calculation suggests that the study was significantly underpowered) then perhaps this increase in furin would have reached significance. There is further evidence to support the idea that the BMP-7 detected is latent and may be the remnants' left from the chondrocytes last anabolic stand before the catabolic insurgence took over the joint; with work carried out using osteoblasts suggesting that BMP-7 expression is correlated to ECM production (245). Therefore, with the anabolic/catabolic battle featured in OA it could be speculated that as the catabolic influences take over the joint there is an associated decrease in ECM synthesis and therefore a decrease in BMP-7 expression. This idea would also support the data that was generated reporting a decrease in BMP-7 in the cartilage of those with OA (150). However, discrepancies existing in the literature cannot be ignored with some groups reporting an increase in the levels of BMP-7 in synovial fluid, plasma and cartilage, as disease progresses (153,228), whilst others have published unaltered BMP-7 levels in the synovial fluid of healthy controls and OA patients (151).

The data generated from this study can confirm neither of the previous findings with regards to plasma and synovial fluid expression and would in fact suggest that BMP-7 is not present within the plasma at concentrations detectable with commercially available ELISAs. Most other groups that have measured BMP-7 by ELISA have utilised an in house method (148,151,246,247), with only two other groups having used commercialised ELISA kits in quantifying the protein. Honsawek *et al* show that BMP-7 could be detected using an R&D systems kit, although they report values below the limit of detection for the assay used, (153). They also state that the synovial fluid levels, as well as the plasma levels, are increased in OA compared to the control. However, the only control samples appear to be from the plasma and therefore the synovial fluid had no appropriate control and a true comparison could not be made. Schmal *et al* used another R&D systems kit and reported detection the protein, however, it must be considered that their samples were taken from ankle OA which may be different with respect to BMP-7 expression. The remaining groups used non commercially validated self-designed ELISAs; three of the groups use a method that is detailed in a publication by Chubinskaya in 2002 (148), utilising 2 capture antibodies, a polyclonal and a monoclonal antibody raised against BMP-7, thus perhaps giving rise to a more sensitive method of detection. The fourth group used an alternative protocol for their in house ELISA (247). Chubinskaya *et al* detail the validation of their assay through the generation of a standard curve with recombinant BMP-7, however the other group do not demonstrate the use of appropriate standards or controls. Some of these self-designed ELISAs report BMP-7 levels in the regions of 50 ng/ml in OA, which has been reported as comparable to control expression levels (151). They further report that BMP-7 protein expression is increased in RA, 100 ng/ml, when compared to the OA and control fluids. Additionally those reporting success in quantifying BMP-7 protein expression using the commercially available kits have yielded concentrations of approximately 50 pg/ml, giving a discrepancy of 3 log orders. Therefore, once again, there appears to be disparity in the literature over the quantifiable levels of BMP-7 in both the plasma and synovial fluid.

4.4.4 Alternative Roles for BMP-7 – BMP-7s Role in the Vasculature

From the data generated in this study alone, the most likely source of the BMP-7 is the synovium, as both the transcript and protein were readily detectable in the tissue using qPCR and IHC respectively. This staining is predominantly in the endothelial cells of the blood vessels. Despite the published literature detailing the transcript expression of BMP-7 in the synovium (248), this is the first time that the localisation of BMP-7 has been shown using IHC. BMP-7 has been shown to up-regulate the expression of vascular endothelial growth factor (VEGF) at both the transcript and protein level in both human and bovine ovarian granulosa cells (249,250) therefore, its presence in the inflamed, highly angiogenic OA synovium (46) may not be all that unexpected due to VEGFs involvement in angiogenesis.

In health, it is possible that the presence of BMP-7 in the vasculature may be a protective mechanism preventing vascular calcification. An inverse relationship has been reported between single nucleotide polymorphisms (SNPs) in the BMP-7 gene and the associated components of both vascular calcification and reduced bone mineral density (252). In addition, it has been demonstrated that BMP-7 offers protection against vitamin D induced vascular calcification (253). The results from this chapter report that qualitatively there appears to be more BMP-7 staining in the vasculature of OA patients compared to RA patients, thus suggesting that mechanisms involved in RA associated angiogenesis may be different to that of OA. These differences may contribute to the strong correlation between RA and cardiovascular disease (254). The fact that there appears to be more BMP-7 present in the vasculature of the OA synovium is consistent with the fact that the prevalence of coronary heart disease is only 27% in OA compared to 49% in RA (255). Despite the lower prevalence in OA, work has begun to try and better understand CVD and OA using cross sectional and longitudinal studies. Overall most have reported a correlation between OA and heart disease, however, it must be taken into account that some groups have not corrected their analysis to reflect BMI (256). It has also been shown that obesity is a strong determining factor for hand OA, and may be the contributing factor to the CVD co-morbidity (257). A recent study noted that

when data was adjusted for several risk factors including BMI, a correlation between OA and CVD still existed. However, the study relied on patient self-reported data and therefore lends itself to bias from false positive and negative results (256). Even with these caveats, a relationship between OA and CVD appears to be consistent and is an area where investigation into biological mechanism is required. With a lack of non-diseased synovium, no analysis can be conducted into altered BMP-7 expression in the endothelial cells in OA.

4.4.5 MiR24 Dysregulation as a Driving Factor of Disease Progression.

The expression of the three miRNA's of interest miR24-3p, miR342 3p and miR342 5p were unaltered in the cartilage when compared to a non-weight bearing internal control. This may not be surprising as all the cartilage was from an OA disease environment and the femoral notch is only a true control for mechanical loading on the cartilage. If OA origin stems from a more global cause, then it would be suspected that all of the cartilage would be affected by the driving factors of disease and not just the areas exposed to excessive loading. These may include age related changes in gene expression within the cartilage (222) or obesity leading to increased fat accumulation, adipokine production and pro-inflammatory mediator secretion (258).

Studies have been carried out to investigate the expression of miR24 in the cartilage of OA patients and healthy controls, these show that there is a disease associated decrease in miR24 expression (195). They go on to further suggest that a decrease in this miRNA is a driving factor in disease. It is stated that a decrease in miR24 results in a reciprocal increase in the cell senescence marker $p16^{INK4a}$ and thus an increase in the senescence-associated secretory phenotype SASP secretome (Section 1.4.4.4). This can contribute to enhanced cartilage degradation through the expression of matrix degradation enzymes such as MMP-1 and MMP-3. In this chapter, the level of plasma miR24 is consistent with the finding of Philipot *et al*, however, the functional data generated within this chapter does not support their hypothesis. The observed over-expression of miR24 3p in this chapter resulted in the down-regulation of two BMP receptors, ALK2 and BMPR1B, both of which are involved in anabolic aspects of matrix

remodelling. Further to this in the OA patients investigated, a significant negative correlation existed between the expression of miR 24 and the receptor ALK2. Therefore, if this miRNA were suppressed in the cartilage, it would suggest that the cartilage is attempting to increase anabolism in order to combat on-going destruction. Whereas Philipot *et al* would suggest dampening of this miRNA is a driving factor perpetuating OA. The caveat to both studies is the use of *ex vivo* chondrocyte culture systems, therefore, the true disease environment can no longer be recapitulated as OA is a multifactorial disease of the joint not just of cartilage.

Philipot *et al* claim the initial decrease in the miR24 controlling the $p16^{INK4a}$ is a result of aberrant IL-1β expression. However, in other experiments conducted in this study (data shown in chapter 5), no changes in the expression of miR24 were observed following IL-1β stimulation. It is possible that culture method of the chondrocytes could lead to differing results, as the chondrocytes in this study were cultured in plastic as opposed to pellet cultures which were used by Philipot *et al*.

In order to begin to understand the complex role this miRNA may play in disease, a summary of the work carried out in this study and the data published by Philipot *et al* was generated (Figure 4.25A). Firstly Philipot *et al* suggest that miR24 is expressed in normal cartilage tissue and has a role in regulating $p16^{INK4a}$, which in turns dampens the SASP secretome (Figure 4.25A) (195). In disease states, such as OA, an initial increase in IL-1β, either from an acute tissue injury or from other disease associated molecular dysregulation, results in senescent chondrocytes which facilitate an IL-1β mediated down-regulation of miR24. This results in increased $P16^{INK4a}$ and therefore an increase in the SASP secretome (Section 1.4.4.4), leading to catabolic destruction of the joint. The hypothesis generated from this study is based on the miR24 mimic data, as healthy cartilage could not be obtained. This suggests that at the end stage of disease miR24 3p is able to target the BMP receptors thus blocking BMP signalling (Figure 4.25B). As previously mentioned, there are strong negative correlations between the miRNA expression and the expression of the target mRNA, ALK2, in the cohort investigated in this study; supporting this *in vitro* observation.

However, data regarding expression of the targets in healthy cartilage remains a vital yet unanswered piece of the story.

Both studies focus predominantly on end stage disease and therefore have missed another massive piece of this disease-derived jigsaw. At some point between the onset of disease and the symptomatic diagnosis of OA there is a battle between anabolic and catabolic processes within the joint. Here the anabolic factors work to suppress the increasing catabolic destruction. The levels of miR24 at these early stages of disease would be of great interest, as it would provide pivotal information as to the overall role of this miRNA in the disease process.

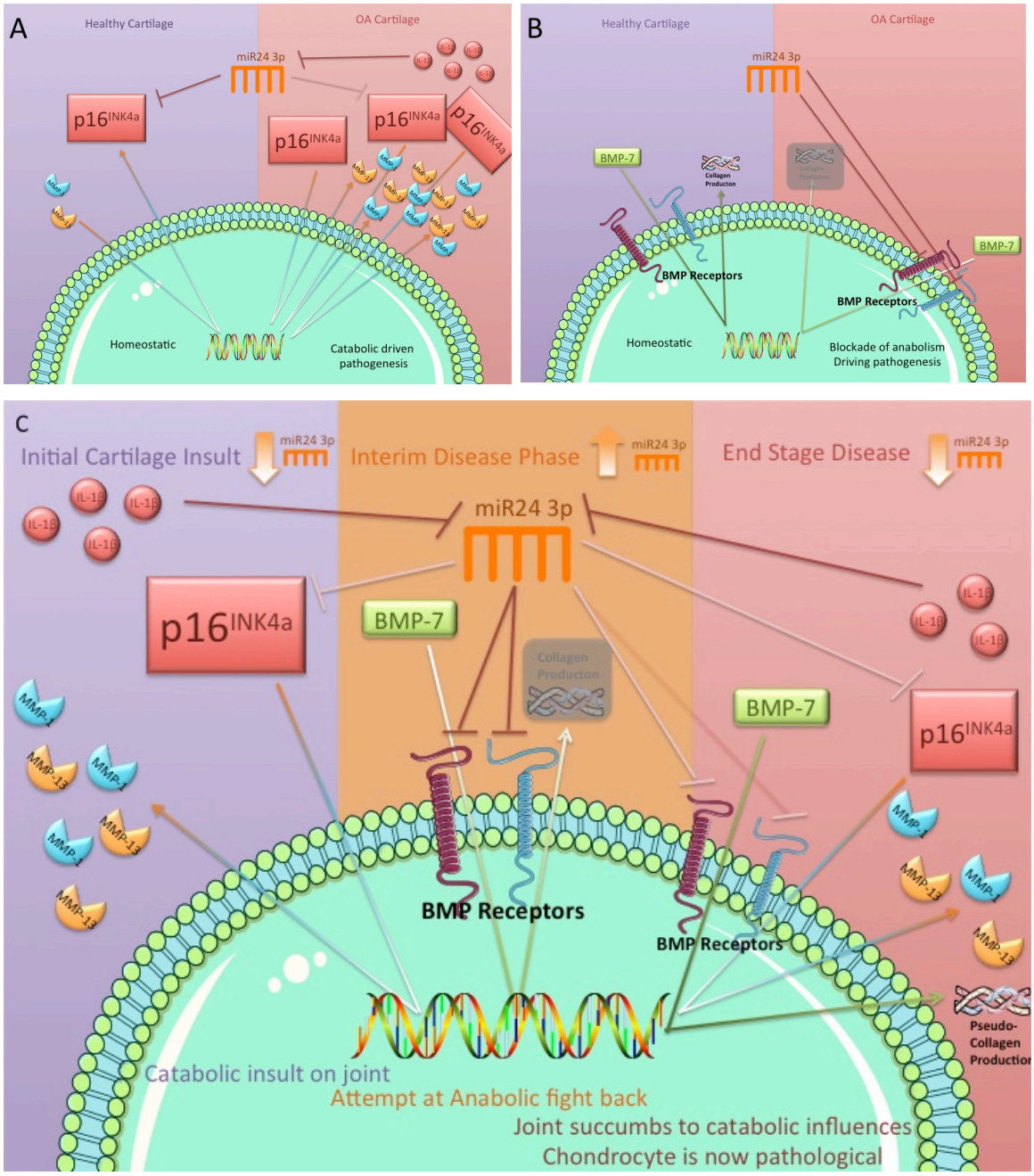


Figure 4.25 Predicted Functions of miR24 3p in Healthy and OA Cartilage.
A) MiR24 3p's regulation of $p16^{INK4a}$ in both healthy and OA chondrocytes. MiR24 3p is able to repress the expression of $p16^{INK4a}$ in healthy cells, however in OA expression of miR24 3p is dampened by IL-1β leading to increased $p16^{INK4a}$ and therefore increased MMP-1 and MMP-3. Schematic is based on work carried out by Philipot *et al* (195). B) MiR24 3p regulation of BMP receptors leading to a decrease in anabolic capacity in OA compared to healthy cartilage via diminished BMP-7 signalling. Schematic based on the data generated in this study and speculation of miR24 3p regulation of the BMP receptors from the miR24 expression data in healthy cartilage provided by Philipot *et al.* C) Potential differential mechanisms of miR24 3p control over the course of OA disease from onset through to end stage disease. MiR24 3p is potentially down-regulated by an initial insult to the joint (IL-1β mediated) leading to an initial increase in catabolic activity. During the interim phase of disease it is possible that the expression of the miRNA is increased in order to dampen the increasing anabolic activity (through the BMP receptors) that is trying to outcompete the catabolism occurring within the joint space. Finally, by the point of end stage disease the miRNA expression has again been decreased facilitating both anabolic and catabolic activity depending on the cells producing the miRNA. It would be assumed by this stage that some of the chondrocytes may be apoptotic and no longer able to signal effectively.

It is possible that the data generated from both this study and Philipots *et al* may in fact hold true with regards to OA, despite the fact they appear to be contradictory (Figure 4.25C). Firstly, it must be considered that the miRNAs result in the fine tuning of protein expression and furthermore it has been suggested that they may have various opposing functions depending on the microenvironment that they are exposed to (251). Therefore, it would be hypothesised that initial trauma to the joint would result in IL-1β production as suggested by Philipot *et al*, this could dampen miR24 expression allowing a pathological environment to arise that facilitates an increase in the SASP secretome via by $P16^{INK4a}$ (first panel, purple). It is then well established that the joint attempts to withstand this catabolic insult for as long as possible. If the catabolic mediators are to continue outcompeting this anabolic fight back, subtle alterations in the local environment may be beneficial. To this end increased expression of miR24 could lead to a decrease in BMP receptor expression and ultimately the dampening anabolic signalling events; thus giving the catabolic mediated erosion an advantage (second panel, orange). By the point of end stage disease, it is hard to determine if the articular chondrocytes are still fully functional, therefore, it must be considered that not all chondrocytes will behave in a homogenous fashion. The cells from the most eroded areas may be taking advantage of the miR24 dampening by up-regulating BMP signalling and trying to facilitate anabolic actions, as supported by the modest increase in receptor expression observed in the eroded cartilage shown in this chapter. However, other cells may be perpetuating the catabolic activity by increasing $P16^{INK4a}$ and its associated catabolism (third panel, red). This hypothesis is supported by data detailing altered expression of miR24 in the OA synovial fluid in early and late stage disease (210). The data suggested that miR24 has lower expression in early disease which would agree with the Philipots increased SASP secretome. However, in late stage disease the expression of miR24 is shown to be greater therefore this increase may dampen anabolic signalling via targeting of the BMP receptors supporting the data shown in this study (210).

To eradicate any bias in the culture system it would be interesting to investigate the role of miR24 in OA using chondrocyte specific, miR24 conditional knock out mice. This would allow confirmation of whether the miRNA is a confounding or

protective factor in OA progression. Using these mice, investigation into both the expression of $p16^{INK4a}$ and the BMP receptors BMPR1B and ALK2 could be examined over the course of the disease.

It is possible and very likely that miR24 targets all of these transcripts. Work has been carried out suggesting that a miRNA may have opposing roles on the expression of a transcript that are dependent on the cell cycle stage (251). It has been suggested that certain miRNA in proliferating cells repress the expression of TNFα, whereas cell cycle arrest facilitates translational activation. Two features have been suggested for this alternative regulation by the miRNA; firstly under cell cycle arrest recruitment of micronuclear proteins are required for interaction with the mRNA and secondly, argonaute 2 (AGO2) tethering which is required to facilitate translational activation (251). It could therefore be considered that miR24 may have a dual capacity, differentially regulating transcripts and therefore disease stage may not be the only factor that is controlling miR24 expression or its effector function. Cell cycle stage could also have a role in controlling the function of the miRNA, however this remains to be confirmed. The work undertaken here along with that of Philipot *et al* would suggest that miR24 3p is decreased at end stage disease when chondrocytes may no longer be functioning properly, or more likely not functioning in a homogeneous fashion, which could potentially result in some cells responding to the down regulation in an anabolic manner and others in a catabolic manner. It must be taken into consideration that neither study has looked at the expression of this miRNA at disease onset or over the course of the disease. This is likely due to our inability to pinpoint the onset of disease in humans, which to date remains one of the major challenges in the field. It therefore falls to animal models of disease to elucidate if this miRNA is differentially regulated throughout disease progression.

4.4.6 Circulating miRNA Signature in OA

The OA field is in need of biomarkers that are indicative of disease onset before symptomatic disease develops. Currently intervention only occurs at the point of diagnosis (which is when symptoms develop) and may not be as successful due to the extent of damage already exerted on the joint. Here, four miRNA's have

been shown to be downregulated in the plasma of OA patients with end stage disease compared to aged matched healthy controls, providing a valid non-diseased control to make a comparison against, something that has been lacking throughout all of the chondrocytic analysis conducted so far. This by no means should be taken as a biomarker signature at this current juncture; however, with further work and validation it may prove useful in the future. Some of the miRNA's used in this study had previously been shown to be associated with OA, such as the Let7e, which has been highlighted as a negative indicator in the need for arthoplasty, (209). Here it was found that this miRNA was significantly down-regulated in patients under-going knee arthoplasty compared to healthy control, validating the findings of Beyer *et al*. Furthermore it has been suggested that miR342 is actually increased within the cartilage of OA patients compared to healthy controls (186), where in this study a converse finding has been observed within the plasma. It is worth noting that the isoform of the miRNA that was investigated by Jones *et al* is not disclosed making it very difficult to draw a real comparison. It must also be considered that the expression in the circulation may not always match that of the cartilage due to tissue specific microenviromental influences on miRNA expression. The data presented above also did not corroborate the findings regarding miR342 3p expression from the Beyer *et al* paper, as they reported no changes in the expression in OA (209) in the plasma. The data generated here, with regards to miR24 3p expression, was recapitulated in the cartilage by a study carried out by Philipot *et al*. Synovial fluid analysis of miR24 3p in early and late stage disease did not show the expected decrease, but instead displayed an increase of the miRNA in the OA synovial fluid as the disease progressed (210). miR140 was the last miRNA in the potential signature to be uncovered. It was primarily investigated since it has been implicated in cartilage homeostasis and OA (191,232). It was also highlighted as a miRNA of interest with regards to OA in the Beyer cohort. Here it has been shown to be down-regulated in OA compared to age matched control plasma. This would be expected, as if this miRNA does indeed play a role in the maintenance of the cartilage, patients presenting with end stage OA are most likely not going to display characteristic well controlled cartilage homeostasis.

There are several hurdles to be overcome before these miRNAs can be considered a 'signature'. Firstly, their expression in other musculoskeletal joint diseases including RA, AS and PsA would need to be interrogated, establishing the disease specificity these findings. Also, contributing factors to OA such as obesity, age and acute joint damage would have to be investigated to assess their impact on the expression of these miRNA in isolation. Finally, it must be understood that all data presented here represents end stage disease, it would be hoped that this signature may be observed at an early stage in disease onset, thus allowing it to be used to identify those with disease before irreversible joint destruction occurs. All together these data provide insight into miRNA deregulation in the plasma of OA patients that one day may help to inform a diagnostic circulating signature in OA, however, there is still a vast amount of work to be undertaken before this would be clinically relevant.

4.4.7 Limitation of the Study

The study is limited in several areas. Firstly, the study is considerably underpowered for determining significant trends in molecular components of the BMP-7 signalling pathway. Due to cost and practical constraints a larger cohort was regrettably not obtainable. Furthermore, lack of a control tissue (non-diseased cartilage) was also a major hindrance to the study, as without this resource only internal control comparisons could be made. Several attempts were made at obtaining a source of non-OA cartilage but none were ever brought to fruition.

Relative quantification was possibly not the optimal qPCR technique to screen transcript expression. Instead absolute quantification may have been a more useful and informative tool, as this would have given exact values for expression of the transcripts straight from their physiological environment. Cartilage adhesion to the IHC slides was also an inhibitory factor within the study, as linked patients analysis was impossible due the random nature by which the sections adhered to the slides.

Finally, due to time constraints it was not possible to validate the mRNA transcripts as direct targets of miR24 3p through luciferase assays, which would have given greater significance to this finding in this study.

4.4.8 Conclusion

From this chapter it can be confirmed that there is still no conclusive evidence that BMP-7 is produced at the transcript level by primary human chondrocytes. What was apparent, however, was the localisation of the BMP-7 protein in the cartilage, especially in areas of typical OA cell clustering (240) . It has been suggested here, through *in silico* analysis, that TTP degradation of the transcript may play a role in the evasiveness of the transcript from most of the molecular detection methods employed here. It is possible that BMP-7 protein has remained bound to the ECM by the 'pro' domain of the protein, thus the protein is detectable and not the transcript. Furthermore, subtle changes in BMP-7 signalling family members were observed within the OA cartilage when comparing eroded and non-eroded sections of tissue. As with most of the study, a lack of healthy control tissue was a major hindrance in the interpretation of the data. The study was also underpowered and therefore the statistical significance of these changes could not be elucidated. MiRNAs that were predicted to target members of this signalling family of interest were also expressed within the cartilage. Moreover, miR24 3p has now been shown to regulate BMP receptors and has also been shown to have a significant negative correlation to their expression in a cohort of OA patients. Finally, plasma analysis of disease-associated miRNAs has revealed a deregulation of 4 miRNAs in OA patient plasma, however the disease specificity remains to be clarified.

5 Elucidating the Impact of Exogenous BMP-7 on Chondrocytes.

5.1 Introduction

In the previous chapter, characterisation of the BMP-7 signalling family in end stage OA cartilage was undertaken and suggested that all of the necessary components are present in OA patients for subsequent response to BMP-7 therapy. Whilst it is essential to understand if individuals have the capacity to respond to BMP-7, it is also essential to understand this response at a molecular level, as this adds to the current understanding of OA and may also help in the development of future therapeutics.

Exogenous BMP-7 has been shown to be an effective therapeutic agent in several animal models of OA and cartilage damage (158,159,160,220), however, the precise mechanisms responsible for this BMP-7 mediated protection remain unclear. One mechanism that has been suggested is BMP-7 mediated up-regulation of collagen, thus helping to maintain cartilage integrity (158,159,220,233). BMP-7 has been reported to up-regulate the CD44 receptor and hyaluronic acid synthase, both of which are known to be implicated in the production of extracellular matrix (156). Moreover, if CD44 is knocked down then a higher concentration of BMP-7 is required to elucidate an anabolic ECM synthetic response from chondrocytes(156). Therefore, CD44 mediated ECM production may be one of the ways in which BMP-7 is able to maintain the ECM.

BMP-7's role in maintaining ECM and as a therapy for OA does not seem to be solely based in the anabolic properties of this protein. An anti-catabolic effect of BMP-7 has been proposed and may have a comprehensive role in preventing disease development through suppression of catabolic activity. Takahashi *et al* suggest that prophylactic BMP-7 can prevent the initial IL-1ß response that occurs in the first 24 hours after tissue insult with zymosan. They also suggested that continued treatment with BMP-7 results in an increase in the immumomodulatory cytokine IL-10, which is often regarded as having anti-inflammatory properties. Furthermore, their data suggests that the addition of

BMP-7 into the joint can up-regulate BMP-7 protein in articular chondrocytes and within the synovium. BMP-7 was also reported to prevent macrophage infiltration into the synovium, which occurred following zymosan injection in the control animals (158).

The observation that BMP-7 can work to supress IL-1ß (158) is of great interest, as it is one of the most studied pro-inflammatory cytokines in OA. The importance of its role has been highlighted in mouse models, where monoclonal antibodies that block both IL-1ß and it's receptor IL-1Rα are sufficient to prevent disease progression. Additionally, IL-1ß rapidly (i.e. within 4 hours) up-regulates several early response genes including the matrix degrading enzyme MMP-13 (260). It was observed that these early responses are maintained for up to 24 hours post stimulation (261) which may account for an IL-1ß driven catabolic attack on the joint leading to pathological changes and ultimately disease. Furthermore, an inductive role for IL-1ß has been shown (263), where IL-1ß will promote an up-regulation of its own transcript. This can be important in certain circumstances, such as wound healing(262), however, it must be regulated so as not to lead to chronic IL-1ß signalling that could be damaging and aid disease progression (264).

Elucidating the role of exogenous BMP-7 with regards to chondrocytes is one of the primary aims of this study. There are a variety of cell lines that can be used to mimic the actions of chondrocytes: CHON-001 a foetal derived long bone cell line, ATDC5 a teratocarcinoma mouse cell line and SW1353, a human derived chondrosarcoma cell line. One major advantage of using a cell line for preliminary work as opposed to primary cells is the homogeneity and proliferative capacity. The SW1353 chondrosarcoma cell line has been shown to be an ideal cell line for investigating catabolic regulation of chondrocytes. For example, their response to IL-1ß is the same to that of primary chondrotyes (265). Moreover, the expression profile of catabolic genes such as MMPs 1,3 and 13 is similar in both SW1353 and primary cells(265). However, it is important to note that some characteristic chondrocytic genes (i.e. extracellular matrix components including collagens type II, III and XI as well as aggrecan) are not expressed in this cell line and thus they cannot mimic the actions of the primary cell completely (265). As primary chondrocytes are a limited resource and

cannot be cultured long term due to dedifferentiation (Chapter 3), a cell line is an ideal starting point for the generation of preliminary data.

It is unlikely that the exogenous effect of BMP-7 will be restricted to chondrocytes alone, and it is possible that other cells in the OA joint will also respond to exogenous BMP-7. This could include fibroblast-like synoviocytes (FLS), endothelial cells or infiltrating leukocytes (i.e. monocytes/macrophages). Interestingly, it has previously been shown that BMP-7 can skew the THP-1 cell line (monocytic cells) to an M2-like (anti-inflammatory) macrophage (144). THP-1 cells express the BMP receptor BMPR2, which is subsequently up-regulated following stimulation with exogenous BMP-7(144). Furthermore, upon stimulation with BMP-7 these cells secrete the anti-inflammatory cytokine, IL-10(144). Despite all the work into investigating BMP-7 mediated macrophage polarisation, there is nothing in the literature that indicates if this cell line phenomenon manifests itself in primary human macrophages.

5.2 Aim

Whilst the roles for endogenous BMP-7 have already been intensively studied in animal models within the literature, there is still much that is not understood about the exogenous effects of this growth factor in humans. This study hypothesised that BMP-7 may not only regulate CD44, but may also have an auto-regulatory impact promoting the up-regulation of itself and its own signalling family. Additionally this study further hypothesised that BMP-7 may have an inhibitory effect on any IL-1ß modulation that may occur with BMP-7 itself or the related signalling family members. Finally due to the work carried out by Roche *et* al, it has been hypothesised that BMP-7 will polarise primary macrophages towards an M2 anti-inflammatory phenotype. Accordingly, this chapter aims to:

1. Delineate BMP-7's effect on both BMP-7 and the BMP-7 signalling family in the SW1353 cell line and primary chondrocytes.

2. Deduce if BMP-7 is protective against IL-1ß mediated catabolic destruction in the SW1353 cell line and primary human chondrocytes.

3. Define any role for BMP-7 in the polarisation of primary macrophages.

5.3 Results

5.3.1 Effects of Exogenous BMP-7 on the BMP-7 Signalling Cascade: SW1353 Cell Line

It has previously been demonstrated that the chondrocytic cell line SW1353 is a particularly useful tool in investigating catabolic processes (265). However, the usefulness of this cell line for the interrogation of the BMP-7 signalling family has yet to be established. It was therefore critical to characterise the endogenous expression of the BMP-7 signalling family members prior to performing any additional studies. Accordingly, the expression level of BMP-7, ALK2, BMPR2 and furin -along with accessory components, follistatin and CD44- were established in the SW1353 cell line. Effector components (type II collagen along with two catabolic mediators, (MMP-13 and IL-1ß) were also investigated. QPCR revealed that all of the genes were detected (Figure 5.1) within the confines of the assay (i.e. have values less than that of the NTC), with the exception of type II collagen as it is not expressed in the cell line (265) and IL-1ß, which was detected at similar levels to the NTC- a finding that had already been reported with the cell line (265).

Having established that the SW1353 cell line expresses members of the BMP-7 signalling family it was also important to validate the catabolic response of the cell line. This was achieved by stimulating the cells with IL-1ß and verifying the induction of MMP-13. Stimulation of SW1353 with 10, 50 and 100ng/ml IL-1ß resulted in an increase in MMP-13 transcript expression (Figure 5.2), which was most consistently up-regulated at both 50 and 100ng/ml. Thus, in all subsequent studies, stimulation with IL-1ß at 100ng/ml was used. Furthermore, due to the variation seen in IL-1ß mediated MMP-13 up-regulation (Figure 5.2), it was decided that MMP-13 was a necessary positive control in each experiment to allow validation of each stimulation with this cytokine.

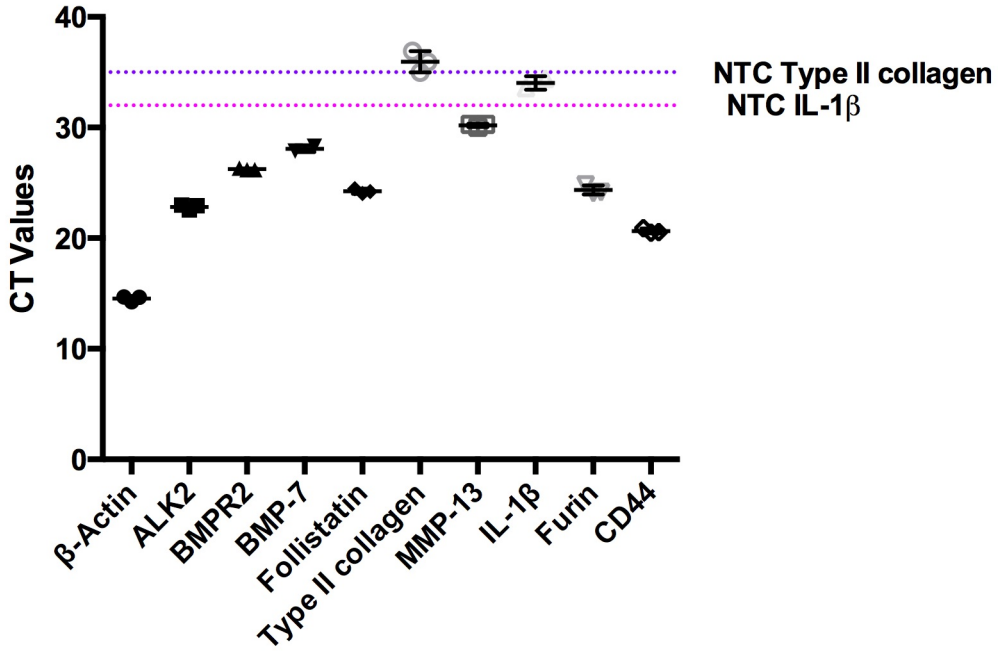


Figure 5.1 Endogenous Expression of BMP-7 Signalling Family Members and Other Associated Transcripts in the SW1353 Chondrosarcoma Cell Line.
Gene analysis for β-actin, ALK2, BMPR2, BMP-7, follistatin, type II collagen, MMP-13, IL-1β, furin and CD44 in the SW1353 cell line. Cells from 1 passage were plated in triplicate, each of these technical replicates are shown on the graph as CT values. NTC values shown for both type II collagen and IL-1β as the observed CT values for these genes were very close to the NTC.

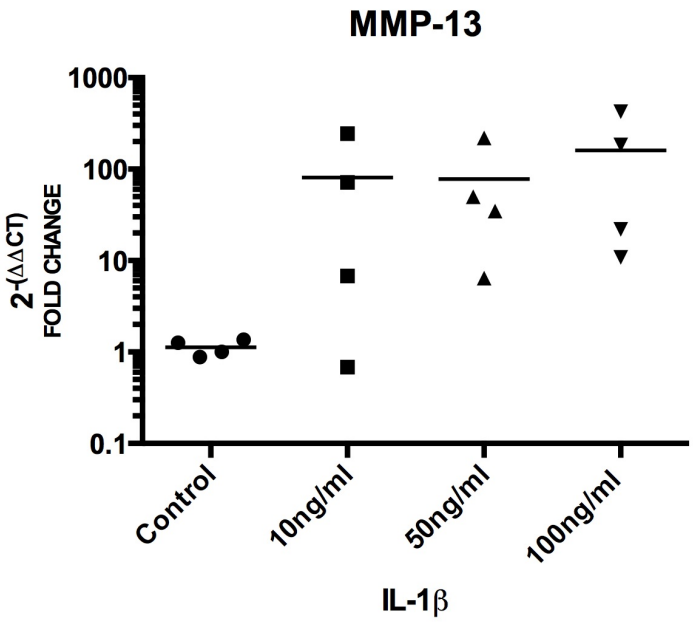


Figure 5.2 IL-1 β Induction of MMP-13 is Observed in SW1353 Cells.
MMP-13 transcript analysis for SW1353 cells stimulated with 10ng/ml, 50ng/ml or 100ng/ml IL-1β for 24 hours. Data is shown as fold change ($2^{-\Delta\Delta CT}$) using the unstimulated cells as a baseline. Cells from 4 different passages were stimulated in 4 separate experiments, technical replicates were pooled, for each passage, in order to generate 1 point, N=4. Statistical analysis was conducted using Kruskal Wallis, Dunn's multiple comparison post test.

To investigate if the modulation of the BMP-7 signalling family by a variety of cytokines that have been related to OA, was an early or late response mechanism, cells were stimulated for 3 or 48 hours with either: IL-1ß, BMP-7, TNFα or IL-6. Validation of the cells lines response to stimulation was assessed via IL-1ß up-regulation of MMP-13 as discussed previously. At the 3 hour time point, the only early response gene significantly up-regulated was the IL-1ß transcript in response to IL-1ß stimulation (Figure 5.3A). Interestingly, BMP-7 stimulation caused an up-regulation of IL-1ß transcript in the cell line (between 2 and 20 fold increase), however, due to the variance in this response it did not reach significance. BMP-7 had no effect on any of the other genes at this time point. TNF and IL-6 also had no significant impact on any of the transcripts investigated at the three hour time point. At the 48 hour, late response, time point IL-1ß displayed a significant increase in its own transcript expression, with the transcript increasing between 10 and 50 fold (Figure 5.3B). MMP-13 was also up-regulated between 8 and 10 fold by IL-1ß stimulation as expected. BMP-7, TNF and IL-6 had no significant effect on any of the transcripts investigated at the late response time point.

As BMP-7 was unable to modulate it's own anabolic signalling pathway in the SW1353 cell line, it was decided that its ability to prevent IL-1ß's up regulation of catabolic (MMP-13) and inflammatory (IL-1ß) mediators should be investigated. Furthermore, as IL-1ß had previously displayed the potential to increase BMP-7 expression, BMP-7 transcript analysis was also carried out with regards to co-stimulation. Cells were stimulated with IL-1ß, BMP-7 or IL-1ß plus BMP-7, and RNA was harvested at 8, 24 or 48 hours post stimulation to evaluate transcripts expression (Figure 5.4). As expected, IL-1ß stimulation led to an up-regulation of both MMP-13 and its own transcript at all three time points. Furthermore, at the 8 hour time point IL-1ß stimulation induced a 2-fold increase on BMP-7 expression (Figure 5.4B), which was consistent with the previous experiment (Figure 5.3B). BMP-7 alone had no impact on the expression of MMP-13, however at 24 hours post stimulation it down-regulated IL-1ß expression by 25%. BMP-7 also appeared to cause a 50% inhibition of its own transcript expression at 24 and 48 hours, which was not observed in the previous stimulations. Co-stimulation with both IL-1ß and BMP-7 had no impact on BMP-7 expression. However, after 48 hours BMP-7 co-stimulation inhibited IL-1ß

mediated MMP-13 up-regulation by 50% and was able to completely inhibit the expression of IL-1ß. In order to elucidate if the inhibition of IL-1ß was specific to BMP-7, the cells were co-stimulated with another TGFß superfamily member, $TGFß_3$ (Figure 5.4 D). The SW1353 cells responded as expected to various concentrations of IL-1ß. However, co-stimulation with a range of concentrations of both IL-1ß and TGFß3 had little effect on the MMP-13 expression. This suggests that the dampening of IL-1ß induced MMP-13 up regulation is not a global TGFß superfamily mechanism.

The data produced by the cell line was not consistent, with BMP-7 having different effects. It either had no effect or caused a 50% inhibition of it's own transcript and that of IL-1ß between different experiments (Figure 5.3/Figure 5.4). Significant variation between replicates in each experiment could also be observed. Further work was undertaken in order to reduce this variation; cells were synchronised in relation to cell cycle stage, using serum starvation, however this had no impact on the variation in the results (data not shown). Cells from a lower passage were also obtained and again had no bearing on the variation of the results (data not shown). In order to overcome this inconsistency with the cell line, primary cells were used in subsequent experiments for in vitro analysis.

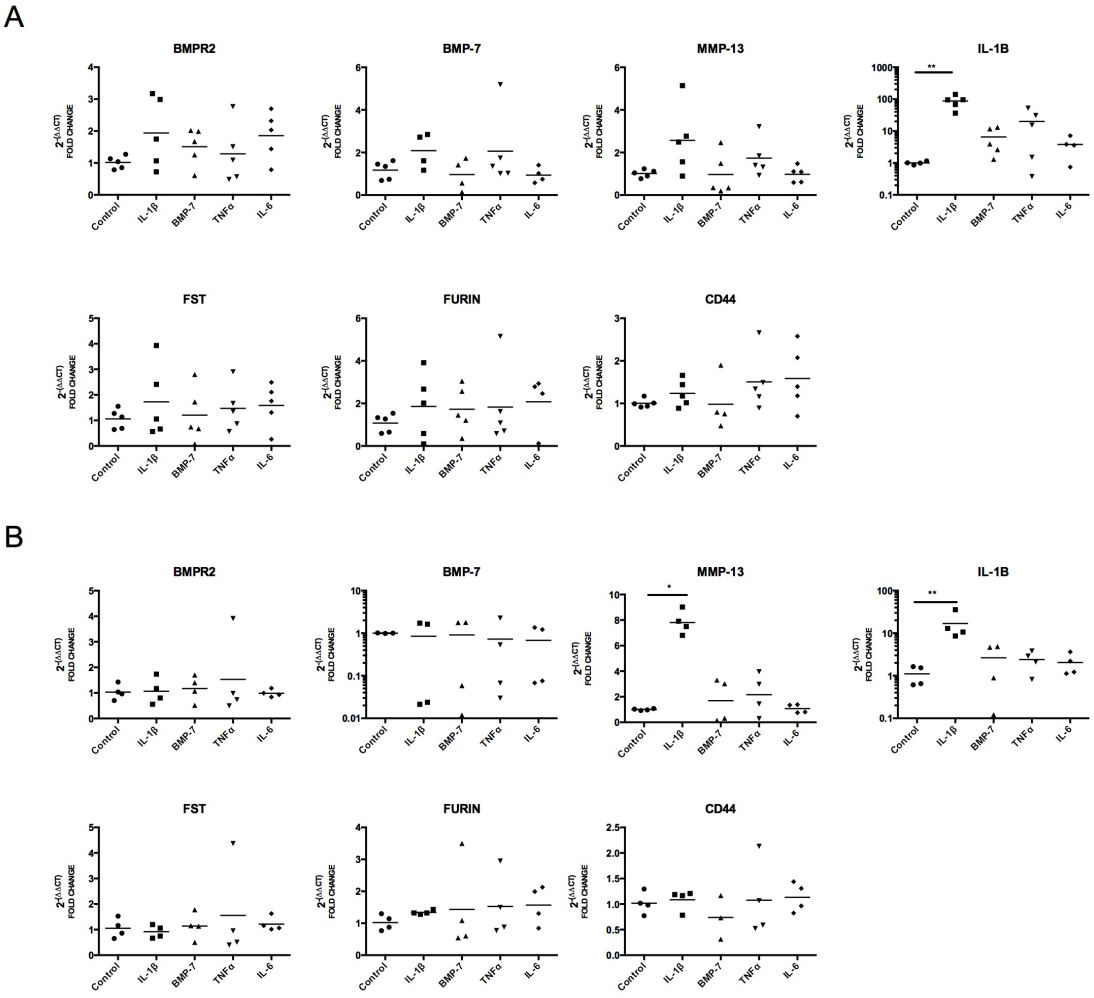


Figure 5.3 SW1353 Early and Late Response to Cytokine Stimulation.
SW1353 cells stimulated with either IL-1β, BMP-7, TNFα or IL-6, all at 100ng/ml, for either A) 3 hours time point with 5 different passages of the SW1353 cells were run in technical triplicates then pooled, N=5 or B) 48 hour time point) 4 different passages of the SW1353 cells were run in technical triplicates then pooled N=4. Cells were stimulated in two batches; the average of the unstimulated cells from each batch was used in order to generate a baseline value for fold change analysis ($2^{-\Delta\Delta CT}$). The following genes were analysed using SYBR green qPCR: BMPR2, BMP-7, MMP-13, IL-1β, follistatin (FST), furin, CD44. Statisitcal analysis was conducted using Kruskal Wallace test with Dunn's multiple comparison post test. ** P<0.01, * P<0.05

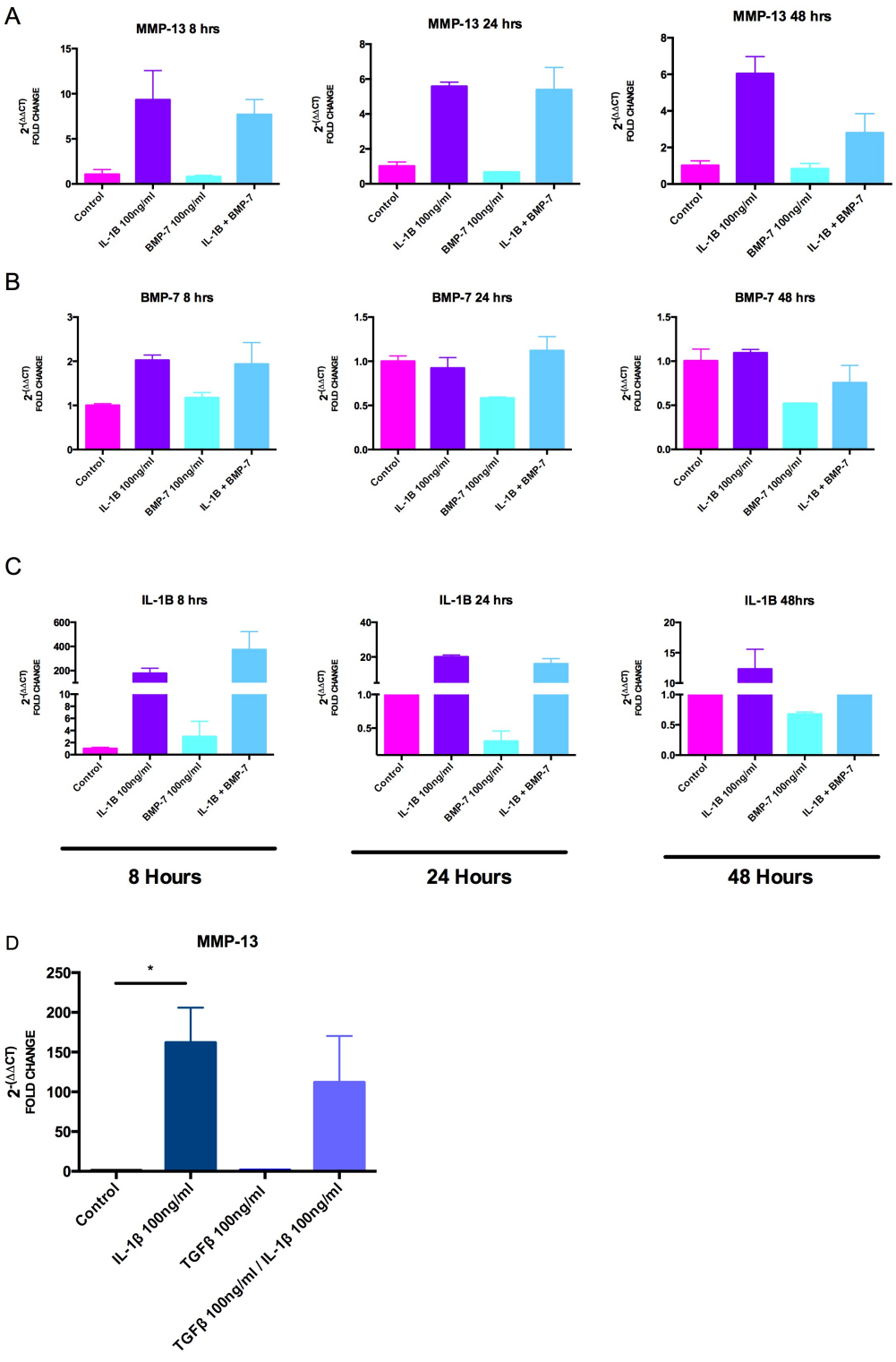


Figure 5.4 SW1353 Responses to Co-Stimulation with IL-1β and BMP-7.
SW1353 cells stimulated with either IL-1β (100ng/ml), BMP-7 (100ng/ml) or both together for either, 8 hours, 24 hours or 48 hours, N=2 and the following genes were quantified using qPCR (SYBR green): A) MMP-13, B) BMP-7 and C) IL-1β. The data is shown as fold change over the unstimulated cells ($2^{-\Delta\Delta CT}$). D) SW1353 cells were also stimulated with 100ng/ml, IL-1β or $TGF\beta_3$ or both. MMP-13 expression was quantified using qPCR (SYBR green) and data shown as fold change ($2^{-\Delta\Delta CT}$) using the unstimulated cells as baseline, N=3. Kruskal-Wallace, Dunn's multiple comparison post test was used to asses statistical significance, * $P<0.05$

5.3.2 Effect of Exogenous BMP-7 in Primary Articular Chondrocytes

The cell line provided limited, variable and non-reproducible data as well as expressing only a limited number of genes. It was therefore decided that all further investigations should be carried out in primary human chondrocytes. Non-eroded cartilage (Outerbridge score 0-1) was taken from OA patients (who had given informed consent) at the time of total knee replacement surgery at the Glasgow Royal Infirmary. The cartilage was digested (section 2.2.2), releasing the chondrocytes from the matrix, which were then washed and plated for 48 hours, allowing acclimatisation before stimulation, described in chapter 3.

Initial experiments examined the effect of exogenous BMP-7 on the BMP-7 signalling family members. Previous studies have shown that the BMP-7 protein was up-regulated by addition of exogenous BMP-7 in an animal model of OA (158). These studies investigated protein expression via IHC and thus did not determine whether or not this was due to transcriptional enhancement or just exogenous BMP-7 up-take by chondrocytes at the time of injection. In order to address this, analysis of the BMP-7 transcript was carried out following stimulation with BMP-7. Here it has been hypothesised that BMP-7 will not only induce its own expression, but also that of the transcripts of its own signalling family. To this end, cells were stimulated with BMP-7 at 100ng/ml for either 8 or 24 hours, before being placed in cytokine free media. Analysis was carried out using qPCR in order to allow the necessary verification of this auto-regulatory mechanism at the level of the transcript (Figure 5.5A). To take into account the very low levels of endogenous BMP-7 transcript, which is close to the non-template control samples, all data generated for this gene is displayed as $2^{-\Delta CT.}$ In explanation, fold change ($2^{-\Delta\Delta CT}$) analysis requires the unstimulated control to be within the normal range for the assay, as this would be used to calculate a baseline value. If this lies to close to the NTC then the data cannot be transformed without introducing error. The $2^{-\Delta CT}$ shows that 8 hours post stimulation there are no changes in the expression of the BMP-7 transcript. However, at the 24 hour time point almost all of the donors displayed an up-regulation in BMP-7 transcript (Figure 5.5B); although this increase did not reach significance.

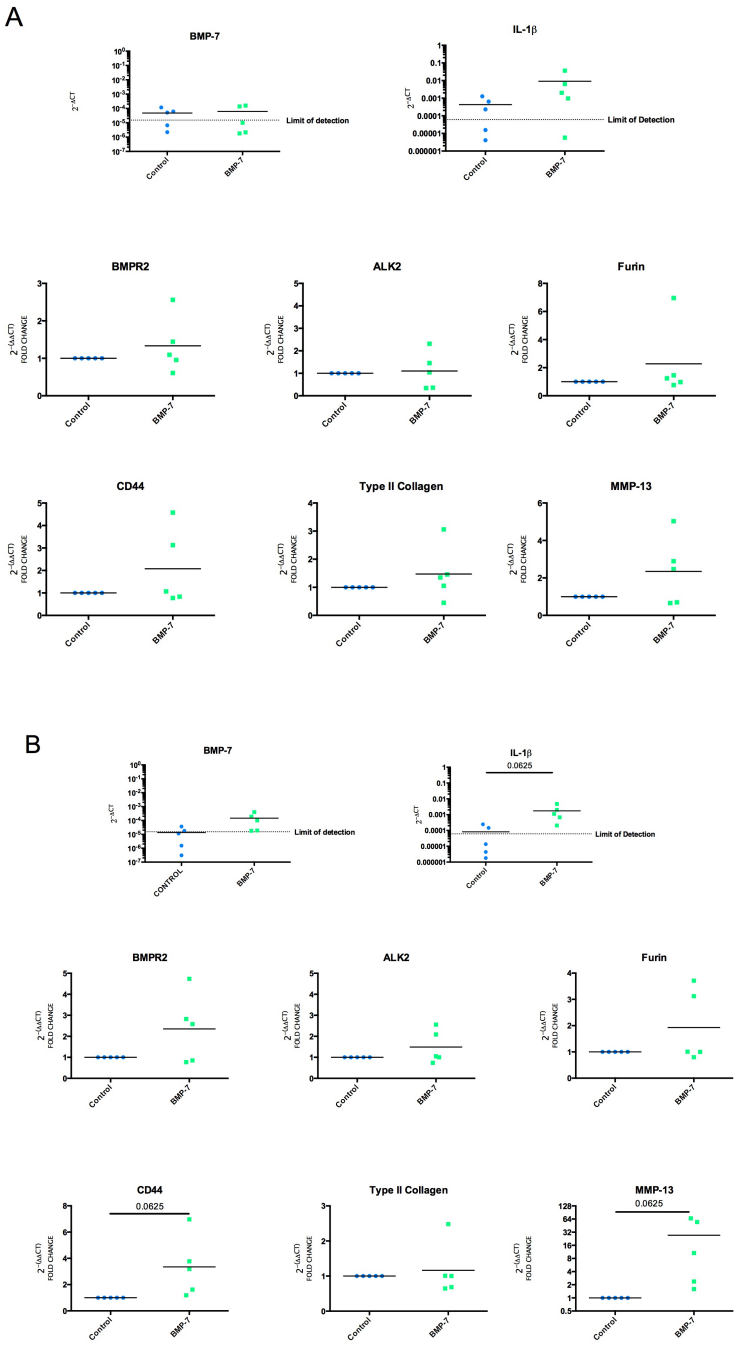


Figure 5.5 Effect of BMP-7 on the Expression of BMP Signalling Family in Primary OA Chondrocytes.

Primary chondrocytes plated at 1.5X10^5/well were stimulated with 100ng/ml, BMP-7 for 8 hours before media was replenished with a cytokine free replacement. Cells were lysed at either A) 8 or B) 24 hours post stimulation in Qiazol™. Each dot on the graph represents an individual chondrocyte donor from which cartilage was taken at the time of TKR surgery from areas of Outerbridge score ≤I. Both BMP-7 and IL-1β transcript analysis is shown as $2^{-\Delta CT}$, with limit of detection defined by the average NTC values of all the experiments conducted for that gene of interest. The limit of detection for BMP-7 is $2^{-\Delta CT}$ = 0000153 and for IL-1β limit of detection is $2^{-\Delta CT}$ = 00006104. Transcript analysis for ALK2, BMPR2, furin, CD44, Type II collagen and MMP-13 are all shown as fold change ($2^{-\Delta\Delta CT}$) using the unstimulated cells at each time point as the baseline value. Statistical analysis was carried out using Wilcoxon T-Test.

It could therefore be concluded that the chondrocyte response to BMP-7 is subtle and a larger cohort would be required in order to gain significance. IL-1β was the next transcript to be analysed. In most of the donors an up-regulation of the IL-1β transcript was observed 8 hours post BMP-7 stimulation (Figure 5.5A). After 24 hours, IL-1β was up-regulated in all of the donors, however, this increase did not reach significance (Figure 5.5B).

Having noticed that BMP-7 has the ability to up-regulate its own transcript in a subtle manner, it was decided to investigate how other members of the BMP-7 signalling family would respond to 8 or 24 hour BMP-7 stimulations (Figure 5.5A/B). These genes included BMP receptors, BMPR2 and ALK2, along with the BMP-7 cleavage enzyme furin. Accessory components related to extracellular matrix synthesis, CD44 and type II collagen were also investigated along with the cartilage degradation enzyme, MMP-13. BMP-7 stimulation had little effect on all of the transcripts 8 hours post stimulation. Some of the transcripts, BMPR2, CD44 and furin, were modulated in a patient dependent manner 24 hours post stimulation. The BMP-7 mediated increase observed in MMP-13 expression was surprising as previous publications had reported that BMP-7 can inhibit MMP-13 expression (266). BMP-7 increased MMP-13 expression in all of the donors, ranging from a 1.5 - 66 fold increase, however, due to the variance in response this did not reach significance.

This suggests that exogenous BMP-7's effect on its own signalling family and other catabolic mediators varies between donors. This is supported by the data generated in the previous chapter (Figure 4.17), which showed that the expression of the BMP-7 signalling components varied between patients.

In the previous chapter it was shown that miR24 3p, miR342 3p and miR342 5p were present in the articular cartilage. Further to this it was also shown that miR24 3p mimic was able to supress the expression of BMP receptors. Thus, it was decided to investigate whether or not exogenous BMP-7 affects the expression of these miRNA in primary chondrocytes. The results concluded that there were no effects on the expression of the miRNA following stimulation with 100ng/ml BMP-7 for either 8 or 24 hours (Figure 5.6).

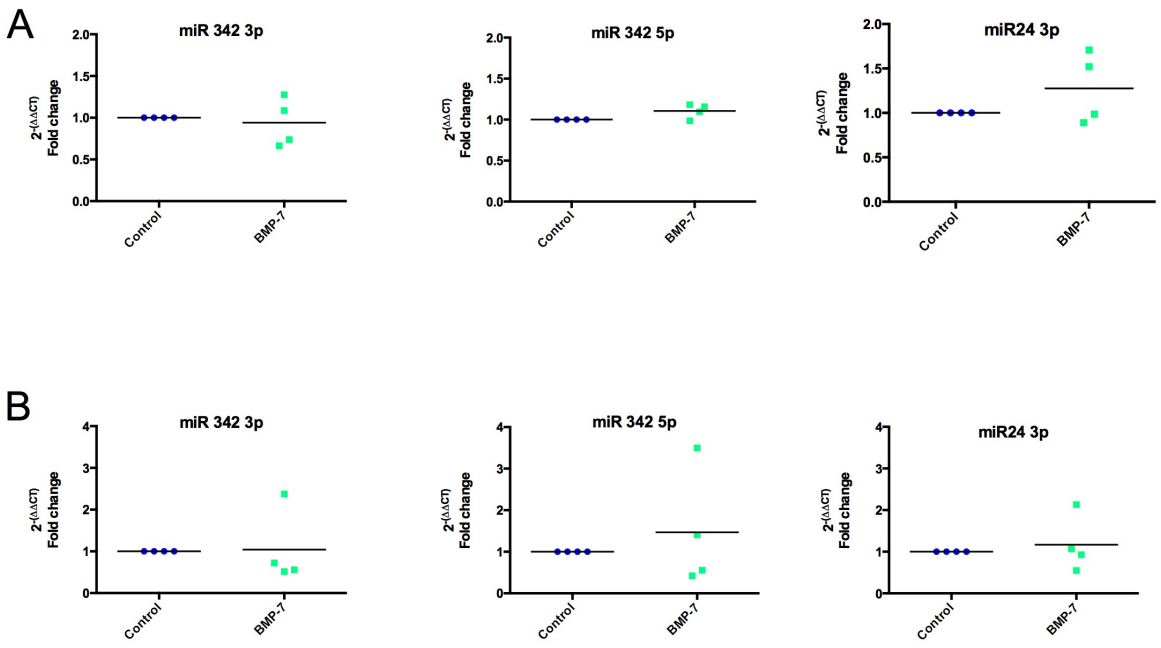


Figure 5.6 Effect of BMP-7 on the Expression of BMP Associated miRNA in Primary OA Chondrocytes.
Primary chondrocytes plated at $1.5X10^5$/well were stimulated with 100ng/ml, BMP-7 for 8 hours before media was replaced with a cytokine free alternative. Cells were lysed at either A) 8 or B) 24 hours post stimulation in Qiazol™. Each dot on the graph represents an individual chondrocyte donor from which cartilage was taken at the time of TKR surgery from areas of Outerbridge score ≤I. Transcript analysis for miR342 3p, miR342 5p and miR24 3p, shown as fold change ($2^{-\Delta\Delta CT}$) using the unstimulated cells at each time point as the baseline value. Statistical analysis was carried out using Wilcoxon T-Test.

5.3.3 BMP-7s Impact on IL-1β Mediated Effects in Primary Articular Chondrocytes

As BMP-7 had shown wide variation in it's direct regulatory capacity for some of the genes of interest in this study, it was decided to further investigate BMP-7's indirect regulatory capacity. Accordingly, investigation into BMP-7's ability to negate any modulation to genes of interest that may be caused by IL-1ß was conducted. It was hypothesised that IL-1ß would have a negative effect on the anabolic transcripts of interest as it is considered a pro-inflammatory cytokine that is catabolic in the context of OA. It was further hypothesised that BMP-7 would be able to inhibit any IL-1ß induced suppression of the BMP-7 related anabolic genes. This hypothesis was originally tested on the SW1353 cell line, however, due to a number of issues that arose with the cell line satisfactory conclusions could not be drawn (Figure 5.4).

Primary chondrocytes were stimulated for 8, 24 and 48 hours and again endogenous levels of BMP-7 and IL-1ß mRNA were close to that of the NTC and thus $2^{-\Delta CT}$ was used to evaluate their expression (Figure 5.7A). Unexpectedly, IL-1ß stimulation resulted in an increase in BMP-7 transcript expression in a donor specific manner 8 hours post stimulation (Figure 5.7A) and not the hypothesised decrease. This appeared to be an early response, as there were no effects observed at either 24 or 48 hours post stimulation (Figure 5.7 C/D). Upon analysis of the IL-1ß transcript (following IL-1ß stimulation) the expected increase was observed at all time points, although this increase was noted to be variable depending on the donor that the chondrocytes were isolated from (all donors did display an increase in transcript in at least one of the three time points, analysis not shown). Again, contrary to the hypothesis, BMP-7 had no impact on the IL-1ß mediated increase of its own transcript. IL-1ßs inability to negatively regulate the BMP-7 transcript was surprising due to it's association with OA disease progression. Therefore, it was hypothesised that IL-1ß may play a role in the down-regulation of BMP-7 signalling components, thus causing disruption to BMP-7 mediated anabolic signalling events. The data again show that IL-1ß does not have the expected negative impact on the transcripts but rather, increases the expression of almost all of the transcripts of interest. Specifically, CD44 is significantly increased following stimulation with IL-1ß for

either 8 or 48 hours. The same trend is also observed at the 24 hour time point, however this did not reach significance. MMP-13 was also increased following stimulation with IL-1ß at all time points as had already been shown in the literature (260). Other genes of interest such as ALK2 and BMPR2 were notably increased in some of the donors at both 24 and 48 hours post stimulation. Furin also displayed increased expression in some of the donors 48 hours post stimulation. The only gene that was down regulated via IL-1ß was type II collagen and again this down regulation was only seen in some of the patients 48 hours post stimulation. Co-stimulation with BMP-7 had no effect on the transcripts investigated (Figure 5.8). Taken together this data suggests that in this *in vitro* system IL-1ß is not a negative regulator of the BMP-7 signalling family. However, there appears to be an IL-1ß induced decrease in type II collagen expression in some of the donors.

As previously mentioned, IL-1ß is thought to be a major driver of catabolic and pro-inflammatory events in OA disease pathogenesis. Therefore, as the preceding data (Figure 5.8) demonstrated that IL-1ß stimulation has no deleterious effects on the expression of BMP-7 signalling family members, it was hypothesised that perhaps IL-1ß does not directly affect the expression of these transcripts. Rather, it may modulate miRNAs that have been predicted to target members of the BMP signalling family. In chapter 4 it was shown that miR24 3p inhibits the expression of BMP receptor ALK2. Thus, it is of interest to investigate IL-1ß's ability to modulate any of the miRNA that have been identified as of interest in this study, specifically, miR24 3p, miR342 3p and miR342 5p. It was found that IL-1ß stimulation has no impact on the expression of any of the miRNA investigated at any of the time points analysed (Figure 5.9). Furthermore, co-stimulation with both IL-1ß and BMP-7 also had no impact on the expression of miR24 3p, miR342 3p or miR342 5p (Figure 5.9). Thus neither BMP-7 (Figure 5.6) nor IL-1ß (Figure 5.9) regulate these miRNA in this *in vitro* system.

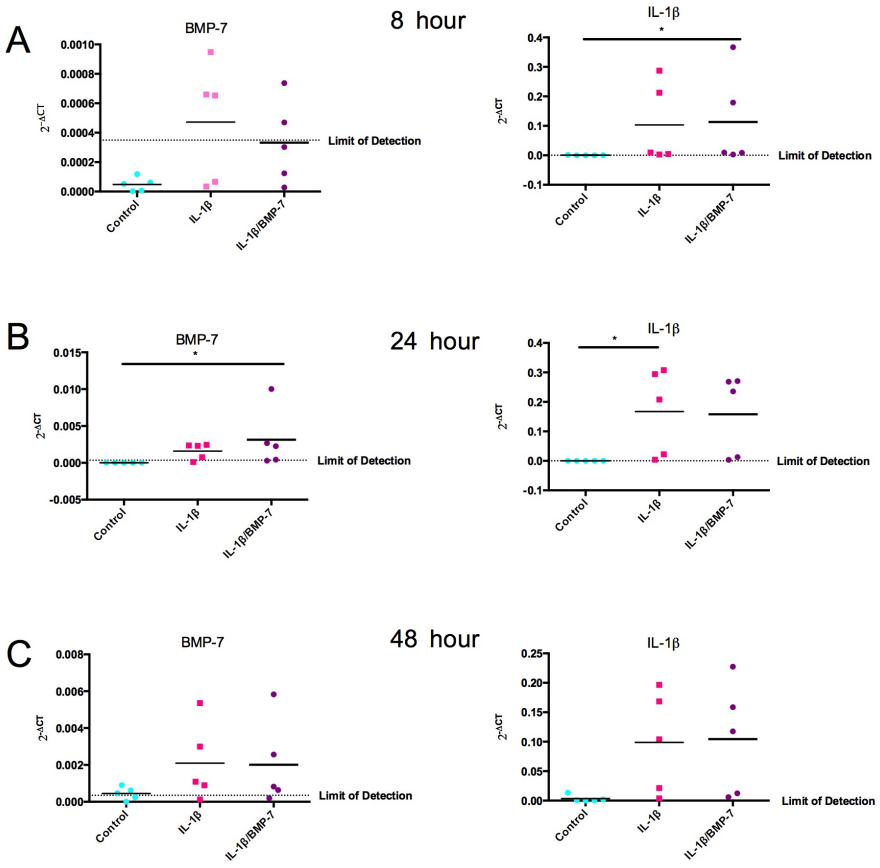


Figure 5.7 Effect of BMP-7 on IL-1β Mediated Alterations of the BMP-7 and IL-1β Transcripts in Primary OA Chondrocytes.
Primary chondrocytes plated at $1.5X10^5$ were stimulated with either 100ng/ml IL-1β or 100ng/ml IL-1β and BMP-7 for 8 hours before being washed and cultured in cytokine free media. Cells were lysed at either A) 8, B) 24 or C) 48 hours post stimulation. Each dot represents a different chondrocyte donor from which cartilage was taken at the time of TKR surgery from areas of Outerbridge score ≤I, N=5. BMP-7 and IL-1β transcript analysis shown as $2^{-\Delta CT,}$ with limit of detection defined by the average NTC values of all the experiment for that gene then applying the formula $2^{-\Delta CT}$ to the averaged value. Limit of detection for BMP-7 is $2^{-\Delta CT}$ = 0000153 and the limit of detection of IL-1β is $2^{-\Delta CT}$ = 00006104. Statistical analysis; Friedman ANOVA, Dunns multiple comparisons post test, *= P<0.05.

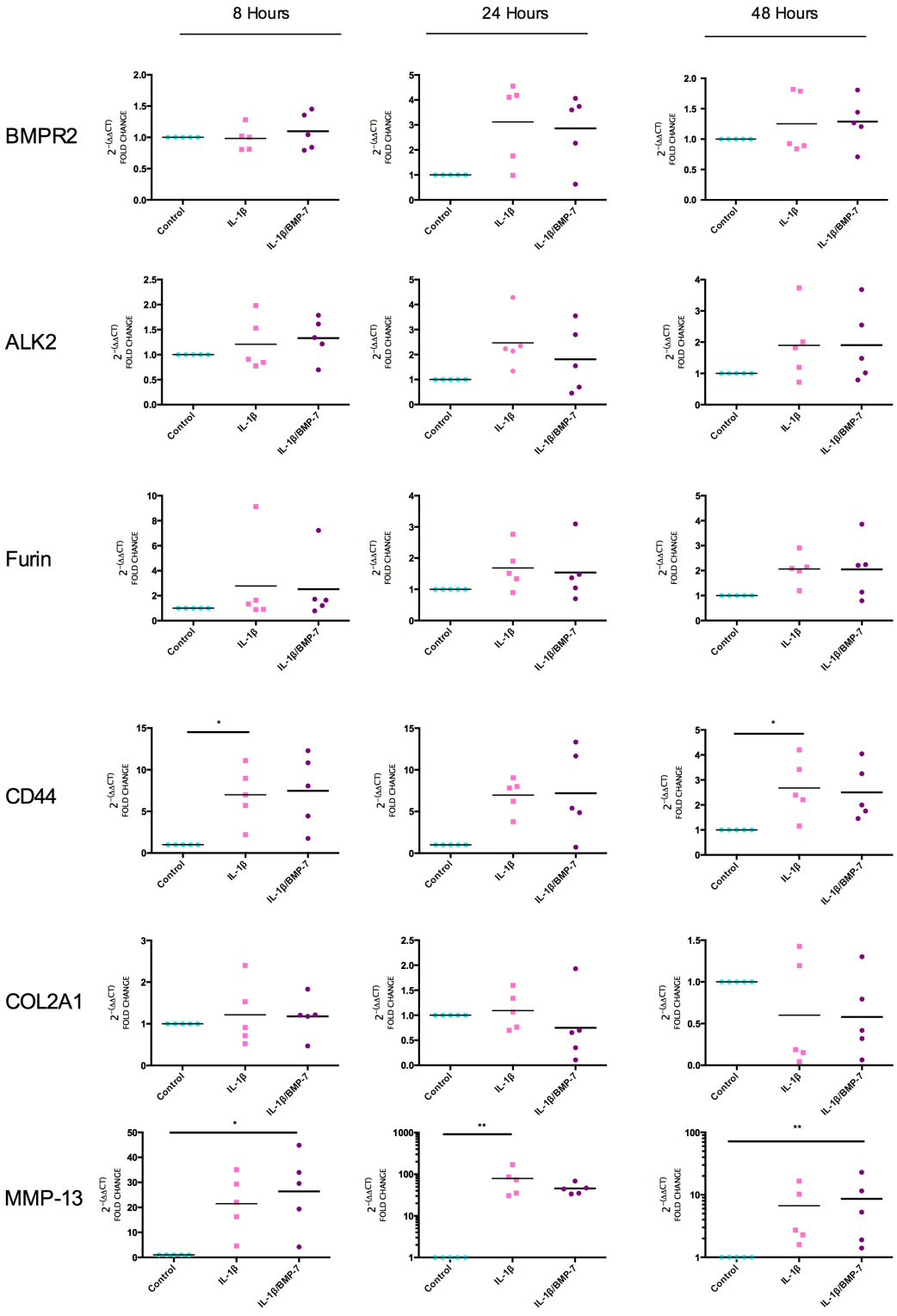


Figure 5.8 Effect of BMP-7 on IL-1β Mediated Alterations of the BMP-7 Signalling Cascade and Accessory Components on Primary OA Chondrocytes.
Primary chondrocytes were stimulated with either IL-1β or IL-1β/BMP-7 all at 100ng/ml for 8 hours before media was replaced with cytokine free media. Cells were then lysed at either 8 hours, 24 hours or 48 hours. Data was analysed as fold change ($2^{-\Delta\Delta CT}$) over the unstimulated cells (baseline = 1). Each dot represents one chondrocyte donor, from which cartilage was taken at the time of TKR surgery from areas of Outerbridge score ≤I, N=5. Genes analysed following stimulation were BMPR2, ALK2, furin, CD44, COL2A1 (type II collagen) and MMP-13. Statistical analysis; Friedman ANOVA, Dunns multiple comparisons post test,* = P<0.05. ** = P<0.01.

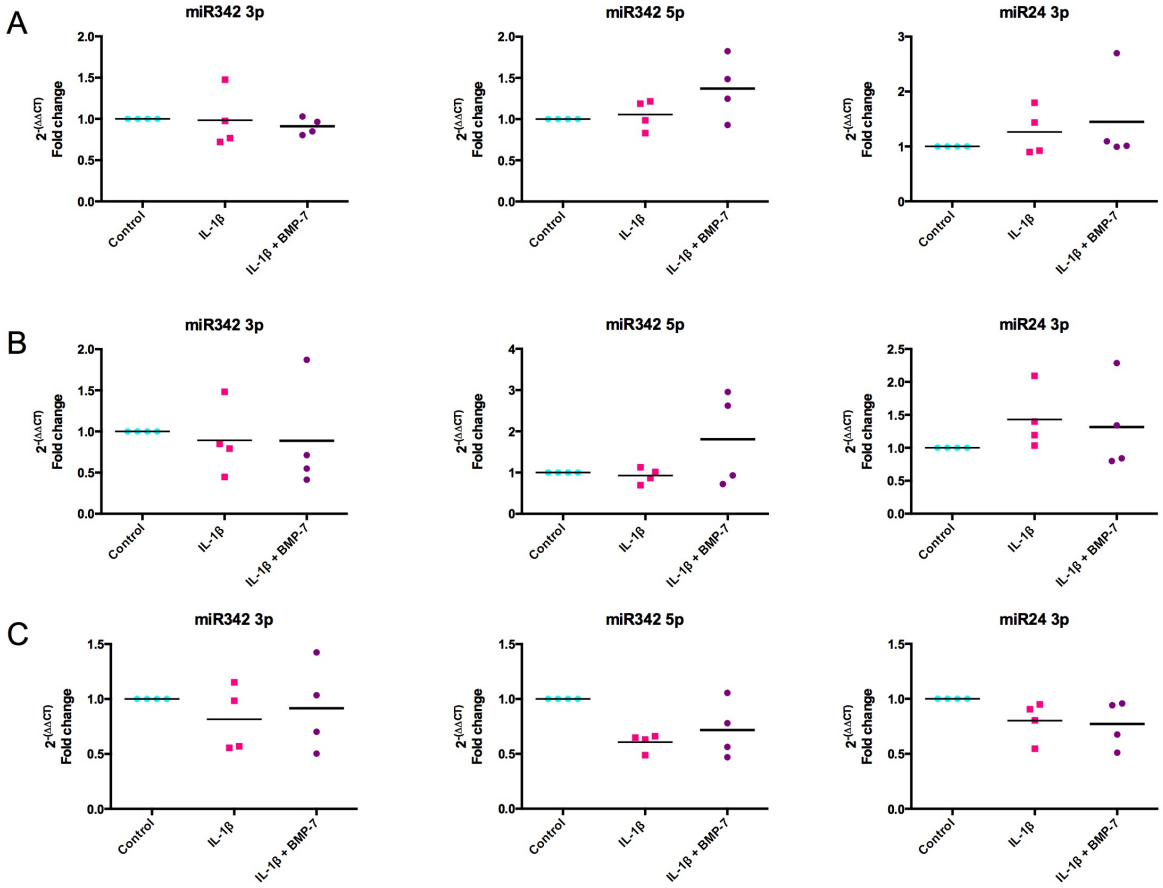


Figure 5.9 Effect of BMP-7 on IL-1β Mediated Alterations of the BMP-7 Associated miRNA in Primary OA Chondrocytes

Primary chondrocytes stimulated with either IL-1β or IL-1β/BMP-7 all at 100ng/ml for 8 hours before cells were placed in cytokine free media. Cells were then lysed at either A) 8 hours, B 24 hours or C) 48 hours. Genes analysed following stimulation were miR 342 3p, miR342 5p and miR24 3p. Data was analysed as fold change ($2^{-\Delta\Delta CT}$) using the unstimulated cells as a baseline. Each dot represents one chondrocyte donor, from which cartilage was taken at the time of TKR surgery from areas of Outerbridge score ≤I, N=4. Statistical analysis; Friedman ANOVA, Dunns multiple comparisons post test,

5.3.4 The Effect of BMP-7 Stimulation on Pro-inflammatory Cytokine Production from Chondrocytes.

Due to the interesting observation that BMP-7 can mediate the induction of both MMP-13 and IL-1ß (Figure 5.5), further investigation into the ability of BMP-7 to modulate other pro-inflammatory cytokines was carried out. A Luminex assay was used in order to examine a panel of pro-inflammatory cytokines, determining if BMP-7 stimulation had any effect on their release from the primary chondrocytes. The supernatants were taken from the unstimulated cells and those stimulated with BMP-7 for 24 hours. The following cytokines were quantified by Luminex: IL-1ß, IFNα, IL-6, IL-12, RANTES, MIP-1α, MIP-1ß, GM-CSF, MCP-1, IL-1Ra, IL-17, IP-10, IL-2R and IL-8. The effect of BMP-7 was not consistent across all of the donors investigated but it appeared to promote the production of several immune-modulatory cytokines, including IFNα, IL-6, IL-12, MCP-1, IL-1Ra, IL-7, IP-10 and IL-2R (Figure 5.10), in a donor dependent manner. The interleukin 1 receptor antagonist (IL-1Ra), which is a natural inhibitor of IL-1ß, was the only mediator significantly up-regulated by BMP-7.

The effect of BMP-7 on the IL-1ß induced up-regulation of these mediators was also investigated (Figure 5.11). As expected, IL-1ß significantly up-regulated all of the proteins of interest, however BMP-7 displayed no anti-inflammatory effects against the IL-1ß mediated cytokines.

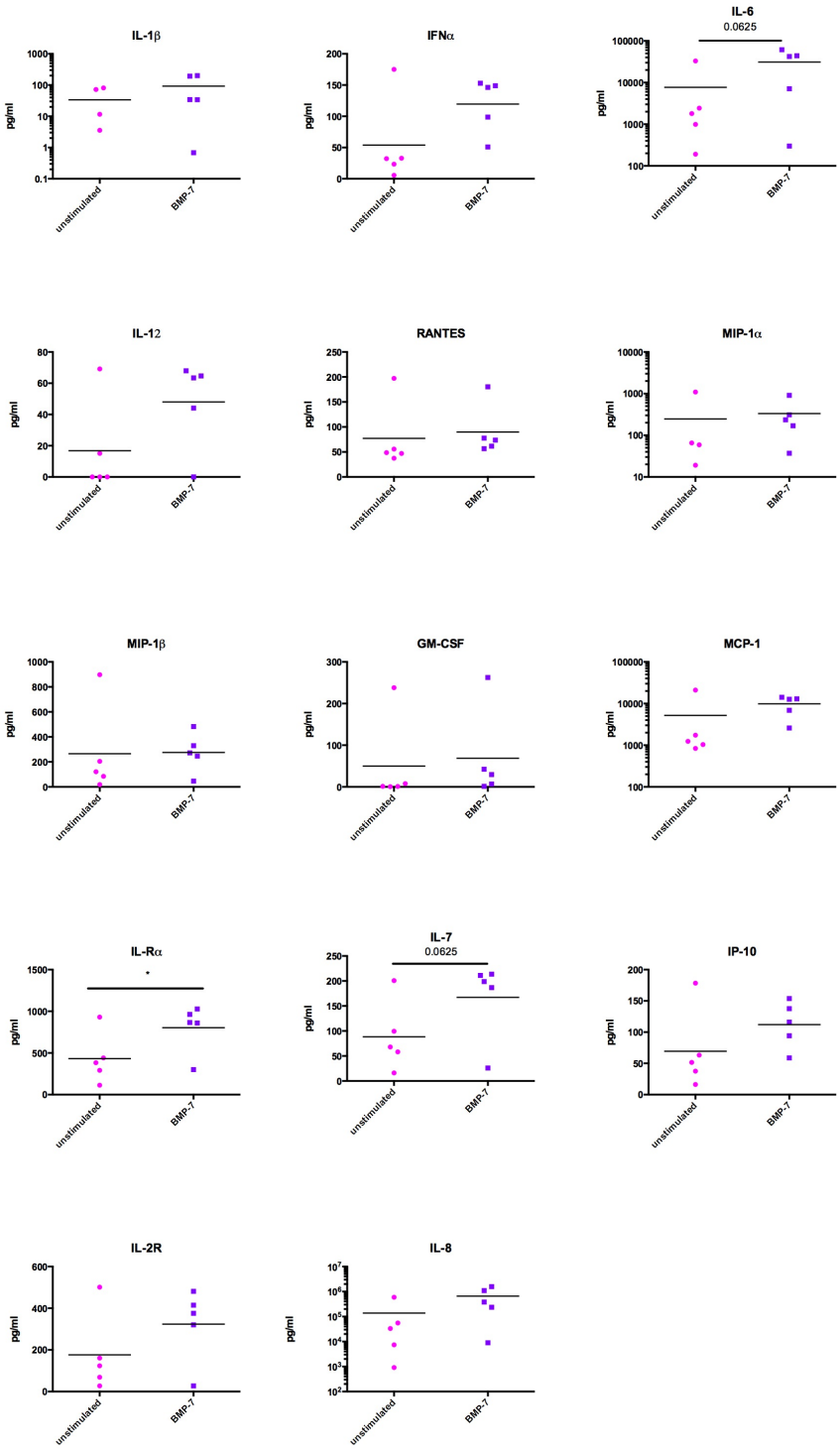


Figure 5.10 Secretion of Pro-Inflammatory Cytokines Following BMP-7 Stimulation of Primary OA Chondrocytes.
Chondrocytes stimulated with BMP-7, 100ng/ml for 8 hours, before media is replaced with cytokine free media. Supernatants were harvested 24 hours post stimulation and added onto a 30-plex luminex plates for analysis. Data is shown as pg/ml. Proteins analysed: IL-1β, TNFα, IL-6, IL-12, RANTES, MIP-1α, MIP-1β, GM-CSF, MCP-1, IL-Rα, IL-17, IP-10, IL-2R and IL-8. Statistical analysis was carried out using a Wilcoxon T-test, * P<0.05

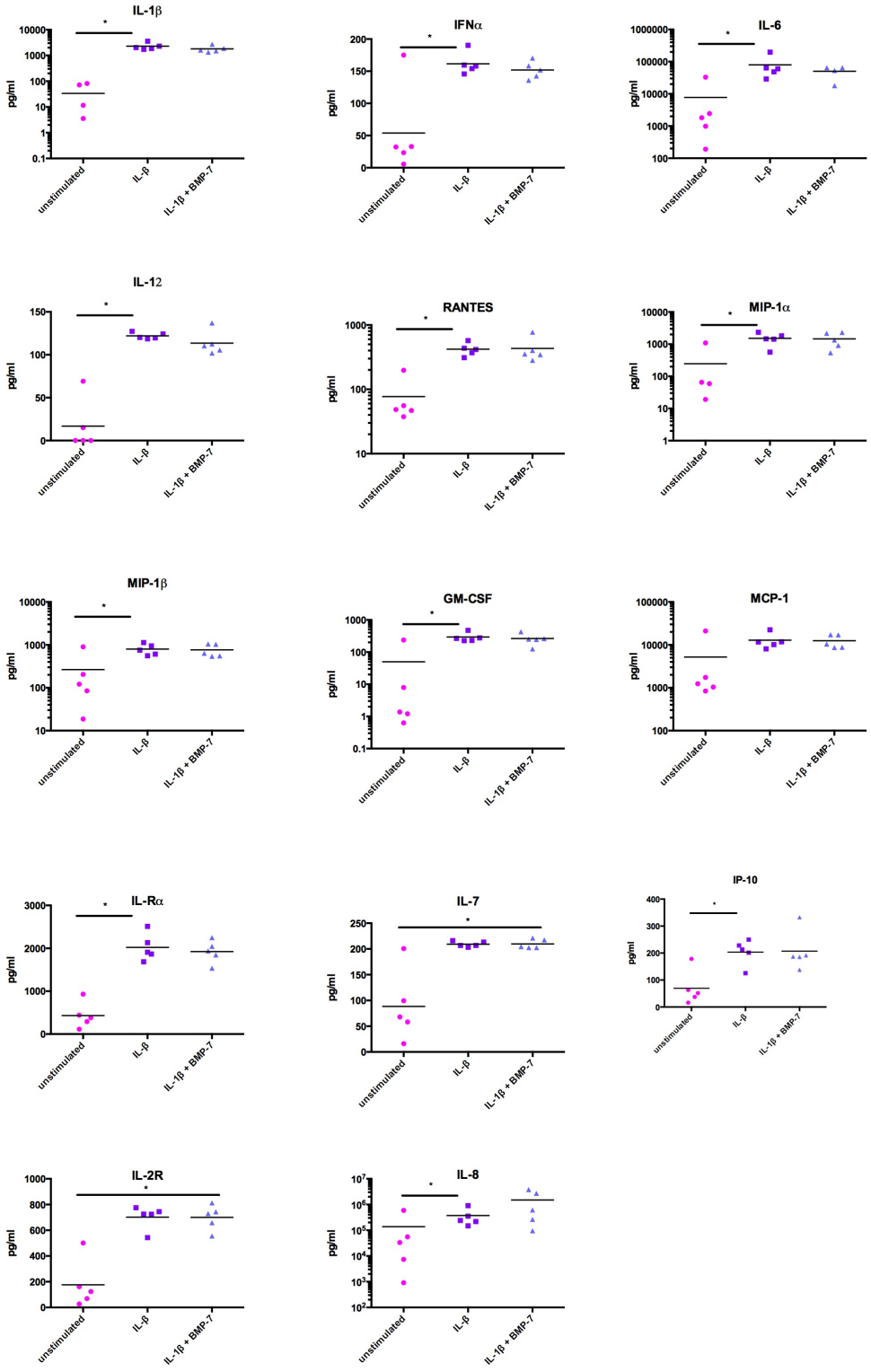


Figure 5.11 Secretion of Pro-Inflammatory Cytokines Following IL-1β±BMP-7 Stimulation of Primary OA Chondrocytes.
Chondrocytes stimulated for 8 hours with IL-1β or IL-1β/BMP-7 all at 100ng/ml, media is replaced after 8 hours with cytokine free media. Following 24 hours stimulation the supernatants were harvested and added onto a 30-plex luminex plates for analysis. Data shown as pg/ml. Protein analysed: IL-1β, TNFα, IL-6, IL-12, RANTES, MIP-1α, MIP-1β, GM-CSF, MCP-1, IL-Rα, IL-17, IP-10, IL-2R and IL-8. Statistical analysis, carried out using Friedman ANOVA, Dunns multiple comparisons post test, * P<0.05.

5.3.5 Effect of BMP-7 on Primary Human Macrophages.

Previously published data demonstrates that BMP-7 can drive THP-1 cells towards an anti-inflammatory M2 phenotype (144). To determine if the findings in the THP-1 cells could be reproduced in primary cells, an investigation into the phenotype of primary macrophages exposed to BMP-7 was conducted. Firstly, the presence of agranular leukocytes (monocytes or macrophages) was confirmed in the cartilage using IHC to identify any CD68 positive staining. CD68 is a known marker of monocytes and macrophages (

Figure 5.12), thereby demonstrating the presence of these cells in OA cartilage. For the purpose of this investigation, macrophages were assigned into 3 different groups: inactivated (M0) cells, classically activated pro-inflammatory (M1) cells (exposed to IFNγ) or alternatively activated anti-inflammatory (M2) cells (exposed to IL-4). In order to elucidate the cellular response to BMP-7 in macrophages, it was critical to ensure that the receptors required for signal transduction were also present on the cells. Accordingly, BMP receptors, BMPR2 and ACVR1 (ALK2), were visualised with end point PCR (Figure 5.13A). Both receptors were present in all three types of macrophage.

In order to illuminate any quantitative differences in receptor expression between these phenotypes of macrophage, qPCR analysis was also conducted (Figure 5.13). It was shown that the M1 macrophages had the most abundant expression of both receptors and of BMP-7 transcript. The CT values were also shown (Figure 5.13C), with the BMP receptors having an average CT value of 23 and 24 for the ACVR1 (ALK2) and BMPR2 receptors respectively. The BMP-7 transcript was less abundant with a CT value of 31.

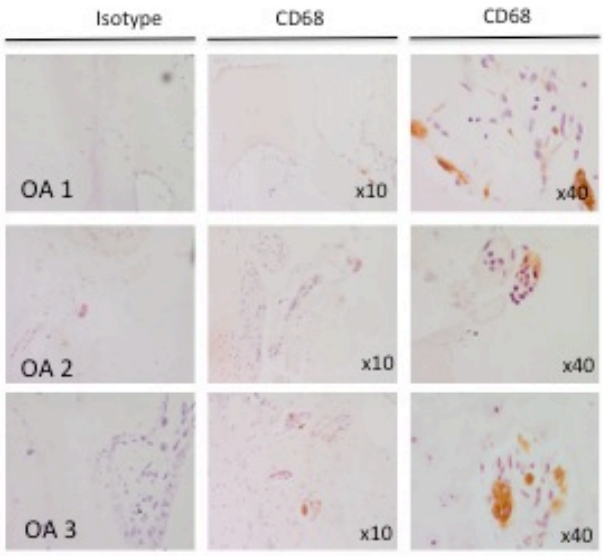
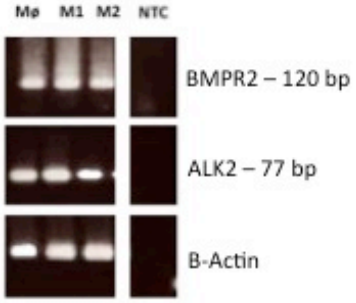
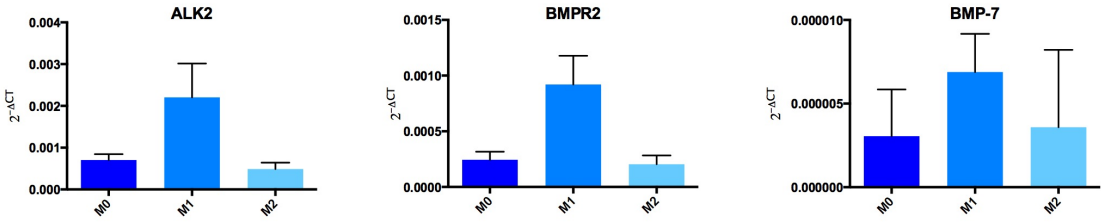


Figure 5.12 CD68 Positive Cells Present in the Cartilaginous Lesions in OA.
Sections were prepared for staining using the UNITRIEVE antigen retreival technique. CD68 (monoclonal mouse antibody - anti-human CD68, clone PG-M1, LOT 0052978) was used at a concentration of 1µg/ml. CD68 positive staining can be seen in brown with DAB substrate solution. The isotype was a Mouse IgG1 (DAKO, LOT 200117491), shown as 10X magnification. CD68 images are shown as both 10x and 40x magnification. All images were taken with an Olympus BX41 microscope and analysed using Olympus cell B imaging software.

A



B



C

	CT value		
	M0	M1	M2
ALK2	23	23	23
BMPR2	24	24	24
BMP-7	31	31	31

Figure 5.13 BMP Receptor Expression on M0 (inactivated), M1 (inflammatory) and M2 (Anti-Inflammatory) Macrophages.
$CD14^+/CD16^+$ cells were isolated from buffy coats (by either Simone Kidger or Lewis Rodgers) and plated at $1x10^6$/ml. Cells were matured for 6 days in M-CSF 100ng/ml. On day 6 M1 cells were treated with IFNγ O/N. On day 7 the cells were either given 100ng/ml M-CSF (M0), media alone (M1), or IL-4 (M2). A) cDNA was generated for the cells, M0, M1(+LPS), M2 M0+LPS, M2+LPS, using AffinityScript. End point PCR was conducted using primers for BMPR2 (120Kb) and ALK2 (77kB) and house keeping gene βactin, products were run on an ethidium bromide agarose gel and visualised using Gel logic 200imaging system. qPCR analysis (with SYB green) was also condicted in the M0, M1 and M2 cells for B) ALK2 (ACVR1), C) BMPR2 and C) BMP-7. All qPCR data was analysed as 2^- Statistical analysis was carried out using Frieidman test (ANOVA), with Dunn multiple comparison test.

Next the impact of a gram negative bacterial cell wall component, lipopolysaccharide (LPS) was assessed on the expression of BMP-7 and BMP receptors (Figure 5.14). LPS is a toll-like receptor 4 (TLR4) ligand and is a well established activator of monocytes and macrophages. An increase in both receptors (BMPR2 and ALK2) was observed following LPS stimulation in the M0's Figure 5.14A), however, LPS had no robust bearing on the expression of BMP-7. LPS addition to the M1 pro-inflammatory cell was only able to induce BMPR2 expression (Figure 5.14B). It is plausible that up-regulation OF BMP-7 AND ACVR1 (ALK2) in this phenotype may not have been possible due to the baseline expression in the M1 cells (induced by IFNγ) already being higher than that of the other subtypes of macrophages. Finally, the impact of LPS on the M2 anti-inflammatory cells was interrogated (Figure 5.14C). Again there was a consistent up-regulation of both ALK2 and BMPR2. BMP-7 transcript was also up-regulated in two out of three of the macrophage donors in response to LPS. It can be deduced from these data that macrophages have the ability, through adequate receptor expression, to respond to BMP-7. Additionally, LPS can further induce the expression of both receptors in the M0 and M2 (anti-inflammatory) macrophages and to a lesser extent the M1 (pro inflammatory) macrophages.

With the macrophages expressing the appropriate receptors to respond to BMP-7 stimulation, it was of interest to investigate the cytokines that would be produced following BMP-7 stimulation. Previously it has been reported that the macrophage-like cell line, THP-1, adopt an anti-inflammatory phenotype following BMP-7 stimulation. The macrophages were matured for 6 days before being polarised with BMP-7 (Figure 5.15). The BMP-7 polarised cells were found to secrete the same concentration of IL-6, IL-10 and TNFα as LPS stimulated cells. Therefore the primary chondrocytes would appear to behave in a different manner when compared to the THP-1 cells exposed to BMP-7.

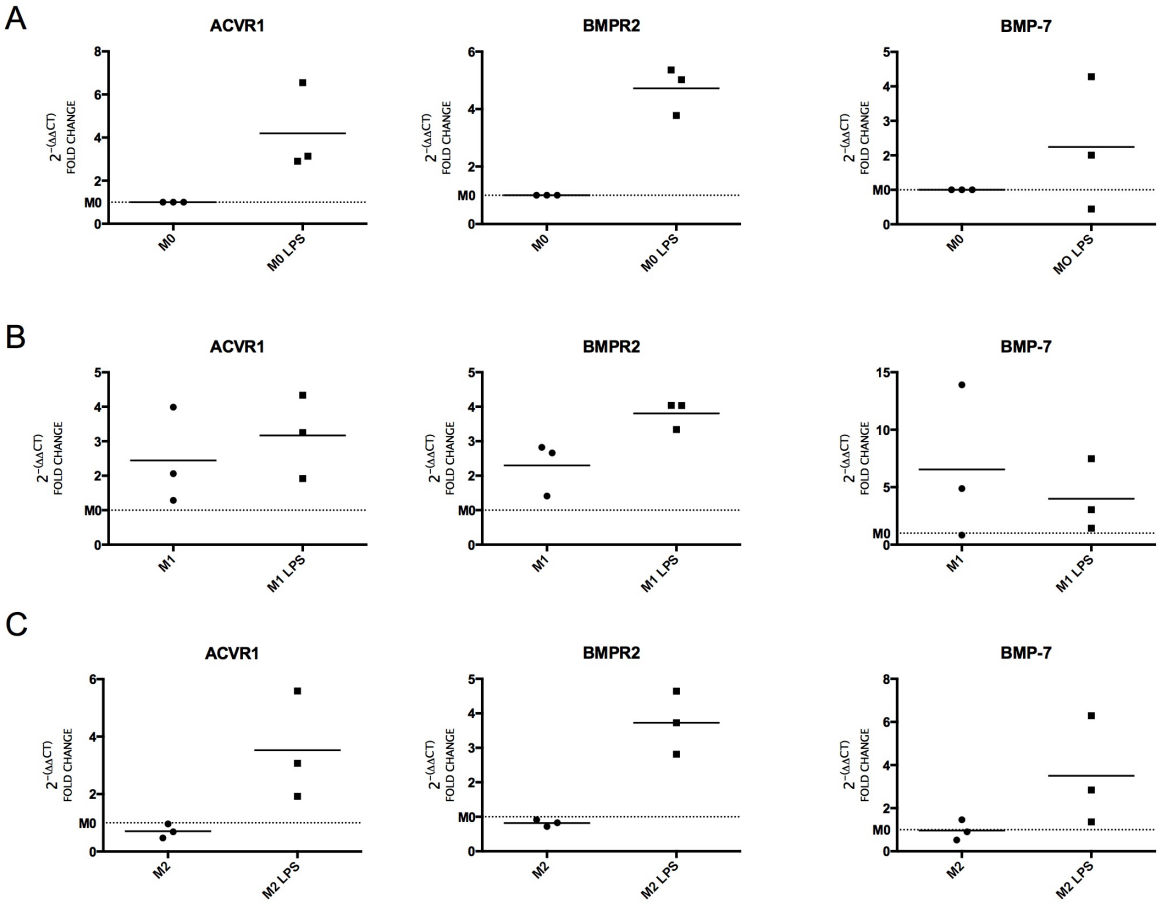


Figure 5.14 LPS Induction of BMP Receptors in M0 and M2 Macrophages
$CD14^{+}/CD16^{+}$ cells were isolated from buffy coats (by either Simone Kidger or Lewis Rodgers) and plated at $1x10^{6}$/ml and matured for 6 days in M-CSF 100ng/ml on day 6 M1 cells were treated with IFNγ O/N. On day 7 the cells were either given 100ng/ml M-CSF (M0), media alone (M1), or IL-4 (M2). If LPS stimulation was included it was also added at this point at 15ng/ml. qPCR analysis (using SYBR green) was conducted in the A) M0, B) M1 and C) M2 macrophages for ALK2 (ACVR1), BMPR2, BMP-7. Statistical analysis; Wilcox t-test.

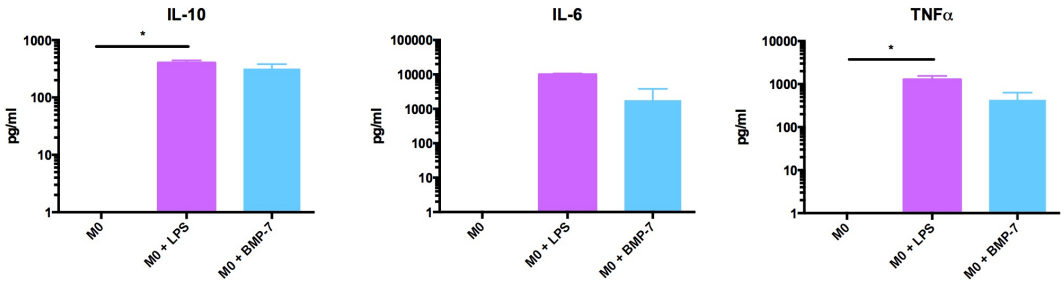


Figure 5.15 BMP-7 Polarisation of Macrophages Resulted in the Same Cytokine Secretion as LPS Stimulation.
$CD14^{+}/CD16^{+}$ cells were isolated from buffy coats (by either Simone Kidger or Lewis Rodgers) and plated at $1x10^{6}$/ml and matured for 6 days in M-CSF 100ng/ml on day 6. On day 7 the cell were either given 100ng/ml M-CSF M0, 100ng/ml M-CSF and 15ng/ml LPS or BMP-7 100ng/ml. Supernatants from the macrophages cultures were harvested on day 8 and subject to ELISA analysis for either IL-10, IL-6 or TNFα. Statistical analysis was carried out using Friedman test (ANOVA), with Dunn multiple comparison test. * = $P<0.05$.

5.4 Discussion

5.4.1 Summary of Results

This chapter aims to elucidate potential mechanisms of action for exogenous BMP-7 including; regulation and modulation of its own transcript and those of its associated signalling family. Furthermore, the idea that BMP-7 may be an anti-catabolic agent was also addressed. Overall it was found that BMP-7 could modulate its own expression as well as that of some accessory signalling molecules in both chondrocytes and macrophages. In chondrocytes this regulation was patient specific and therefore not observed to the same extent in all donors. BMP-7 has shown both catabolic and anabolic effects: induction of IL-1β and catabolic enzyme MMP-13 along with increased expression of glycoprotein CD44 receptor (which is key in mediating ECM production). BMP-7 also regulates macrophage polarisation resulting in the production of pro-inflammatory TNFα, IL-6 and anti-inflammatory IL-10.

5.4.2 SW1353 Cell Line Response Differs from Primary Chondrocytes.

The SW1353 cell line was initially utilised as a valuable research tool due to the fact that it can often be difficult to get adequate numbers of primary chondrocytes. It has been established that the cell line SW1353 can reliably reproduce the IL-1β mediated up-regulation of catabolic factors such as MMP-13 (265). In this current study, some initial optimisation issues arose with the IL-1β stimulations as the SW1353 cells did not respond as expected with MMP-13 transcript up-regulation - neither cell cycle phase nor age of the cells had an impact on these results. It must also be considered that when compared to the primary chondrocyte, response to BMP-7 stimulation was consistently different in the SW1353 cells. Reassuringly the more robust finding from the cell line data - induction of IL-1β transcript by BMP-7 was also confirmed in the primary cells.

It is possible that the SW1353 cells, due to their oncogenic background, simply do not respond in a similar fashion to the primary cells with regards to BMP-7 signalling components.

5.4.3 Exogenous BMP-7 – Mechanism of Cartilage Protection.

5.4.3.1 Indirect ECM Induction via CD44 Up Regulation

Historically, extensive research has been carried out investigating the effect of exogenous BMP-7 on primary cells *in vivo,* however, without the use of *in vitro* cultures it is hard to unravel all the potential mechanisms that may be targeted for further pharmaceutical intervention. CD44 is a key candidate for BMP-7 mediated chondro-protection, resulting in ECM repair and maintenance. The data generated in this study show that there is indeed a BMP-7 mediated up-regulation of CD44 at a transcriptional level in 3 out of the 5 chondrocyte donors. This suggests that there may be donor specific variation in the response to BMP-7. One of the shortcomings of this study would be that there was no validation of BMP-7's effect on hyaluronic acid synthase or hyaluronic acid as both are required for CD44 mediated ECM synthesis (156). Furthermore, translation of the CD44 transcript into functional protein following stimulation with BMP-7 should also be carried out in future studies. From the data presented above, there was a strong positive correlation between the expression of the CD44 and BMP-7 transcript (data not shown), however a larger cohort would be required in order to confirm this. Previous publications also report that inhibition of BMP-7 via antisense oligonucleotides resulted in an decrease in CD44 transcript (155) with other studies supporting this concept, as an increase in CD44 expression is evident following incubation with BMP-7 for 1, 3 or 7 days (156,259). Validation of CD44's role in BMP-7s signalling has previously been carried out, demonstrating that knocking down CD44 resulted in increased concentrations of BMP-7 ligand being required to induce Smad phosphorylation (267). The increased concentration that was required in order to induce phosphorylation in the absence of CD44 was also not sufficient enough to induce aggrecan synthesis. In order to see an increase in aggrecan, an even higher concentration of BMP-7 had to be used. (267). Taken together this strongly suggests that both BMP-7 and CD44 work synergistically in order to produce ECM. This work has shown that BMP-7 can mediate CD44 production in some

individuals at the level of the transcript in the cartilage. Therefore, this study supports previous finding that BMP-7 therapy may be beneficial in increasing CD44 receptor expression which could subsequently increase ECM synthesis in a patient specific manner.

5.4.3.2 Direct Up Regulation of BMP-7 signalling components.

To date, this is the first study to show the auto-regulatory capacity of BMP-7 on its own transcript and receptor, specifically BMPR2 (Figure 5.5) by addition of exogenous ligand. This mechanism however, appears to be donor dependent (as demonstrated by the variation within the data) and therefore was not found to be significant. Previous studies show that BMP-7 protein is up-regulated in the cartilage following therapeutic injection in animals (158,159), however, this is the first study to confirm transcriptional up-regulation, therefore showing that this is not just a result of latent protein potentially being trapped following therapeutic administration. It is therefore plausible that exogenous BMP-7 may be able to regulate its own signalling pathway, kick-starting the endogenous signalling that was speculated to be lost with the progression of the OA (153). Furin, the enzyme required for the cleavage of pro BMP-7 into its mature and active form is again up-regulated by over 3 fold in three out of the five chondrocyte donors (Figure 5.5) (one after 8 hours and 2 after 24 hours) helping to facilitate BMP-7 signalling within the cartilaginous environment. As a functional readout to determine if increased furin along with BMP-7 and BMP-7 receptors could lead to increase ECM production, type II collagen expression was analysed, however, no consistent trends in transcript expression were observed. It must be noted that previously aggrecan has been the ECM component analysed with regards to BMP-7 stimulation, despite the fact that type II collagen has also been reported to respond to BMP-7 (220). It would therefore have been beneficial to quantify the expression of aggrecan as well as type II collagen in this study, thus allowing a more in-depth analysis of the ECM component response to exogenous BMP-7 stimulation.

5.4.4 Modulation of Catabolic Factors and Pro-Inflammatory Cytokines in Primary Chondrocytes.

5.4.4.1 IL-1β Driven Anabolic Process in Chondrocytes.

As BMP-7 is thought to be down-regulated as a result of OA pathogenesis (150), it was considered that IL-1ß may be the key regulator of this inhibition, however, this study has generated no data that supports this hypothesis. Conversely, it was found that IL-1ß induced BMP-7 transcript expression, validating largely ignored data that was published over a decade ago using rabbit chondrocytes (268) and work carried out in human cells where low dose IL-1ß results in an increase of both BMP-7 transcript and protein (246). In these human studies, high dose IL-1ß results in a greater increase in transcript but not protein, as it is surmised that the active protein in this case is secreted into the surrounding media (246). Taken together, these data suggest that IL-1ß may not be responsible for disease-associated diminution of BMP-7. Based on these results, further studies were undertaken to investigate whether or not a lack of BMP-7 signalling components may result in altered BMP-7 signalling associated with OA. Therefore, if the receptors required for signalling were down-regulated by IL-1ß then this would in effect break the signalling loop. To this end the effect of IL-1ß on the expression of these signalling components was investigated and demonstrates that IL-1ß induced each component (Figure 5.8) as opposed to the hypothesised inhibition. Additionally, co-stimulation with IL-1ß and BMP-7 had no additional impact, neither synergistic or inhibitory. These data would therefore suggest that IL-1ß is not involved in the diminished BMP-7 signalling, either by directly inhibiting BMP-7 or indirectly, via inhibition of the BMP-7 signalling family, in the short term *in vitro* system used here. The observed induction of BMP-7 signalling components following IL-1ß stimulation in these short term cultures may be a normal response to tissue damage. The idea that IL-1ß induces anabolic factors has already been suggested in the inflammatory response to tissue injury, thus these findings should actually not come as that much of a surprise (269). Whereas chronic exposure to IL-1ß, or possibly a milieu of cytokines over decades during disease progression may cause alterations in BMP signalling. Due to study design, only short term cultures with IL-1ß could be conducted and not the more appropriate long term cultures. It is possible that

long-term chondrocyte cultures would have been more informative in validating the role of chronic IL-1ß exposure on primary chondrocytes, which would have been more representative of OA. Long-term cultures of chondrocytes are however, not practical as the cells dedifferentiate over time as shown in chapter 3. Stem cell differentiation into chondrocytes within ECM scaffolds may also have been an appropriate platform in order to understand the long-term effects of IL-1ß, as it has been demonstrated that these cells are more likely to hold their phenotype; giving rise to more viable, phenotypically relevant cells in long-term culture (270-272).

5.4.4.2 BMP-7 Driven Inflammatory and Catabolic Responses in Chondrocytes.

This study not only suggests that BMP-7 does not have an anti-inflammatory/catabolic effect, but goes onto suggest that BMP-7 may drive an inflammatory/catabolic response in chondrocytes. A review published in 2007 stated that (to date) there has been no evidence of BMP-7 mediated MMP-13 modulation (234). However, there is disparity in the literature as a study in 2003 used luciferase reporter assays in immortalised chondrocytes in order to demonstrate that BMP-7 could prevent IL-1ß induced MMP-13 up-regulation (273). The data presented in this chapter agrees with neither of these previous statements, as no such inhibition of IL-1ß induced MMP-13 was observed. Instead, here it has been demonstrated for the first time that BMP-7 itself has a robust impact, increasing MMP-13 expression in primary human chondrocytes (Figure 5.5), with the peak response observed 24 hours post stimulation. The extent of the up-regulation was donor dependent, ranging from a 1.5-fold to a 66-fold increase. BMP-7's inability to inhibit any IL-1ß mediated effects during co-stimulation could be a result of the high concentration of IL-1ß used in the assay, therefore it is possible that if the cytokine had been titrated then BMP-7 may have had an impact.

Additionally, BMP-7 also modulated the expression of IL-1ß (Figure 5.5), a phenomenon observed in all of the chondrocyte donors. This observation is contradictory to previously published data that show a significant decrease in IL-1ß in the synovium of rat joints treated with BMP-7 (158). This attribute of BMP-7 has also been observed in proximal tubule epithelial cells from the kidney

(274). It is possible that this anti-IL-1ß effect is only present in certain subsets of cells, such as the ones previously reported in the literature (fibroblast like synoviocytes and proximal tubule epithelial cells), thus suggesting that chondrocytes are simply a cell that cannot produce this response. As the data here does not support the literature and the synovium was not investigated in this particular study, the potential of BMP-7 to protect the joint environment via modulation of IL-1ß remains unconfirmed.

This BMP-7 modulation of catabolic processes may be considered to be part of normal homeostatic regulation. It is likely to be a perfectly normal physiological reaction from the chondrocytes in the same way that IL-1ß production of anabolic process is likely to be part of the normal physiological response to injury. From the literature, BMP-7 is thought to be up-regulated in order to maintain tissue homeostasis and repair. As part of this role, promotion of moderate inflammation and catabolic activity in order to restore cartilage homeostasis following acute injury, would be expected.

It is also interesting that a significant BMP-7 mediated up-regulation of IL-Ra (a natural inhibitor of IL-1ß) was shown (Figure 5.10). Further highlighting BMP-7s homeostatic role as not only can it promote the expression of IL-1ß, aiding in the repair of the tissue but also promotes the termination of this response through the up-regulation of inhibitors for these mediators, such as IL-Ra. Thus preventing chronic IL-1ß signalling that could promote pathological destruction.

5.4.5 BMP-7 Activation of Macrophages

Discrete CD68 staining was observed in the cartilage of several of the OA patients investigated in this study. CD68 is classically regarded as a macrophage marker, however, it has also been shown that this glycoprotein is also expressed in fibroblast like synoviocytes (275). Unfortunately, without further analysis it is impossible to determine if these are infiltrating macrophages, cleaning up the debris left by the catabolic processes, or invasive FLS that have begun to intertwine with the cartilage aiding the destruction process. FLS have been reported to invade the cartilage in RA and this is thought to be in part due to local disease associated pro-inflammatory mediators, including IL-1 and TNFα (276). Further to this, RA fibroblasts have been shown to maintain their invasive

phenotype once removed from the joint. This was also shown in OA, however the extent of the invasiveness was significantly reduced when compared to RA (277).

If macrophages are present in the cartilage, then the literature would suggest that BMP-7 may have the potential to differentiate these cells into an anti-inflammatory M2 cell (144). Despite all of the efforts that have been deployed into defining the effect of BMP-7 on the phenotype of THP-1 cells, there is no published data of the effects on BMP-7 on primary macrophage differentiation. Data in this chapter (Figure 5.15) would suggest that BMP-7 stimulated macrophages are similar to that of LPS challenged cells (Figure 5.15), and not the previously stated M2 – like cells. There are clearly discrepancies between the data generated in this study and the previously published literature, however, it must be considered that the previously published data was carried out on a cell line and also a concentration of 660ng/ml BMP-7 was used to stimulate the cells. In this current study a much lower concentration of 100ng/ml was used on primary macrophages. Therefore it has been concluded that macrophages that are exposed to BMP-7 have the same cytokine output as those who have been exposed to LPS *in vitro,* thus they do not behave in the same manner as the THP-1 cell line in response to BMP-7.

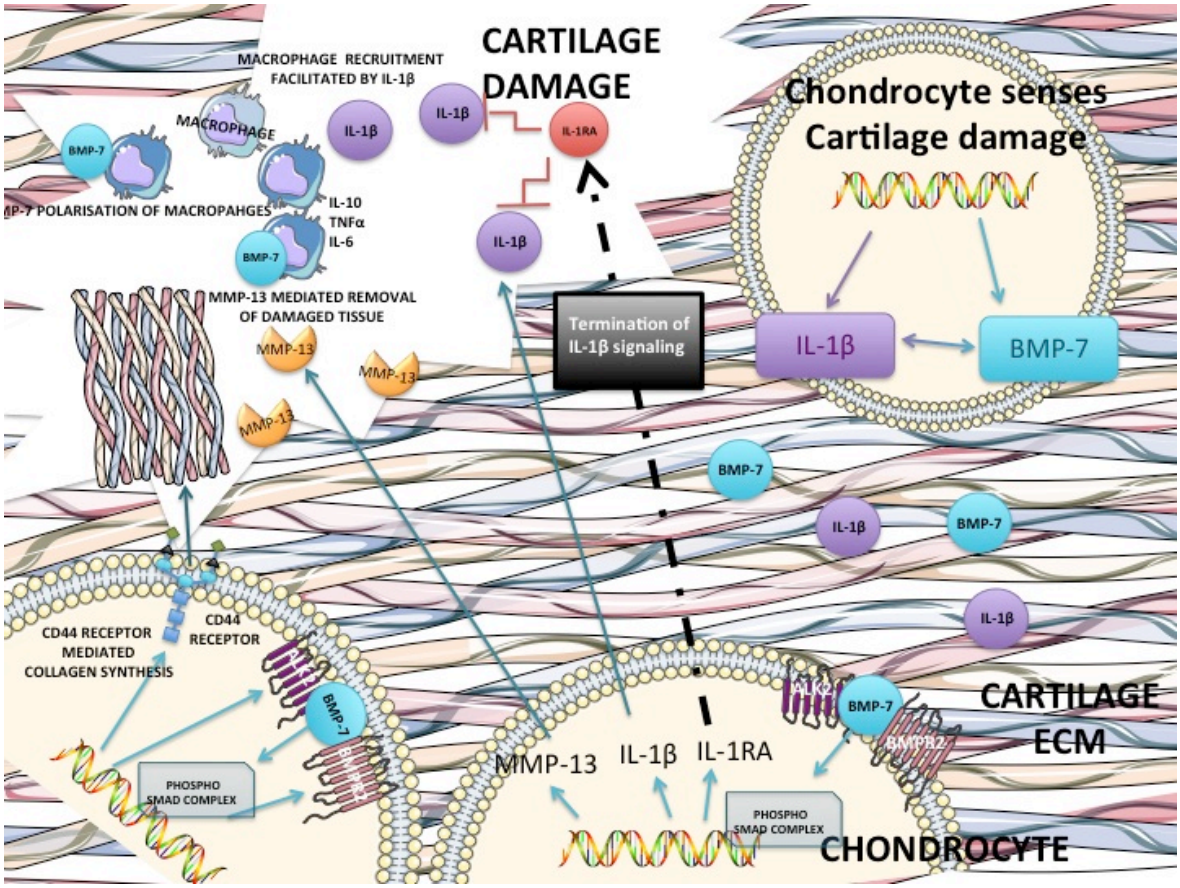


Figure 5.16 Potential mechanisms of Action for Exogenous BMP-7 Therapy.
Schematic depicting the potential mechanisms of action exerted by BMP-7 at sites of acute tissue damage**:** BMP-7 and IL-1β have been shown to work in a similar fashion to resolve injury and repair the tissue as well as to both promote the expression of each other. Anabolic factors, BMP-7 and BMPR2 are up-regulated by BMP-7, these anabolic factors are thought to work synergistically with CD44 to produce ECM. BMP-7 can also modulate an inflammatory response via IL-1β in the area of damage which will aid the recruitment of the appropriate immune cells in an attempt to further repair the tissue. BMP-7 has also been shown to promote MMP-13 that will help to remove any damage tissue. Here BMP-7 can promote the production of TNFα and IL-6 from macrophages, which will help in the repair efforts for the tissue. Furthermore, BMP-7 polarised macrophages can also produce IL-10, which may be of importance in dampening the IL-1β response following the resolution of the damage. IL-1RA can also be induced by BMP-7 from the chondrocytes, further helping to terminate IL-1β response following resolution. This in turn may attenuate the up-regulated BMP-7 signalling that was increased in response to damage.

5.4.6 BMP-7's Role in Response to Tissue Injury.

This study set out to investigate the role of exogenous BMP-7 signalling in the modulation of anabolic events within the context of OA. However, the data presented in this chapter has highlighted a role for BMP-7 in the promotion of inflammatory and catabolic events from the chondrocytes. The chondrocytes from this study have been excised from an OA joint, but here it has not been confirmed if they maintain a disease like phenotype after isolation from the joint, as no healthy chondrocytes were available as a comparator. Also with a number of mediators and external factors (i.e mechanical stress) missing the *in vitro* studies have not recapitulated the disease environment and this system has consequently not closely mimicked OA. Therefore, the studies conducted in this chapter are more likely to be investigating how chondrocytes would react under normal circumstances to BMP-7 or IL-1ß stimulation. Thus, in exposing the chondrocyte to short term cultures, it is possible that the cells are responding as they would to a short burst of inflammatory stimuli than they would be exposed to in the event of mild to moderate tissue injury and not a chronic disease state.

These data have shown that BMP-7 may have anabolic potential (via modulation of its own signalling family and CD44 mediated ECM synthesis) however, it would appear that BMP-7 also modulates the expression of the pro-inflammatory cytokine IL-1ß, along with catabolic mediator MMP-13. Furthermore, BMP-7 has the capacity to modulate other pro-inflammatory cytokines and activate macrophages to the same extent as LPS. Under normal physiological circumstances, it is likely that this pro-inflammatory recruitment is a mechanism of homeostatic repair that BMP-7 may have the ability to 'switch off' via the up-regulation of IL-1ß inhibitor IL-1Ra.

Cartilage can be damaged in three ways: disruption of the matrix, fissuring of the cartilage causing a partial defect, or erosion of the cartilage through to the subchondral bone resulting in a full thickness defect (278). In the first instance it has been suggested that if the cartilage is disrupted the remaining viable chondrocytes are able to repair the ECM, however as with all sites of injury, this most likely involves some form of immune intervention. From the data generated, it can be surmised that IL-1ß may up-regulate the expression of

BMP-7 from the chondrocytes in the initial insult. From here, BMP-7 has the capacity to mediate anabolic processes via CD44 leading to ECM production. However, as with most tissue insults, damage associated molecular patterns (DAMPS) are released leading to the recruitment of immune cells such as monocytes to the site of injury (with the most likely point of entry for the immune cells being the highly angiogenic synovium). BMP-7 has also been shown to modulate IL-1ß expression, which may lead to the downstream generation of chemotactic cytokines such as: RANTES, MIP-1α, MIP-1ß, MCP-1 and IP-10, also facilitating the recruitment of these cells to the site of injury. These cells can then further produce cytokines on exposure to BMP-7, that may aid the healing process, such as IL-6, which has been shown to promote healing in the skin via regulation of leukocytes and collagen accumulation (279). These cells may also help in the resolution of the inflammatory and reparative process as the macrophages have been shown to produce IL-10 under the influence of BMP-7, which has been shown to down-regulate IL-1ß expression in monocytes (280). Further to this, BMP-7 can also promote the IL-1ß antagonist IL-1Ra (Figure 5.10). Together these would help to dampen the pro-inflammatory cytokine signalling as well as hindering IL-1ß's ability to promote BMP-7 expression, thus terminating the signalling loop. This model has been summarised in Figure 5.16. This type of response may also occur in partial cartilage defects, however, full repair of the cartilage is unlikely to occur in this sort of injury (278), with the reasoning for this incomplete repair remaining elusive.

5.4.7 Limitations of the Study.

As outlined above, one of the main experimental drawbacks of this study was chondrocyte culture time. In order to recapitulate a disease environment, long-term cultures would have to be established. However, due to chondrocyte phenotypic drift in an *in vitro* setting, this is problematic. Hence why short-term cultures were chosen for this study. As a result, answering questions regarding BMP-7's role in OA proved difficult, however, elucidation of BMP-7s potential role in dealing with acute injury in the chondrocytes was possible and proved to be rather fruitful in aiding our current understanding. Other limitations included the narrow repertoire of genes analysed throughout the

study. Due to limited time and finance it was decided to focus on a small cohort of genes of interest. If further resources had been available, then a wider range of genes may have been analysed to fully elucidate the role of BMP-7 outwith its own signalling family.

5.4.8 Conclusion

Here it has been demonstrated that BMP-7 is able to modulate its own expression and that of the BMP-7 signalling family, showing for the first time that BMP-7 can modulate its own receptor (Figure 5.5) in primary human chondrocytes from a subset of individuals. Furthermore, the role of BMP-7 in the modulation of IL-1ß and MMP-13 was also demonstrated in this study. Taken together this evidence suggests a more comprehensive role for BMP-7 in the homeostatic regulation of cartilage; not only as an inducer of anabolic processes but also the inflammatory and catabolic processes that must also be implemented for repair and homeostasis to occur. In addition to the chondrocyte centric pathway, it was observed that there was a cross-talk between chondrocytes and macrophages, which has already been reported in the literature. Here, however, it is suggested that via potentiation of IL-1ß, BMP-7 may attract monocytes/macrophages to sites of cartilage damage. BMP-7 may also be able to switch off this signalling loop via the production of IL-1ß anatagonist IL-1Ra. Here further evidence to support a complex signalling loop has been presented with BMP-7 and IL-1ß being key in the maintenance of cartilage integrity.

6 General Discussion

This thesis has investigated the role and regulation of BMP-7 and its signalling family in OA. Previous studies have pointed towards a disease associated decrease of BMP-7 (153), however data generated here suggests that BMP7 transcript is not robustly expressed in the OA cartilage. BMP-7 family member transcripts however, are readily detected in the cartilage and further to this here it has been shown that disease associated miR24 3p can repress the expression of the BMP receptor ALK2. Additionally, a correlation was reported between the expression of ALK2 and miR24 3p in the OA cohort investigated in this study. Thus giving disease significance to this observation. Analysis of primary chondrocytes revealed heterogeneity in both the endogenous expression and stimulated modulation of the BMP signalling family members. Despite this heterogeneity in response to stimulation, BMP-7 was still shown to regulate IL-1ß and its inhibitor IL-1Rα in a robust manner. In this thesis, due to the short nature of the *in vitro* stimulations, conditions were unlikely to recapitulate those seen in an OA setting. Instead, acute inflammatory insults were carried out, investigating the role of the BMP-7 family in this setting. The data display a role for BMP-7 in the regulation of anabolic and catabolic processes along with macrophage polarisation in acute response to inflammatory insults.

6.1 Low Level BMP-7 Expression in Primary Chondrocytes.

The transcriptional and protein expression of BMP-7 in OA was extensively investigated in chapter 4. It was found that only very low levels of BMP-7 mRNA were expressed in a donor dependent manner in OA patient cartilage and synovial tissue. This was further confirmed using nested PCR analysis. The discrepancies observed in the studies undertaken in this thesis and those previously reported in the literature could be due to the sensitivity of the scientific technique employed for detection. Post-transcriptional regulation of BMP-7 may also allow for the differences observed. One plausible explanation was discussed in chapter 4, BMP-7 transcript contains AU rich elements (AREs) in the 3'UTR that are predicted to contribute to transcript stability. The zinc finger

protein TTP can bind these AU rich regions and induce rapid degradation of the transcript (Figure 4.13). Further studies are required in order to confirm this, including a luciferase assay to measure the direct binding of TTP to the AU rich elements in the 3'UTR of the transcript. If TTP can directly bind to the 3'UTR of BMP-7 then it would be important to investigate whether there are any variations in the 3' UTR of BMP-7 transcripts in chondrocytes from patients with OA. Transcript variation could occur via alternative splicing or mutations in the AU rich region. Thus the splice variant(s) or mutations produced by the cell could have an impact on how many ARE sites are available for binding via TTP, which would impact transcript stability. A technique known as rapid amplification of cDNA ends (RACE) could be utilised in order to look at the variance in the 3'UTR of the BMP-7 transcripts found in the chondrocytes. In this way, BMP-7 from OA and healthy chondrocytes could be compared to look for disease associated variation that could have an impact on transcript stability and expression. The presence of splice variants could also alter the way in which the transcripts respond to miRNA regulation. Studies in this thesis have shown that both IL-1ß and BMP-7 are down-regulated in two out of three chondrocyte donors when transfected with miR24 3p mimic. It is possible that this response to miR24 3p is due to patient specific 3'UTR variation.

6.2 Heterogeneity in the Expression of the BMP-7 Signalling Family in OA Cartilage.

At the beginning of this study it was hypothesised that BMP-7 would stimulate its own expression and also induce the expression of other BMP-7 related transcripts such as BMPR2, ALK2, CD44, FST, GREM and type II collagen. Based on the data presented in chapter 5, this hypothesis could not be confirmed. The impact of BMP-7 on these genes varied between donors. The variability in the results observed from both the BMP-7 stimulation and the analysis of the transcript expression in the OA cohort suggest that there is significant heterogeneity with regards to these genes in OA patients. Despite this variation, a significant positive correlation was found between the expression of BMPR2, ALK2 and type II collagen in the patients investigated. This suggests that some of the individuals in this study have intact anabolic BMP-7 signalling that results in collagen production, whilst other do not possess this capacity. This gives rise to

the hypothesis that molecular responses to the progression of OA may differ from patient to patient. This may be dependent upon the aetiology of the disease or possibly due to variation in genetic susceptibility. Thus highlighting the need for further evaluation of the BMP-7 signalling pathway in OA, as this may provide new targets for treatments or biomarkers for patient stratification.

6.3 Expression and Role of miR24 3p in OA

One of the major findings from this study was the regulation of the BMP receptor ALK2 by miR 24 3p. Despite the variation in the transcript expression observed in chapter 4, a significant negative correlation was discovered in OA patients between the expression of miR24 3p and ALK2. One limitation of the work undertaken in this thesis is the lack of healthy cartilage, as the disease specificity of this correlation remains undetermined. However, the functional studies performed in primary cells confirm an interaction between miR24 3p and ALK2. OA chondrocytes transfected with synthetic miR24 3p led to a 40% - 50% decrease in expression of the receptor. Thus for this first time this study has highlighted ALK2 as a target for miR24 3p. To confirm the regulatory role of direct binding of the miRNA to the 3'UTR of the target mRNA further work should be undertaken, such as luciferase assays. Nonetheless, based on the fact that this miRNA targets a receptor that is required for anabolic signalling, the current data does provide support for its role as a driver of disease pathogenesis. It should be appreciated, however, that contradictory data does exist (195). Although, it is feasible that both interpretations are in fact true (Figure 4.25), this miRNA may indeed be decreased in the end stage of disease, but this may not reflect the levels of this miRNA in early disease pathology (figure 4.25). More work is required to profile the temporal kinetics of this miRNA during OA evolution. Animal models of OA (DMM, ACLT) could be adopted in order to define the kinetic expression of this miRNA and its targets at both the onset and during the development of the pathology. Furthermore miR24 3p KO or conditional KO mice could be utilised to assess the impact this miRNA may have on driving OA-like disease in mice.

6.4 Impact of BMP-7 on Primary Macrophage

A hypothesis regarding the ability of BMP-7 to inhibit IL-1β mediated effects (on the BMP-7 signalling family) was also proposed in this study, as it has previously been shown that BMP-7 can prevent IL-1β mediated up-regulation of MMP-13(1). The data presented in chapter 5 disagrees with this literature. Importantly, BMP-7 stimulation actually led to an up-regulation of IL-1β at the level of the transcript (Figure 5.5). Moreover, the studies also revealed that macrophages respond to BMP-7 in a similar fashion to LPS stimulation, with increased expression of IL-10, IL-6 and TNFα. Taken together these data suggest a role for BMP-7 in the activation of both pro-inflammatory and anti-inflammatory responses. This has led to the testable concept that BMP-7 may up-regulate anabolic, catabolic and inflammatory mechanisms in homeostatic repair (Figure 5.16).

As BMP-7 has an emerging role in the regulation of immune factors in the joint, it is possible that its role in OA may be more complex than was initially suggested. Its role is not to solely induce anabolic signalling events that would lead to ECM formation, but also to regulate a catabolic and inflammatory responses to damage. With all of the factors working in tandem the tissue can be returned to its homeostatic state after an insult or injury. Further work is needed to expand these findings and to elucidate the entirety of BMP-7s role in the cartilage and joint.

6.5 Future Work

In order to verify these findings, there is a massive need for the acquisition of healthy cartilage in order to have an appropriate control tissue. Here, all of the cartilage used for *in vitro* analysis was taken from areas of cartilage with an Outerbridge score of 0-1. However it is known that OA is a disease of the whole joint and all the cartilage is exposed to a diseased environment for potentially decades before it is surgically removed and used in these studies. This brings into question the validity of using cartilage from a diseased joint as a control. Healthy cartilage would be needed in ordered to compare the phenotype of the disease chondrocytes, observing if they behave differently *in vitro* than the OA

derived cells. Healthy cartilage would also add significance to the patient cohort data that suggests that some changes may occur in the eroded cartilage compared to the non-eroded.

This study initially aimed to investigate the impact of BMP-7 on the associated signalling family, as it had been previously reported that therapeutic BMP-7 up-regulated endogenous BMP-7 protein expression in an animal model. The data generated here showed little impact on either BMP-7 or the associated signalling family following stimulation with exogenous BMP-7. Furthermore, the reported ability of BMP-7 to negate the effects of IL-1ß was also not observed here. Thus, the mechanism by which BMP-7 confers protection remains ill-defined. Further work is required in order to determine exactly how BMP-7 mediates protective effects in the context of OA. In the future a wide scale transcriptomic and proteomic approach could be adopted, to analyse the full effect of BMP-7 across a wider range of genes and proteins. This would give a better indication into the pathways involved in this protection. This approach would be best adopted in the animal models first, as their response to BMP-7 appears to be more homogenous than that seen in primary chondrocytes, perhaps due to genetic similarity. Any findings would then need to be validated in human primary chondrocytes to ensure relevance in humans.

6.6 Conclusions

In conclusion, this thesis has identified that the BMP receptor ALK2 can be regulated by miR24 3p and that its expression is significantly correlated in OA patients. This highlights miR24 3p as a potential regulator of cartilage homeostasis and changes in the expression of this miRNA over the course of OA disease progression may be involved in driving disease pathogenesis. In addition to the reported anabolic role of BMP-7, data herein suggests BMP-7 also has the capacity to promote the expression of IL-1ß and its antagonist IL-1Ra in OA derived chondrocytes. Further to this it also promotes the secretion of IL-10, TNFα and IL-6 in primary macrophage cultures. In combination, this suggests that BMP-7 contributes to the promotion of inflammation and subsequent associated repair as part of the cartilage homeostatic mechanisms

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