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PPARδ AND AMPK PATHWAYS IN CARTILAGE

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ABSTRACT

Osteoarthritis (OA) is a degenerative joint disease characteristic of articular cartilage breakdown. Molecular mechanisms of OA are not well understood. Roles of the PPARδ and AMPK pathways in cartilage have been suggested but are not well defined. We hypothesized that the AMPK and PPARδ pathways control cartilage ECM turnover by altering chondrocyte gene expression. Mouse primary chondrocytes and adult knee joint explant cultures were treated with either a PPARδ (GW1516) or an AMPK agonist (AICAR). Cell culture was analyzed by palmitate oxidation assay, MTT assay and Real-time PCR. Histological stains and immunohistochemistry were used to analyze explant culture tissues. Changes in gene expression of catabolic factors were demonstrated for both treatments, as well as a decrease in matrix proteoglycan staining. GW1516 also increased fatty acid oxidation, while AICAR decreased cell number. Results indicate involvement of both pathways in early OA changes and suggest that both could be novel therapeutic targets.

Keyword: Osteoarthritis, articular cartilage, PPARδ, AMPK, fatty acid oxidation, primary chondrocyte culture, extracellular matrix homeostasis

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ABBREVIATIONS AND SYMBOLS

ADAMTS	A disintergrin and metalloproteinase with thrombospondin motifs
AICAR	5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside
AMP	Adenosine Monophosphate
AMPK	Adenosin Monophosphate Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
DAB	Diaminobenzidine
dH2O	Distilled water
DMEM	Dulbecco's modified eagle's medium
DMM	Destabilization of the medial meniscus
DMSO	Dimethyl Suloxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter Type 4
G1	Globular Domain 1

G2	Globular Domain 2
G3	Globular Domain 3
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HtrA1	High temperature requirement A 1
IHC	Immunohistochemistry
IGD	Interglobular domain
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
IL-1	Interleukin-1
Lpl	Lipoprotein lipase
MEM	Modified Eagle's Medium
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NO	Nitric Oxide
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pen-strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PPAR	Peroxisome Proliferator-Activated Receptor
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor

SEM	Standard error of mean
Sox9	SRY (sex determining region Y) box 9
Sox5	SRY (sex determining region Y) box 5
Sox6	SRY (sex determining region Y) box 6
TCA	Trichloroacetic acid
TGFβ	Transforming Growth Factor Beta
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNFα	Tumor Necrosis Factor Alpha
^{3}H	Tritium
α	Alpha
β	Beta
δ	Delta
γ	Gamma

1.0 INTRODUCTION

1.1 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease and the most common form of arthritis occurring within our population (Poole, 1999; Reginster, 2002; Aigner *et al.*, 2006). Any part of the joint structure can be affected during the disease state. However, one defining feature of OA is the degeneration of articular cartilage (Goldring & Goldring, 2007). As a result of cartilage degeneration, chronic pain and impaired joint function arise as the main symptoms of the disease. Recently, OA has become more prevalent within our society, which can be attributed to a number of factors including an aging population and increasing biomechanical loads as a result of increasing obesity rates (Goldring & Goldring, 2007; Krasnokutsky *et al.*, 2007). For the same reason, the prevalence of OA is expected to rise further in the coming decades.

Despite the high prevalence of this disease and the extensive socio-economical impact it has on our society, the molecular mechanisms that contribute to and ultimately cause the degeneration of cartilage tissue remain mostly unknown. As a result, there has been no cure or long-term pharmacological treatment plan established for this disease. Current therapeutic options focus on managing disease symptoms and are unable to fully address the underlying mechanisms that are leading to the eventual breakdown of cartilage tissue. It is for these reasons that a better understanding of the pathological progression of OA is needed.

1.1.1 Synovial Joint Structure

Synovial joints are specially designed structures that allow for frictionless and coordinated movements (Figure 1.1) (Mow & Lai, 1980). The epiphysis of each articulating bone is covered by a thin layer of cartilage, beneath which lies the subchondral bone. The entire joint is encapsulated by the synovial membrane and its lining cells, the synoviocytes. The synoviocytes are metabolically active cells that maintain the composition of the synovial fluid, which functions in lubricating the joint during movement (Aigner *et al.*, 2006; Schmidt *et al.*, 2007). These joint structures along with supporting connective tissues confer overall stability to the joint. A number of pathological changes are indicative of the onset and progression of OA.

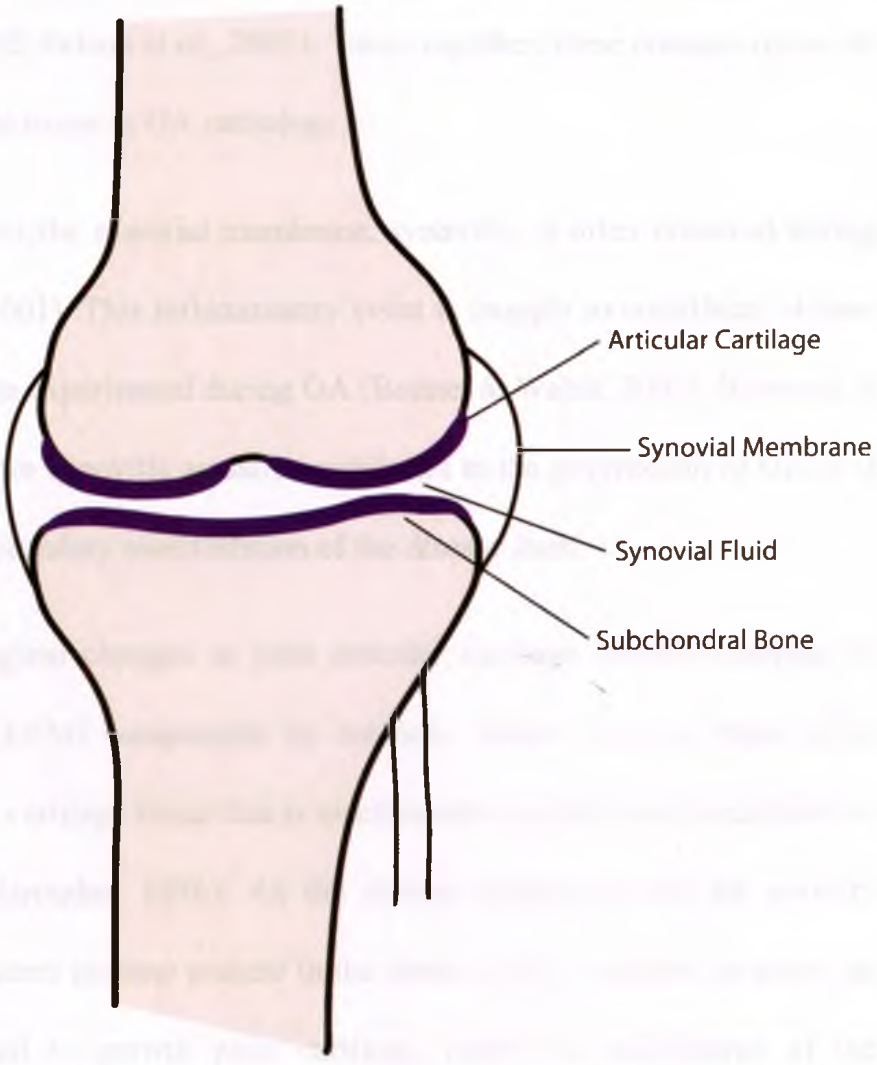
1.1.2 Pathological Changes in Osteoarthritis

Due to the complex architecture of synovial joints, a number of joint tissues can be affected during OA, including the subchondral bone, synovial membrane and articular cartilage (Burr & Schaffler, 1997; Hill *et al.*, 2001; Buckland-Wright, 2004). Furthermore, a number of these pathological changes have also been found to further contribute to the progression of OA by inducing changes in additional tissues, leading to the high degree of complexity characterizing this disease.

A number of subchondral bone changes have been noted to occur during OA with the most predominant of these changes being the thickening of subchondral bone tissue (Amir *et al.*, 1992). Interestingly, the extent of bone thickening has been found

Figure 1.1 Synovial Joint

Within the structure of synovial joints each bone is covered with a layer of cartilage tissue at their articulating ends. Along with the synovial fluid, articular cartilage allows for near frictionless movement. Although a number of joint changes have been noted to occur during OA, extensive and premature degeneration of articular cartilage is a prominent feature of the disease state.


Articular Cartilage
Synovial Membrane
Synovial Fluid
Subchondral Bone

to correlate with the degree of cartilage degeneration (Bobinac *et al.*, 2003). The subchondral bone has also been shown to undergo remodeling events during OA (Hayami *et al.*, 2004). Bone cysts, or lesions in the subchondral bone, become prevalent during OA (Rhaney & Lamb, 1955). Additionally, as OA progresses, bony projections, termed osteophytes, develop along the margins of affected joints (Jeffery, 1975; Amir *et al.*, 1992; Felson *et al.*, 2005). Taken together, these changes reveal an important role for bone tissue in OA pathology.

Inflammation of the synovial membrane, synovitis, is often observed during OA (Pelletier *et al.*, 2001). This inflammatory event is thought to contribute, at least in part, to the joint pain experienced during OA (Bonnet & Walsh, 2005). However, it remains unclear whether synovitis actually contributes to the progression of OA or if it arises merely as a secondary manifestation of the disease itself.

Initial pathological changes in joint articular cartilage involve cleavage of extracellular matrix (ECM) components by catabolic factors. Loss of these ECM components results in cartilage tissue that is mechanically unstable and susceptible to further disruption (Maroudas, 1976). As the disease progresses and the severity increases, vertical fissures become present in the tissue. Lastly, vascular invasion, an event usually confined to growth plate cartilage, results in calcification of the cartilage tissue (Lane *et al.*, 1977; Oegema *et al.*, 1997). As a result of these changes, the overall joint structure becomes compromised as the joint space narrows and bone on bone contact occurs.

1.2 Articular cartilage

Through its intricate composition, healthy articular cartilage functions in providing a smooth articulating surface between bones during the movement of joints. Articular cartilage is an avascular and aneural connective tissue composed of two distinct components, the cartilaginous cells (chondrocytes) and the ECM (Figure 1.2) (Aigner *et al.*, 2006). The chondrocytes, which have an extremely low proliferative rate, function to build and maintain the ECM. In healthy cartilage, there is a balance between matrix synthesis (anabolism) and matrix degradation (catabolism) (Eyre, 2004). However, during the disease state of OA, this equilibrium is shifted in favor of degradative events.

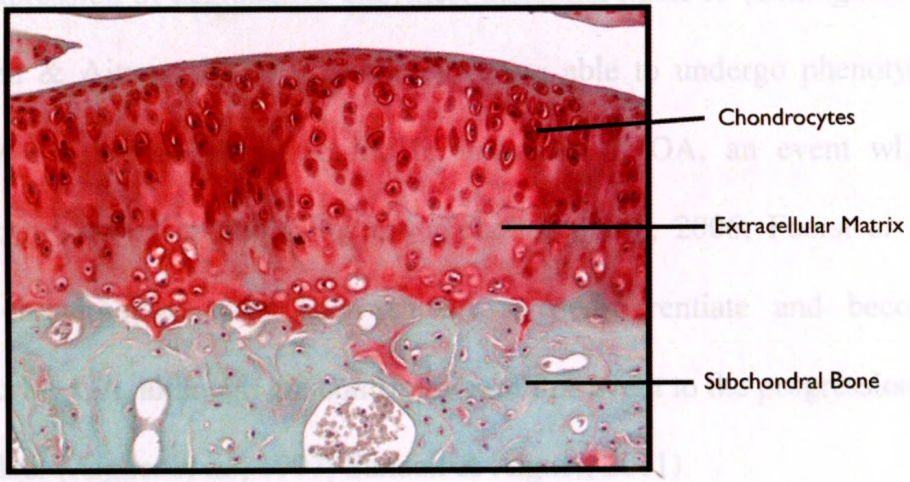
1.2.1 Chondrocytes

Chondrocytes, the sole cell type of cartilage tissue, have an extremely low proliferative rate, yet remain metabolically active within the mature tissue (Archer & Francis-West, 2003). The chondrocytes themselves are responsible for producing and maintaining the cartilage ECM. Physiological remodeling of the cartilage ECM is accomplished through the balanced expression of factors involved in matrix synthesis and those involved in matrix degradation (Eyre, 2004). The chondrocytes are responsible for secreting ECM components and are involved in regulating the overall turnover of the ECM.

Chondrocytes undergo a number of changes during OA, including phenotypic changes and altered cell metabolism. It has been demonstrated that chondrocytes

Figure 1.2 Composition of Articular Cartilage Tissue

Articular cartilage has two distinct components, the cellular component (chondrocytes) and the extracellular matrix (ECM). The chondrocytes are responsible for secreting and maintaining the ECM through a delicate balance of matrix synthesis and matrix degeneration. The cartilage ECM has two major structural components, a collagen fibril network and large aggrecates of proteoglycan molecules. These structural components each confer a distinct property to cartilage tissue and allow it to function in response to joint loading.


Chondrocytes
Extracellular Matrix
Subchondral Bone

begin to proliferate in response to early cartilage degeneration in an attempt to repair the damaged tissue (Rothwell & Bentley, 1973). It has also been noted that many of these cells undergo apoptotic cell death during the early stages of OA, resulting in a loss of viable cells (Adams & Horton, 1998; Blanco *et al.*, 1998). Additionally, chondrocytes begin to increase the synthesis of ECM components (Lippiello *et al.*, 1977; Sandy *et al.*, 1984; Aigner *et al.*, 1992; Gouttenoire *et al.*, 2004). Despite this increase in matrix synthesis, cartilage degeneration continues to take place as there is also increased expression of degradative enzymes, including MMP13 (Billinghurst *et al.*, 1997; Sandell & Aigner, 2001). Chondrocytes are able to undergo phenotypic changes as they can become hypertrophic in instances of OA, an event which increases the expression of catabolic factors (Tchetina *et al.*, 2006; Fuerst *et al.*, 2009). Lastly, chondrocytes have been shown to dedifferentiate and become fibroblast-like during OA, although the contribution of this event to the progression of OA remains unclear (Aigner *et al.*, 1997; Sandell & Aigner, 2001).

1.2.2 Extracellular Matrix

Cartilage tissue is composed of a large amount of ECM and thus the composition of this ECM is extremely important to the overall functional capabilities of this tissue. A large portion of the ECM is composed of a collagen fibril network, primarily type II collagen (Aigner *et al.*, 2006). Additional collagen species that contribute to the composition of the ECM include types IX and XI, which are distributed amongst type II collagen fibrils to provide further structural stability to the

matrix (Mendler *et al.*, 1989). Overall, the collagen network functions to provide tensile strength and stiffness to the cartilage.

Embedded within this extensive collagen fibril network are large aggregates of proteoglycan molecules. In cartilage, these aggregates consist primarily of aggrecan. The overall structure of aggrecan molecules consists of a core protein with numerous glycosaminoglycan side chains, including keratan sulfate and chondroitin sulfate (Hardingham & Fosang, 1992). These proteoglycan molecules are associated with hyaluronan molecules through link protein (Hardingham, 1979). Together these aggregates function in providing the tissue an extensive capability for the binding of water molecules and thus the ability to withstand extensive compressive forces (Bolton *et al.*, 1999).

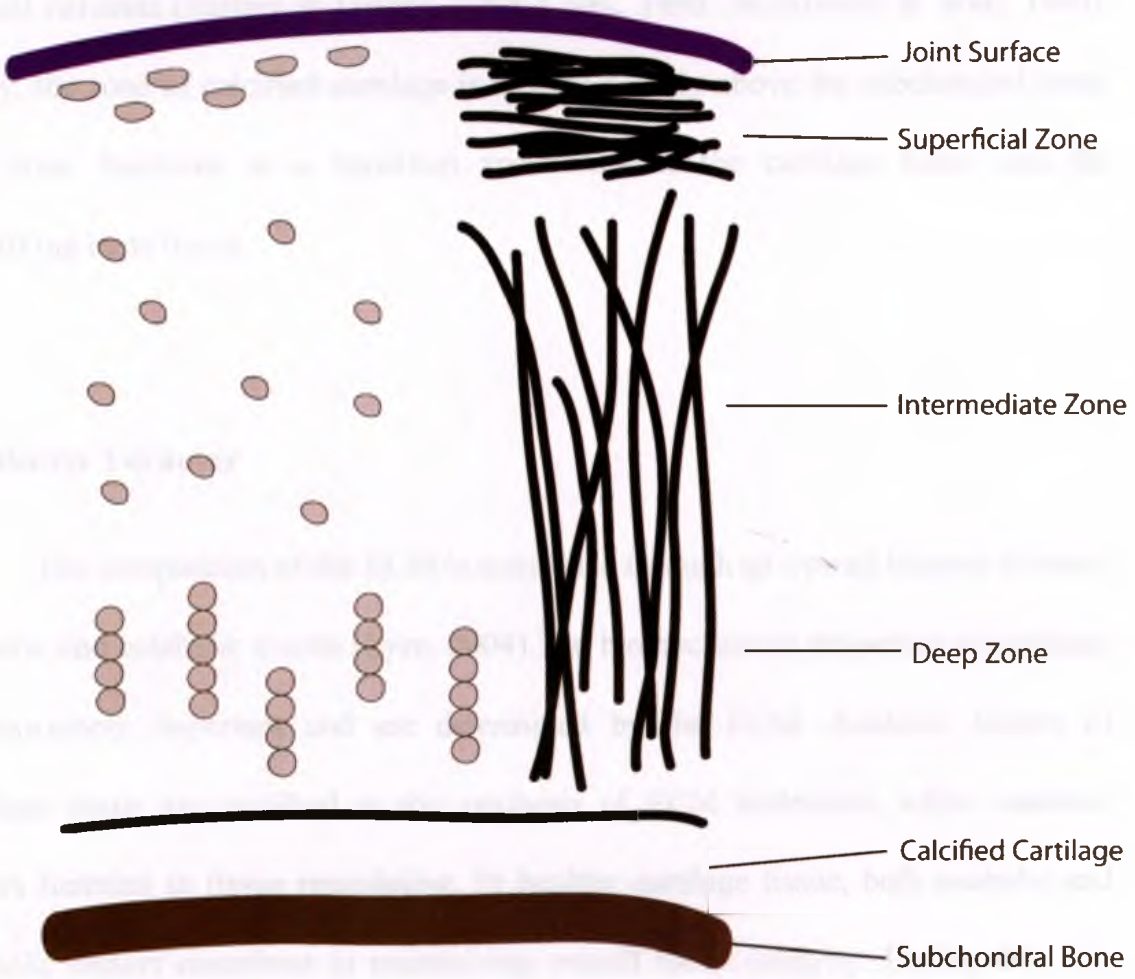
1.2.3 Zones of Articular Cartilage

Articular cartilage is comprised of four distinct zones: the superficial zone, the intermediate zone, the deep zone and the zone of calcified cartilage (Figure 1.3) (Palfrey & Davies, 1966; Ulrich-Vinther *et al.*, 2003). These zones differ in chondrocyte cell shape, as well as the architecture of the ECM. Specifically, the ECM differs between each of these zones with respect to the orientation and abundance of collagen fibers. Together, these zones contribute to the overall functional properties of articular cartilage.

The superficial zone lies nearest the joint surface and has the highest collagen content of all the zones (Hyttinen *et al.*, 2001). Within this zone, the collagen fibers

Figure 1.3 Zones of Articular Cartilage

Four distinct zones contribute to the overall structure of articular cartilage: the superficial zone, the intermediate zone, the deep zone and the zone of calcified cartilage. The distinction between each zone is established by differences in chondrocyte cell shape and collagen fiber orientation. In the superficial zone, both collagen fibers and the chondrocytes are oriented parallel to the joint surface. This zone accounts for the highest collagen content and its resident chondrocytes are flat in shape. In both the intermediate and deep zones, the collagen fibers are arranged vertically. The distinction between these two zones is made based on chondrocyte distribution. Chondrocytes in the intermediate zone are round in shape and are randomly distributed, while deep zone chondrocytes are found in vertical columns. Lastly, the zone of calcified cartilage provides attachment of cartilage tissue to the underlying subchondral bone.


Joint Surface
Superficial Zone
Intermediate Zone
Deep Zone
Calcified Cartilage
Subchondral Bone

are oriented parallel to the surface. The chondrocytes in this superficial zone are elongated in shape and oriented parallel to the surface (Palfrey & Davies, 1966; Wong *et al.*, 1996). In the intermediate and deep zones the collagen fibers are arranged vertically. However, chondrocyte cell shape differs between the intermediate and deep zones. Chondrocytes found within the intermediate zone are randomly distributed and are rounded in shape, whereas deep zone chondrocytes are arranged in vertical columns (Palfrey & Davies, 1966; Clark, 1990; Siczkowski & Watt, 1990). Lastly, the zone of calcified cartilage is located directly above the subchondral bone. This zone functions as a transition zone between the cartilage tissue and the underlying bone tissue.

1.3 Matrix Turnover

The composition of the ECM is controlled through an overall balance between anabolic and catabolic events (Eyre, 2004).The biomechanical properties of cartilage are extremely important and are determined by the ECM. Anabolic factors of cartilage tissue are involved in the synthesis of ECM molecules, while catabolic factors function in tissue remodeling. In healthy cartilage tissue, both anabolic and catabolic factors contribute to maintaining overall tissue integrity. During OA, this physiological matrix turnover is altered and a net loss of matrix molecules results as degradative events become more prevalent.

Loss of aggrecan in the matrix is indicative of early OA as its loss generally precedes loss of the collagen fibril network (Bluteau *et al.*, 2001; Pratta *et al.*, 2003).

Furthermore, it seems that cartilage degeneration is reversible following aggrecanase-mediated cleavage events (Karsdal *et al.*, 2008). The aggrecan component of the ECM is more readily subjected to physiological turnover, whereas the collagen network is extremely stable as extensive fibril cross-linking occurs (Bank *et al.*, 1998). It is for this reason that very little turnover of the collagen network occurs as it requires extensive enzymatic activity (Billinghurst *et al.*, 1997; Cawston *et al.*, 1999). Loss of matrix collagen is indicative of a later and more extensive stage of OA (Little *et al.*, 2002). There are a number of proteolytic enzymes and cytokines that contribute to matrix degeneration, which will be discussed in detail below.

1.3.1 Important Transcription Factors in Cartilage

The primary transcription factor important in the synthesis of the cartilage matrix is SRY (sex determining region Y) box 9 (Sox9) (Cucchiarini et al., 2007). Sox9 exerts its effect by activating many cartilage-specific genes (Takahashi *et al.*, 1998; Cucchiarini *et al.*, 2007). Sox9 has been found to promote the expression of both type II collagen and aggrecan (Bell *et al.*, 1997; Lefebvre *et al.*, 1997; Sekiya *et al.*, 2000; Han & Lefebvre, 2008). The expression of *Sox9* has been shown to decrease in instances of OA, yet this decrease does not seem to correlate directly with type II collagen expression (Aigner *et al.*, 2003). Fittingly, the expression of other Sox genes, mainly *Sox5* and *Sox6*, has also been found to decrease in chondrocytes during OA (Lee & Im, 2011). Sox5 and Sox6 are additional transcription factors that act with Sox9 to activate cartilage-specific genes (Ikeda *et al.*, 2004). These genes are

thought to act mainly by complementing the activity of Sox9, as they are unable to independently alter chondrocyte gene expression (Akiyama *et al.*, 2002). Together, Sox9, Sox5 and Sox6 provide excellent genetic targets for the development of potential treatments for OA.

1.3.2 Anabolic Factors

Anabolic factors of cartilage health are involved in promoting the synthesis of ECM components and act to maintain overall cartilage health. A number of anabolic factors important in cartilage tissue have been described, including transforming growth factor β (TGFβ), insulin-like growth factor-1 (IGF-1) and fibroblast growth factors (FGFs) (Sandell & Aigner, 2001). Many of these factors are secreted by both the synoviocytes and the articular chondrocytes.

The main anabolic activity of TGFβ is to stimulate matrix production by chondrocytes (Yaeger *et al.*, 1997; Blom *et al.*, 2007). More specifically, TGFβ is involved in inducing proteoglycan synthesis within the matrix (van Beuningen *et al.*, 1994). These findings each suggest that TGFβ may be a potential target in the repair of previously damaged cartilage tissue. Complicating the involvement of TGFβ in OA however, is evidence that it may additionally be involved in events leading to matrix degeneration and osteophyte formation (Scharstuhl *et al.*, 2002).

Synthesis of matrix components, both type II collagen and aggrecan, is stimulated by IGF-1 treatment *in vitro* (Yaeger *et al.*, 1997). IGF-1 treatment alone

does not offer a mechanism for repair potential as chondrocyte response to this anabolic factor is decreased during OA (Loeser *et al.*, 2000). However, articular chondrocytes treated with both IGF-1 and TGFβ displayed a synergistic response and increased ECM synthesis accordingly (Yaeger *et al.*, 1997). IGF-1 can further have a beneficial effect in cartilage tissue by acting to decrease catabolic activities (Trippel, 1995).

Lastly, a number of fibroblast growth factors have been identified as anabolic factors in cartilage tissue and are thought to provide therapeutic potential in OA. In particular, both FGF-2 and FGF-18 have been shown to promote the repair of degenerated cartilage tissue (Moore *et al.*, 2005; Kaul *et al.*, 2006). In addition, FGF-2 is able to suppress interleukin-1 (IL-1) activity and inhibit the production of ADAMTS-4 and ADAMTS-5, thus protecting against early degenerative events (Sawaji *et al.*, 2008; Chia *et al.*, 2009). FGF-18 is thought to exert its anabolic activity in part by increasing chondrocyte proliferation (Shimoaka *et al.*, 2002; Liu *et al.*, 2007; Ellman *et al.*, 2008).

To date, anabolic factors have not offered great promise in the treatment of OA as their roles in cartilage tissue are complex and remain to a large degree unknown. Many undesired events, such as production of catabolic factors, also arise with activity of these anabolic factors. A better fundamental understanding of both anabolic and catabolic factors of cartilage tissue is therefore needed.

1.3.3 Catabolic Factors

Pathological matrix degeneration involves the expression of a number of catabolic factors. These catabolic factors may be involved in directly cleaving ECM components or inducing catabolic events by altering gene expression. Furthermore, catabolic factors can be induced by the matrix fragments that result from matrix degeneration in a positive feedback loop (Homandberg, 2001). The main catabolic factors of interest include matrix metalloproteinases, aggrecanases (a disintergrin and metalloproteinase with thrombospondin motifs, ADAMTS), tumor necrosis factor alpha (TNFα) and IL-1 (Sandell & Aigner, 2001).

1.3.4 Matrix Metalloproteinases

Members of the matrix metalloproteinase family (MMPs) are involved in ECM remodeling during normal physiological events, such as development and tissue repair (Johansson *et al.*, 1997; Vu & Werb, 2000). However, these enzymes have also been found to contribute to pathological ECM changes, such as during arthritis (Billinghurst *et al.*, 1997; Takaishi *et al.*, 2008). Numerous MMPs have been implicated in OA including MMP1, 2, 3, 7, 8, 9 and 13 (Yoshihara *et al.*, 2000; Vincenti & Brinckerhoff, 2002). However, biological inhibitors of MMPs exist as tissue inhibitors of matrix metalloproteinases (TIMPs), which act to regulate the activity of MMPs. TIMP3 is also involved in regulating the activity of certain ADAMTS enzymes (Visse & Nagase, 2003).

Extensive research has focused on the involvement of MMP13 in OA, as it preferentially cleaves type II collagen, the main collagen of articular cartilage (Knauper *et al.*, 1996; Billinghurst *et al.*, 1997). The importance of MMP13 in articular cartilage, and subsequently OA, was demonstrated extensively using MMP13 knockout mice (Little *et al.*, 2009). Knockout mice had significantly less cartilage erosion than controls following surgically induced OA, indicating that degeneration had been inhibited. The expression of MMP13 has also been shown to increase during aging in human articular chondrocytes (Forsyth *et al.*, 2005; Loeser, 2009). It is important to note that in addition to MMP13, MMP1 and MMP8 are also able to disrupt the collagen matrix by cleaving the triple helix of type II collagen (Billinghurst *et al.*, 1997).

1.3.5 Aggrecanases in Osteoarthritis

Aggrecanases are major enzymatic contributors to the breakdown of the cartilage ECM as they act to cleave aggrecan. The main aggrecanases thought to be involved in cartilage degeneration during OA are ADAMTS-4 and ADAMTS-5 (Malfait *et al.*, 2002; Stanton *et al.*, 2005; Majumdar *et al.*, 2007). More recently, additional members from the ADAMTS family have been implicated in OA, including ADAMTS-1, -2, -7 and -12, although their specific roles remain unknown (Davidson *et al.*, 2006; Swingler *et al.*, 2009).

Cartilage degeneration during the OA disease state in humans relies on the activity of both ADAMTS-4 and ADAMTS-5 (Malfait et al., 2002; Song et al., 2007).

In comparison, ADAMTS-5 has been identified as the major aggrecanase contributing to aggrecan breakdown in mouse models of the disease (Stanton et al., 2005). The role of aggrecanases in mouse models of OA has been established by the generation of knockout mice for each of these enzymes (Glasson et al., 2004; Glasson et al., 2005; Majumdar et al., 2007). Although the involvement of each of these factors in OA seems to differ between animal models of the disease and the human disease state, it is clear that each are important factors in aggrecan cleavage and subsequently the development of OA.

1.3.6 Additional Catabolic Factors

Degradative events during OA can also be attributed to a number of additional catabolic factors. Increasing evidence has implicated a number of inflammatory cytokines in the progression of OA. These cytokines can induce cartilage degeneration by increasing the synthesis of catabolic factors, decreasing the synthesis of inhibitory factors and by decreasing the synthesis of ECM components (Sandell & Aigner, 2001; Goldring, 2002).

The proinflammatory cytokines IL-1 and TNFα are each increased during instances of OA (Melchiorri *et al.*, 1998; LeGrand *et al.*, 2001; Klooster & Bernier, 2005). IL-1 and TNFα are produced by synoviocytes, as well as by the articular chondrocytes themselves (Fernandes *et al.*, 2002). Each of these cytokines is involved in cartilage degeneration by increasing the gene expression of MMPs, with IL-1 also increasing the expression of ADAMTS-4 *in vitro* (Mengshol *et al.*, 2000; Bau *et al.*,

2002; Goldring, 2002). Additionally, it is well established that IL-1 and TNFα exert their catabolic effect by stimulating the production of reactive nitric oxide species, including nitric oxide, a known mediator of cartilage degeneration (Taskiran *et al.*, 1994; Hashimoto *et al.*, 1998; Melchiorri *et al.*, 1998; LeGrand *et al.*, 2001).

Another noteworthy, yet understudied, catabolic factor in cartilage tissue is high temperature requirement A 1 (HtrA-1). HtrA-1 became associated with OA when it was shown to be increased in articular cartilage following surgical induction of the disease (Hu *et al.*, 1998; Tsuchiya *et al.*, 2005). The exact role that HtrA-1 plays in the pathology of OA remains unclear, however, it has been suggested to be involved in the cleavage of matrix molecules, in particular aggrecan (Chamberland *et al.*, 2009). Additional evidence has suggested that it plays a role in altering chondrocyte metabolism during OA by cleaving a portion of the pericellular matrix (Polur *et al.*, 2010).

1.3.7 Aggrecan Cleavage

As previously mentioned, aggrecan cleavage in the cartilage ECM is a complex event with a number of catabolic enzymes contributing to its breakdown. aggrecan cleavage can be brought about by MMPs, as well members of the ADAMTS family (Fosang *et al.*, 1996a). Many sites for enzymatic cleavage exist along the aggrecan core protein (Sandy *et al.*, 1991; Flannery *et al.*, 1992; Fosang *et al.*, 1996a). The specific structure of the aggrecan core protein is made up of three globular domains, G1 and G2 located at the N-terminus, and G3 located at the C-terminus

(Doege et al., 1991). The interglobular domain (IGD) is found between G1 and G2 and is the major site of protein cleavage. The catabolic enzymes, MMP13, ADAMTS-4 and ADAMTS-5 cleave at different sites within this IGD (Fosang *et al.*, 1996a; Sztrolovics *et al.*, 1997). For this reason, antibodies recognizing specific cleavage products of aggrecan have been developed (Hughes et al., 1995). These antibodies have been crucial in cartilage research as they are able to distinguish between MMP-generated and aggrecanase-generated neoepitopes (Janusz et al., 2004). Therefore, these antibodies help in elucidating the exact molecular mechanisms contributing to aggrecan breakdown during OA.

1.4 Molecular signaling in cartilage

It is becoming increasingly evident that signaling events in cartilage tissue play an important role in the onset and progression of OA. This disease, which has classically been thought of as a normal result of aging and joint loading, is now being discovered to be heavily reliant on extensive molecular signaling. As mentioned above, a number of growth factors, cytokines, proteases, as well as a number of additional factors have all been implicated in cartilage degeneration during OA (Goldring, 2000; Sandell & Aigner, 2001; Glasson *et al.*, 2005; Naito *et al.*, 2007; Takaishi *et al.*, 2008; Beier & Loeser, 2010).

The peroxisome proliferator-actived receptor (PPARδ) and AMP-activated protein kinase (AMPK) pathways are each activated in skeletal muscle during exercise; AMPK playing a role in cellular energy homeostasis promoting the shift to

oxidative metabolism during exercise and PPARδ being mainly involved in skeletal muscle remodeling in response to exercise (Narkar *et al.*, 2008). More recently, each of these pathways has been found to play a role in chondrocyte biology.

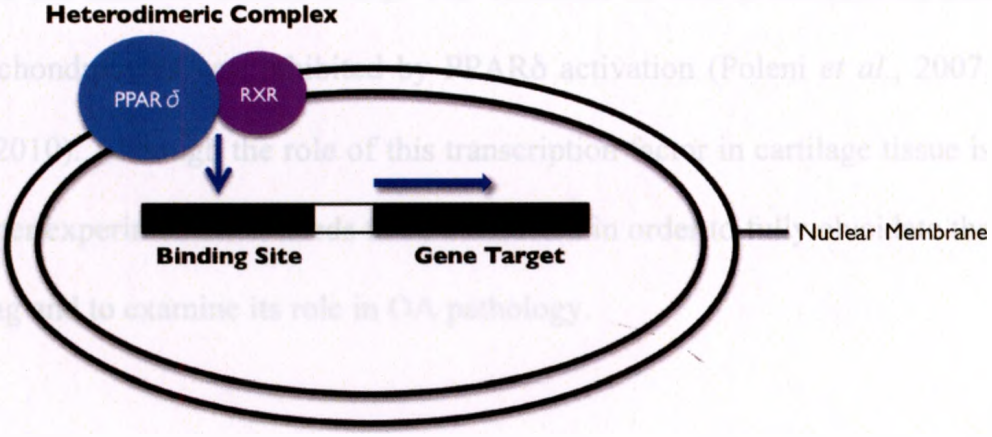
1.4.1 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptor (PPAR) proteins are members of the nuclear receptor family and act as ligand-activated transcription factors, with lipids functioning as the activating ligands (Evans *et al.*, 2004; Fredenrich & Grimaldi, 2005). Three PPAR proteins have been identified: PPARα, PPARγ and PPARδ (Evans *et al.*, 2004). Prior to exerting their transcriptional control, PPAR proteins heterodimerize with the retinoid X receptor (RXR) allowing for subsequent DNA binding to occur (Figure 1.4) (Gearing *et al.*, 1993). PPARδ, which is expressed in a number of tissue types, most notably adipose, liver and cardiac tissue, acts to regulate fatty acid metabolism in these tissues and others (Barish *et al.*, 2006; Seedorf & Aberle, 2007). Knowledge of the downstream targets by which PPARδ is able to exert its role in fatty acid oxidation remains limited. However, very recently the matrix metalloproteinase, MMP9, was identified as a novel downstream target of PPARδ in brain tissue (Chen *et al.*, 2011).

Mice null for the PPARδ receptor have been mainly used to characterize changes in glucose homeostasis and lipid metabolism. Phenotypic changes in these mice include increased glycolysis and increased lipid production in the liver

Figure 1.4 PPARδ function as transcriptional regulator.

PPARδ is a nuclear receptor acting as a ligand-activated transcription factor. This receptor heterodimerizes with the retinoid X receptor before binding to DNA. It is this receptor complex that has the ability to influence transcriptional activities.


Heterodimeric Complex
PPAR δ
RXR
Binding Site
Gene Target
Nuclear Membrane

(Lee *et al.*, 2006). However, these mice also display a decreased size when compared to controls, indicating a potential defect in endochondral ossification.

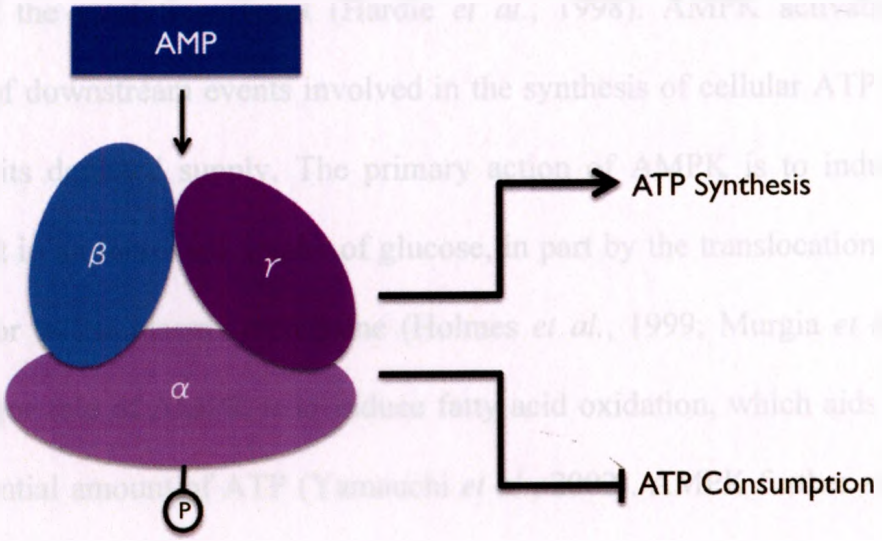
PPARγ and PPARα have been found to exert anti-inflammatory effects in articular cartilage, in part by modulating IL-1 activity (Bordji *et al.*, 2000; Francois *et al.*, 2006). Additionally, PPAR proteins have been detected in growth plate chondrocytes, suggesting that this family of proteins has a role in cartilage tissue (Shao *et al.*, 2005). Preliminary experiments utilizing chondrocytes in culture have provided evidence that activation of PPARs decreases production of ECM components by the anabolic factor, TGFβ. The synthesis of both proteoglycans and collagens by chondrocytes was inhibited by PPARδ activation (Poleni *et al.*, 2007; Poleni *et al.*, 2010). Although the role of this transcription factor in cartilage tissue is apparent, further experimentation needs to be conducted in order to fully elucidate the role it is having and to examine its role in OA pathology.

1.4.2 AMP-activated protein kinase

The adenosine monophosphate-activated protein kinase (AMPK) is a serine-threonine kinase present in almost all tissue types. AMPK is comprised of three subunits: the catalytic α-subunit and the regulatory β- and γ-subunits (Figure 1.5) (Carling, 2004). Additionally, a number of isoforms exist for each of the three subunits: α1 and α2 for the α-subunit, β1 and β2 for the β-subunit, and γ1, γ2 and γ3 for the γ-subunit (Viollet *et al.*, 2009). This protein kinase acts as a master regulator

Figure 1.5 AMPK regulation of cellular ATP.

The AMP-activated protein kinase is involved in regulating levels of cellular ATP. The structure of this enzyme is heterotrimeric with two regulatory subunits (β and γ) and a catalytic subunit (α). The binding of AMP induces phosphorylation of the α-subunit, which then increases events leading to ATP synthesis and inhibits events involving ATP consumption.


AMP
β
γ
α
P
ATP Synthesis
ATP Consumption

of cellular energy homeostasis as AMPK acts to induce ATP synthesis when activated by increasing levels of ADP (Bohensky *et al.*, 2009; Steinberg & Kemp, 2009; Bohensky *et al.*, 2010). Moreover, in many cell types, AMPK has been shown to inhibit the activity of anabolic pathways and to activate less energy demanding catabolic pathways (Carling, 2004; Steinberg & Kemp, 2009; Bohensky *et al.*, 2010).

The process leading to AMPK activation is not completely understood, however it is thought to be accomplished in part by the binding of AMP, which results in phosphorylation of the catalytic subunit (Hardie *et al.*, 1998). AMPK activation induces a number of downstream events involved in the synthesis of cellular ATP in order to replenish its depleted supply. The primary action of AMPK is to induce pathways that result in an increased uptake of glucose, in part by the translocation of the GLUT4 receptor to the plasma membrane (Holmes *et al.*, 1999; Murgia *et al.*, 2009). Another major role of AMPK is to induce fatty acid oxidation, which aids in generating a substantial amount of ATP (Yamauchi *et al.*, 2002). AMPK further aids in enhancing cellular ATP stores by decreasing processes that involve ATP consumption. Fittingly, AMPK has been shown to inhibit the activity of the enzymes acetyl-CoA carboxylace and 3-hydroxy-3-methyl-CoA reductase which are involved in the energy demanding processes of fatty acid synthesis and cholesterol synthesis, respectively (Hardie *et al.*, 1998).

Animal models have become central in elucidating the physiological role that this kinase plays in many tissues. Most studies involving these knockout mouse models have focused on aspects of glucose metabolism (Viollet *et al.*, 2009). Whole-body knockouts of each the α1- and α2-subunits have been generated and reveal that

these isoforms of the catalytic subunit have distinct physiological roles. Surprisingly, the AMPKα1$^{-/-}$ mice displayed no changes in the aspects of glucose metabolism studied (Viollet *et al.*, 2003). The most noted features of the AMPKα2$^{-/-}$ mice were glucose intolerance, both decreased insulin secretion and insulin resistance, and problems with glycogen synthesis in skeletal muscle (Viollet *et al.*, 2003).

Recently, evidence has suggested that AMPK plays a role in cartilage tissue. However, the exact role of AMPK in cartilage homeostasis remains uncertain, as evidence has suggested both a role in protecting against ECM degeneration and conversely also a potential role in cartilage catabolism. AMPK has been found to be involved in activating cell autophagy, a protective mechanism of chondrocytes that is lost during incidences of OA (Bohensky *et al.*, 2010; Carames *et al.*, 2010). Very recent evidence has also demonstrated that AMPK may be involved in decreasing matrix degeneration by the catabolic factors, IL-1 and TNFα (Terkeltaub *et al.*, 2011). However, activation of AMPK is increased during OA, which is thought to be in response to decreased ATP due to the presence of reactive oxygen species such as nitric oxide (NO) (Fermor *et al.*, 2010).

1.5 Objectives and Hypothesis

Based on the limited amount of information on the role of these pathways in chondrocyte biology, **we hypothesize that the AMPK and PPARδ pathways each control extracellular matrix turnover in cartilage by altering chondrocyte gene**

expression. To date minimal research has been conducted in order to determine the specific role that each of these pathways have in cartilage tissue.

The specific objectives of this research project are to:

1) Determine effects of the AMPK and PPARδ pathways on chondrocyte gene expression in cell culture.

2) Investigate effects of the AMPK and PPARδ pathways on matrix homeostasis in articular cartilage using an *ex vivo* knee joint organ culture.

2.0 MATERIALS AND METHODS

2.1 Animals

CD1 mice of various ages were purchased from Charles River Laboratories and were sacrificed for experimentation by carbon dioxide asphyxiation. The use of animals for experimentation was approved by Animal Care and Veterinary Services at the University of Western Ontario.

2.2 Primary Chondrocyte Isolation

Embryos were dissected from timed-pregnant CD1 mice at embryonic day 15.5. Forelimb bones (humerus, radius, ulna) were dissected and placed in organ culture media overnight. Organ culture media consists of Modified Eagle's Medium (MEM, Invitrogen), Bovine Serum Albumin (BSA, Fisher), ascorbic acid, β-glycerophosphate, L-glutamine and Pen-strep (Invitrogen). The following day, cells were isolated through digestion with Trypsin-EDTA (Invitrogen) for 15 minutes at 37°C, followed by digestion with Collagenase P (3mg/ml, Roche Applied Science) in Dulbecco's modified eagle's medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) for 2 hours at 37°C. Subsequently, cells were passed through a 40μm cell strainer to remove portions of mineralized bone that remain undigested. Cells were pelleted by centrifugation at 1000 rpm for 5 minutes and the resulting pellet was re-suspended in cell culture media consisting of 55% F12, 35% DMEM, 10% FBS, 1.25% Pen-strep, 1.25 % L-glutamine (Invitrogen). Cells were counted, diluted and plated according to their experimental use.

2.3 Cell Culture

On the day prior to treatment, primary chondrocytes were placed in cell culture media supplemented with 1% FBS in order to assure that changes occurring could be attributed to the treatment. Cells were treated with either an AMPK agonist (AICAR, Sigma Scientific) or a PPARδ agonist (GW1516, Alexis Biochemicals) diluted in the 1% FBS cell culture media. Agonists were diluted at varying concentrations (0.01 μM, 0.1 μM or 1 μM) and cells were treated for 48 hours. Vehicle controls for experimentation were cells treated with dH_2O or dimethyl suloxide (DMSO) for AICAR and GW1516, respectively.

2.4 Real-Time Polymerase Chain Reaction

Primary chondrocytes were plated at a density of 500,000 cells per well on 6-well plates (Nunc) for RNA isolation. Following the treatment period, total RNA was isolated from cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and diluted to 30ng/μl for analysis by Real-Time Polymerase Chain Reaction (PCR).

Real-Time PCR was performed using One-Step RT qPCR Master Mix kit and TaqMan Gene Expression Assays (Applied Biosystems) for Aggrecan (*Acan*), Type II Collagen (*Col2a1*), Sox9 (*Sox9*), MMP13 (*Mmp13*), ADAMTS-5 (*Adamts5*), ADAMTS-1 (*Adamts1*), ADAMTS-2 (*Adamts2*), and ADAMTS-7 (*Adamts7*) ADAMTS-12 (*Adamts12*), MMP2 (*Mmp2*), MMP3 (*Mmp3*), TIMP3 (*Timp3*), HtrA1

(*Htra1*) and Lipoprotein lipase (*Lpl*). Real-Time PCR analyses included 40 cycles on the ABI Prism 7900 HT sequence detector (PrismElmer Life Sciences). Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Analyses for Real-Time PCR were performed in triplicate for three independent experimental trials and data is expressed relative to vehicle controls. Relative gene expression for each gene of interest was calculated using the delta-delta Ct method (Livak & Schmittgen, 2001).

2.5 MTT Assay

Cell numbers present in culture conditions were examined by MTT assay. A Cell Proliferation Kit I (Roche Applied Science) was used for this assay according to the manufacturer's instructions. Results from our lab have previously validated this assay as an indicator of chondrocyte cell number (Halawani *et al.*, 2004; Wang *et al.*, 2004). Primary chondrocytes were plated in triplicate at a density of 10,000 cells per well in a 96-well plate (Falcon) and cells were treated as described above. After 48 hours of treatment, cells were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid (MTT labeling reagent) for 4 hours at 37°C. Following the incubation period, cells were solubilized overnight and absorbance was read at 550-600 nm.

2.6 Palmitate Oxidation Assay

Primary chondrocytes were plated in duplicate at a density of 160,000 cells per well on 24-well plates (Nunc) for use in palmitate oxidation assay. Cells were

treated as described above. This assay was implemented in order to determine the extent of pathway activation following drug treatment in culture, as each pathway of interest is known to be involved in fatty acid oxidation (Yamauchi *et al.*, 2002; Wan *et al.*, 2010).

Cells were incubated for 1 hour with [9,10, ^{3}H] palmitate (PerkinElmer) at 37°C and the assay was performed as previously described (Huss *et al.*, 2004). The extent of radioactivity in each sample was measured by collecting media and phosphate buffered saline (PBS) wash of cells following the incubation period. Prior to measuring radioactivity, protein present in the collected sample was precipitated by incubation with trichloroacetic acid (TCA, Sigma-Aldrich) for 30 minutes followed by centrifugation at 2200 rpm for 10 minutes at 4°C. Following the spin, the aqueous portion was collected, washed with hexane to extract any unreacted fatty acids (x4), and radioactivity was measured using a Beckman Coulter LS6500 Multipurpose Scintillation Counter.

The amount of radioactivity was normalized to the total protein extracted from each sample. Total protein was extracted from each sample by solubilizing cells with 1N NaOH (Sigma-Aldrich) for 30 minutes at 37°C, followed by neutralization with 1N HCl (Sigma-Aldrich). Protein extracted was quantified by bicinchoninic acid (BCA) assay (Smith *et al.*, 1985).

2.7 Explant Culture

Knee joints from 10-week-old male CD1 wild type mice were isolated and

treated in organ culture. Dissected knee joints were placed in organ culture media and allowed to recover overnight. Both left and right intact knee joints were treated the following day with the same compounds as in cell culture (AICAR and GW1516). Knee joints were treated every 2 days for a total of 6 days, with concentrations of 0.01 μM, 0.1 μM, 1 μM or 10 μM of AICAR and GW1516 diluted in organ culture media. Knee joints treated with dH_20 or DMSO served as vehicle controls for AICAR and GW1516, respectively. Following organ culture conditions, knee joints were fixed in 4% paraformaldehyde (PFA) for 24 hours. Samples were decalcified for 1 week in a 5% EDTA solution (pH 7.0), following which they were sent for processing and paraffin embedding at the Molecular Pathology Facility at the Robarts Research Institute (London, Ontario). Samples were subsequently sectioned for histological analyses.

2.8 Histological Stains

Paraffin sections were de-waxed in xylene and then re-hydrated for staining in a series of graded ethanol solutions (100%, 100%, 95%, 70%), with the final step being water. Sections were stained for proteoglycan content with 0.1% Safranin O and counterstained with 0.01% Fast Green by standard protocols (Wang *et al.*, 2004). Additional sections were stained with Picrosirius Red by standard protocols to visualize fibrillar collagen content (Puchtler *et al.*, 1973; Junqueira *et al.*, 1979).

2.9 Immunohistochemistry

Immunohistochemistry (IHC) was performed on organ culture sections using

primary antibodies against aggrecan neoepitopes and Type II Collagen neoepitopes (donated by John Mort, Shriners Hospital, Montreal, QC), as well as type II collagen (Santa Cruz Biotechnology) by standard protocols (Appleton *et al.*, 2007; Ulici *et al.*, 2008).

Sections were de-waxed and re-hydrated as above and then incubated in 3% hydrogen peroxide in methanol for 15 minutes at room temperature. Antigen retrieval was performed by incubating sections in 10 mM sodium citrate (pH 6.0) for 20 minutes at 95°C (neoepitopes) or 0.1% Triton X at room temperature for 12 minutes (type II collagen). Sections were then blocked in 5% goat serum in PBS (neoepitopes) or 5% donkey serum in PBS (type II collagen) for 1 hour at room temperature.

Primary antibodies were diluted in the blocking solution according to their optimized concentrations: 1:300 for aggrecan neoepitopes, 1:250 Type II Collagen neoepitopes, and 1:200 for Type II Collagen. Sections were incubated overnight at 4°C with the primary antibody. The following day, sections were washed in dH_2O and incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (neoepitopes) or HRP-conjugated donkey anti-goat IgG secondary antibody (Type II Collagen) for 1 hour at room temperature (1:200 antibody:PBS). Sections were washed and visualized using diaminobenzidine (DAB) substrate solution (Dako). All sections were counterstained with Weigert's Iron Hematoxylin. Representative images of knee joint sections were taken using a Leica DMRA2 Microscope (Leica Microsystems) and image analyses were performed using OpenLab 4.0.4.

2.10 Statistical Analysis

All data was collected from at least 3 independent experimental trials and is represented as mean ± SEM. Data was analyzed using GraphPad Prism version 4.0a for MacIntosh by a one-way ANOVA and Dunnett post-test ($*p<0.05$). For histological stains and immunohistochemistry protocols, sections were analyzed in duplicate within each of the independent trials.

3.0 RESULTS

3.1 PPARδ activation increases fatty acid oxidation of primary chondrocytes, but does not significantly affect expression of the lipid metabolism gene *Lpl*

The PPARδ pathway has a well-established role in fatty acid oxidation in a number of tissue types (Wan *et al.*, 2010). Therefore, a palmitate oxidation assay was performed in order to determine the extent of pathway activation in primary chondrocytes following a 48-hour treatment period. Analysis involved tritium-labeled palmitate, which gives rise to tritiated water being released as a result of fatty acid oxidation. The extent of radioactivity thus corresponds to the extent of oxidation that occurred. A significant increase in oxidation was observed for all concentrations of GW1516 treatment (0.01 μM, 0.1 μM, 1 μM) (Figure 3.1A).

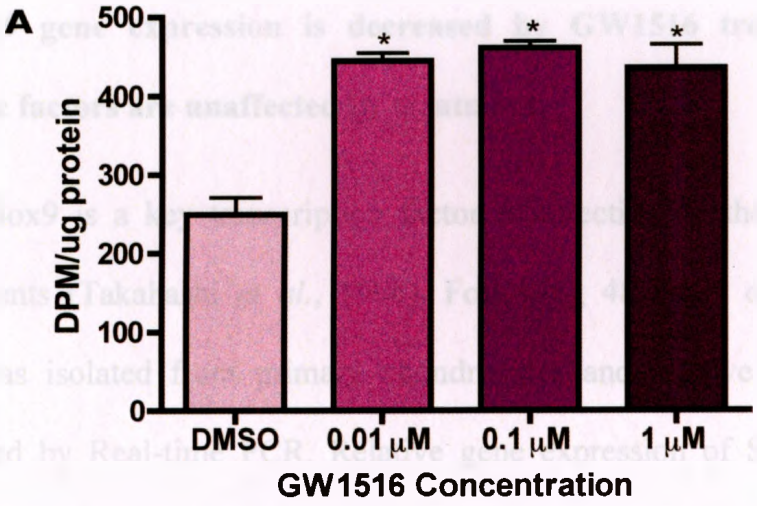
To follow up on this increase in fatty acid oxidation, the gene expression of *Lpl* was analyzed by Real-time PCR. *Lpl* is one of the main genes induced during lipid metabolism (Peterson *et al.*, 1990). Real-time PCR analysis revealed a trend towards increased expression of *Lpl* at GW1516 concentrations of 0.1 μM and 1 μM relative to DMSO control (Figure 3.1B). However, these increases in gene expression were not found to be statistically significant at either of these concentrations.

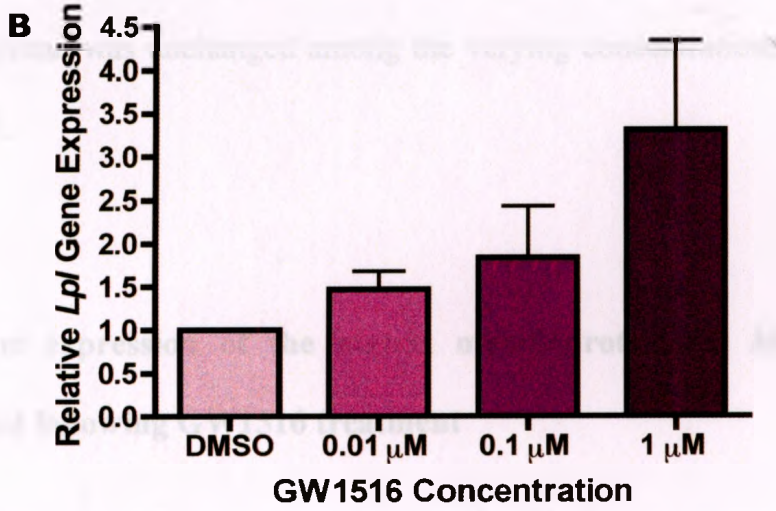
3.2 Primary chondrocyte cell number is unchanged by GW1516 treatment

Chondrocyte cell number in culture was analyzed by MTT assay. This assay has previously been shown to be an accurate measure of chondrocyte cell number (Halawani *et al.*, 2004; Wang *et al.*, 2004). Incubation with the MTT labeling reagent

Figure 3.1 PPARδ agonist treatment increases fatty acid oxidation in culture.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice. Cells were treated in culture with a pharmacological agonist of PPARδ, GW1516, for 48 hours. A serial dilution was performed with concentrations of 0.01 μM, 0.1 μM and 1 μM and cells treated with DMSO served as control. (A) The extent of fatty acid oxidation induced by these treatments was measured using a palmitate oxidation assay (Huss *et al.*, 2004). Cells were incubated with [9,10, ^{3}H-palmitate for 1 hour and the extent of radioactivity was measured in each sample. Data was normalized to total protein in each sample. GW1516 treatment significantly increased fatty acid oxidation at all concentrations of treatment, (n=3). (B) Relative gene expression was measured using Real-time PCR. Relative *Lpl* gene expression was unchanged among treatments although a trend towards increased expression at higher concentrations exists, (n=3)(*$p<0.05$).


A
500
400
300
200
100
0
DPM/ug protein
*
*
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration


B
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative Lpl Gene Expression
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

and subsequent reading of sample absorbance revealed no significant changes in cell number among the GW1516 concentrations (Figure 3.2).

3.3 *Sox9* gene expression is decreased by GW1516 treatment, while other anabolic factors are unaffected by treatment.

Sox9 is a key transcription factor in directing synthesis of cartilage ECM components (Takahashi *et al.*, 1998). Following 48 hours of GW1516 treatment, RNA was isolated from primary chondrocytes and relative gene expression was quantified by Real-time PCR. Relative gene expression of Sox9 was found to be significantly decreased at 1μM of GW1516 treatment (Figure 3.3A). The genes encoding the main structural components of the cartilage ECM, type II collagen and aggrecan, were next analyzed. The relative gene expression of both type II collagen and aggrecan was unchanged among the varying concentrations of GW1516 (Figure 3.3B, C).

3.4 Gene expression of the matrix metalloproteinases *Mmp2* and *Mmp3* is increased following GW1516 treatment

Matrix metalloproteinases are involved in matrix cleavage during normal and pathological conditions. MMPs thus are crucial factors to be analyzed when considering cartilage homeostasis and overall health. In analyzing gene expression of a number of MMPs, it was determined that 0.1 μM of GW1516 significantly

Figure 3.2 PPARδ agonist treatment does not alter cell number in culture.

Primary chondrocytes isolated from the long bones of embryonic day 15 mice were treated with a PPARδ agonist (GW1516) for 48 hours at varying concentrations. Relative cell number in each condition was determined using an MTT reagent with absorbance being measured with a spectrophotometer at 600 nm. Cell number remained unchanged among the varying concentration of GW1516, (n=3 in triplicate)(*$p<0.05$)

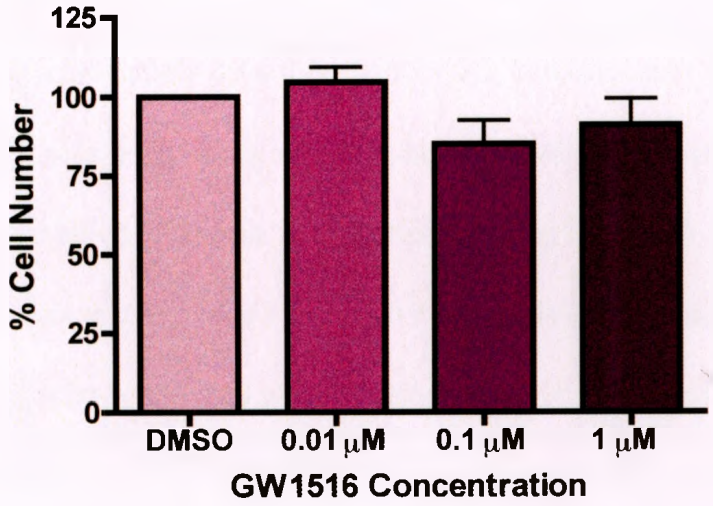
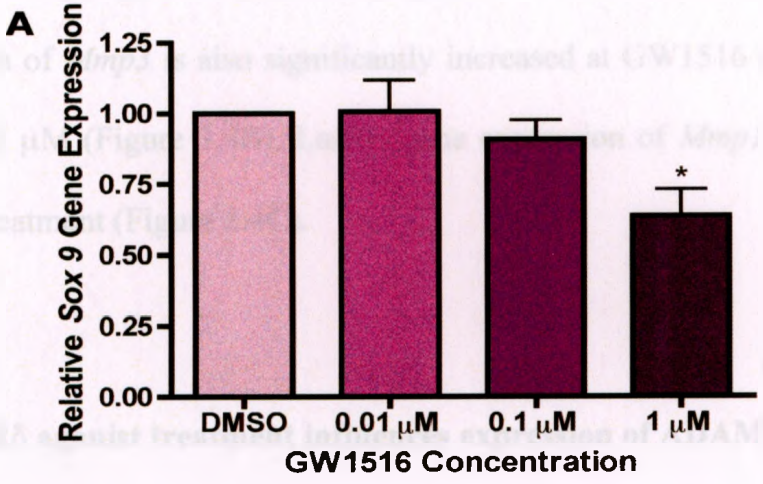
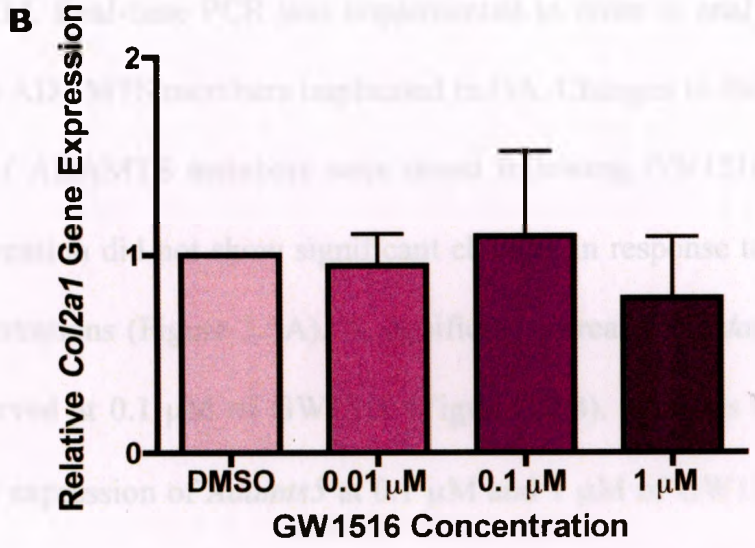
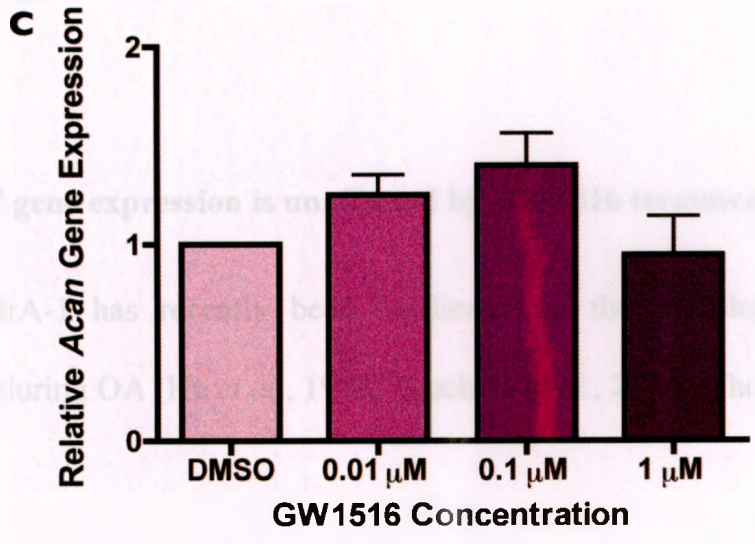

125
100
75
50
25
0
% Cell Number
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

Figure 3.3 PPARδ agonist treatment decreases *Sox9* gene expression but does not influence gene expression of ECM components.

Primary chondrocytes were isolated and treated with a PPARδ agonist (GW1516) at varying concentrations for a 48-hour period. Relative gene expression was quantified using Real-time PCR. (A) Sox9 gene expression was significantly decreased at 1 μM of GW1516. (B) The relative gene expression of Type II collagen was unchanged in cell culture when treated with GW1516. (C) Relative gene expression of Aggrecan was unchanged among the varying concentrations of GW1516 concentrations, (n=3)(*$p<0.05$).


A
Relative Sox 9 Gene Expression
1.25
1.00
0.75
0.50
0.25
0.00
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration


B
Relative Col2a1 Gene Expression
2
1
0
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration


C
Relative Acan Gene Expression
2
1
0
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

increases *Mmp2* gene expression (Figure 3.4A). Additionally, the relative gene expression of *Mmp3* is also significantly increased at GW1516 concentrations of 0.1 μM and 1 μM (Figure 3.4B). Lastly, gene expression of *Mmp13* is unchanged with agonist treatment (Figure 3.4C).

3.5 PPARδ agonist treatment influences expression of ADAMTS genes

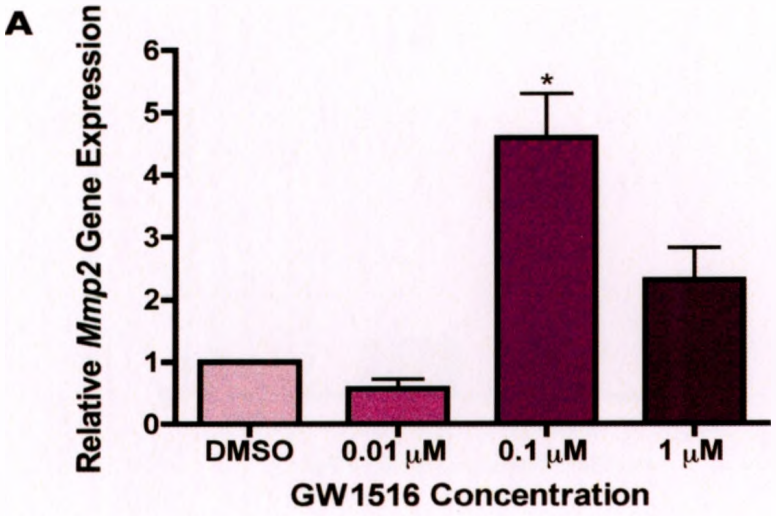
Many members of the ADAMTS family have been shown to cleave aggrecan in the ECM. Real-time PCR was implemented in order to analyze the expression of important ADAMTS members implicated in OA. Changes in the gene expression of a number of ADAMTS members were noted following GW1516 treatment. *Adamts1* gene expression did not show significant changes in response to agonist treatment at all concentrations (Figure 3.5A). A significant increase in *Adamts2* gene expression was observed at 0.1 μM of GW1516 (Figure 3.5B). Analysis revealed significantly increased expression of *Adamts5* at 0.1 μM and 1 μM of GW1516 treatment (Figure 3.5C). The relative gene expression of both *Adamts7* and *Adamts12* are increased at 0.1 μM (Figure 3.6A, B).

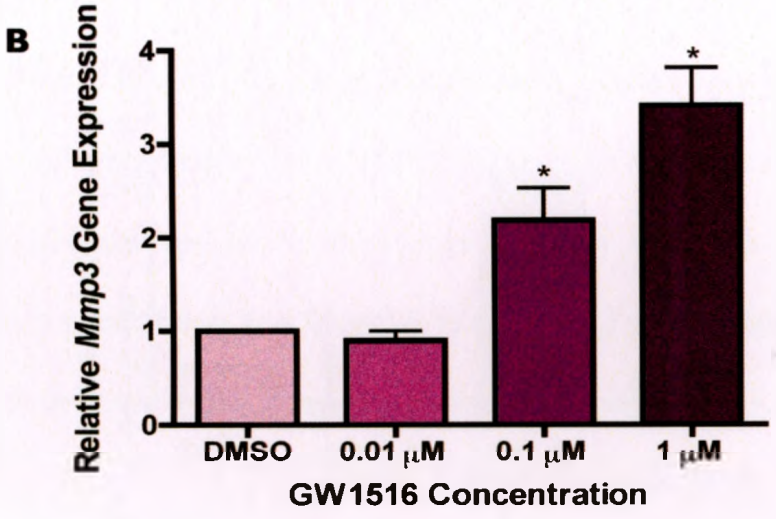
3.6 *Htra1* gene expression is unaffected by GW1516 treatment

HtrA-1 has recently been implicated in the pathological cartilage ECM cleavage during OA (Hu *et al.*, 1998; Tsuchiya *et al.*, 2005). The relative gene

Figure 3.4 PPARδ agonist treatment increases MMP2 and MMP3 gene expression.

Primary chondrocytes were treated with a serial dilution of a PPARδ agonist (GW1516) and relative gene expression was quantified using Real-time PCR. (A) The relative gene expression of *Mmp2* was increased at 0.1μM of GW1516. (B) Gene expression *Mmp3* was significantly increased at GW1516 concentrations of 0.1 μM and 1 μM. (C) The relative gene expression of *Mmp13* was unaffected by agonist treatment, (n=3)(*$p<0.05$).


A
Relative Mmp2 Gene Expression
6
5
4
3
2
1
0
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration


B
Relative Mmp3 Gene Expression
4
3
2
1
0
*
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

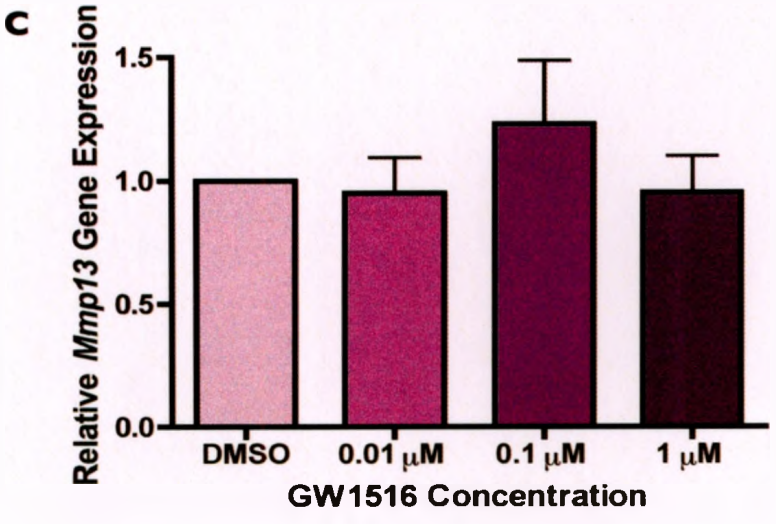
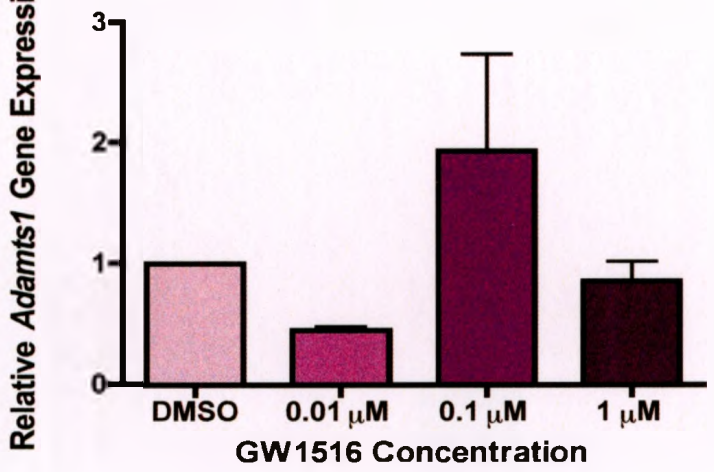

C
Relative Mmp13 Gene Expression
1.5
1.0
0.5
0.0
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

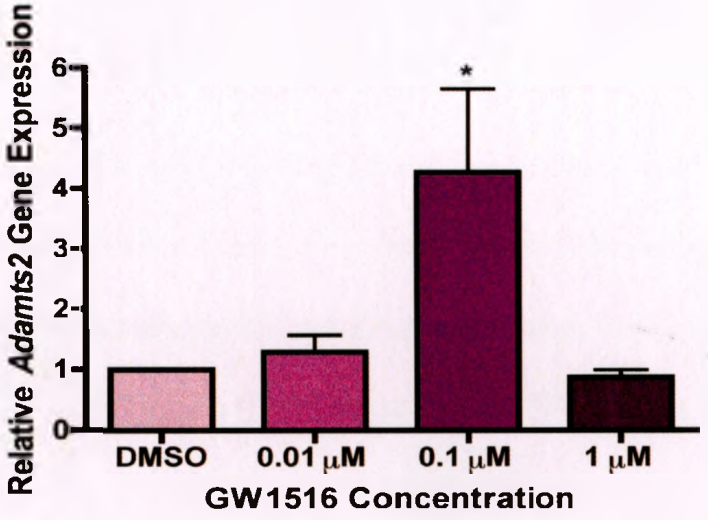
Figure 3.5 PPARδ agonist treatment influences Aggrecanse expression.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice and treated in culture with a PPARδ agonist (GW1516). Relative gene expression was quantified using Real-time PCR. (A) Gene expression of *Adamts1* was unchanged with treatment. (B) The relative gene expression of *Adamts2* was significantly increased at 0.1 μM of GW1516. (C) *Adamts5* gene expression was significantly increased at both 0.1 μM and 1 μM of GW1516 treatment, (n=3) (*$p<0.05$).

A


Relative Adamts1 Gene Expression
3
2
1
0
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

B


Relative Adamts2 Gene Expression
6
5
4
3
2
1
0
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

C

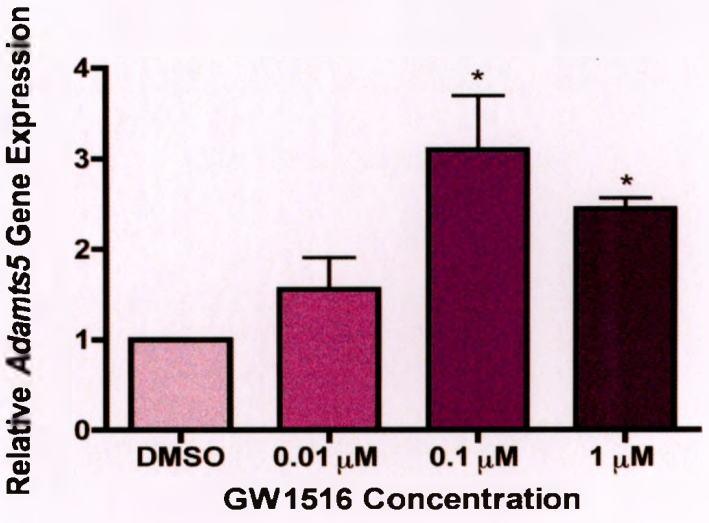
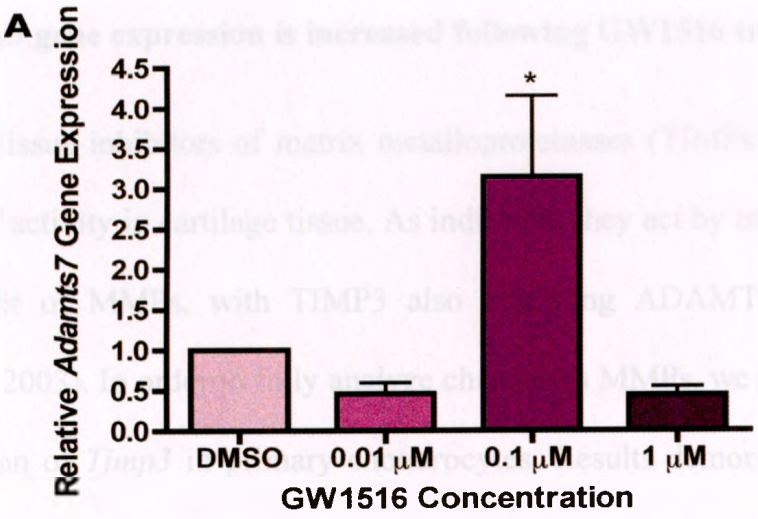
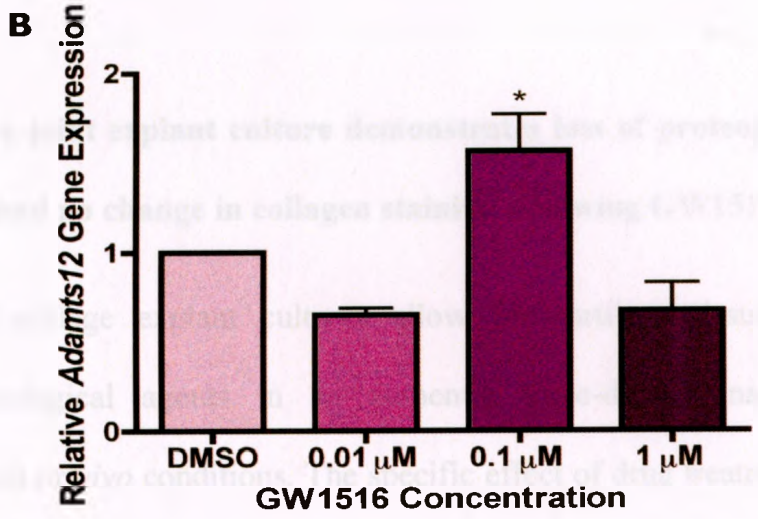

Relative Adamts5 Gene Expression
4
3
2
1
0
*
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

Figure 3.6 PPARδ agonist treatment causes increased expression of *Adamts7* and *Adamts12*.

Primary chondrocytes were isolated and treated with GW1516, a pharmacological agonist of PPARδ, at varying concentrations. Relative gene expression was determined using Real-time PCR. (A), (B) Both *Adamts7* and *Adamts12* displayed significantly increased gene expression at 0.1 μM of GW1516 treatment, (n=3) (*$p<0.05$).


A
Relative Adamts7 Gene Expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration


B
Relative Adamts12 Gene Expression
2
1
0
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

expression of *Htra1* was not significantly changed in primary chondrocyte cultures with all concentrations of GW1516 treatment (Figure 3.7A).

3.7 *Timp3* gene expression is increased following GW1516 treatment.

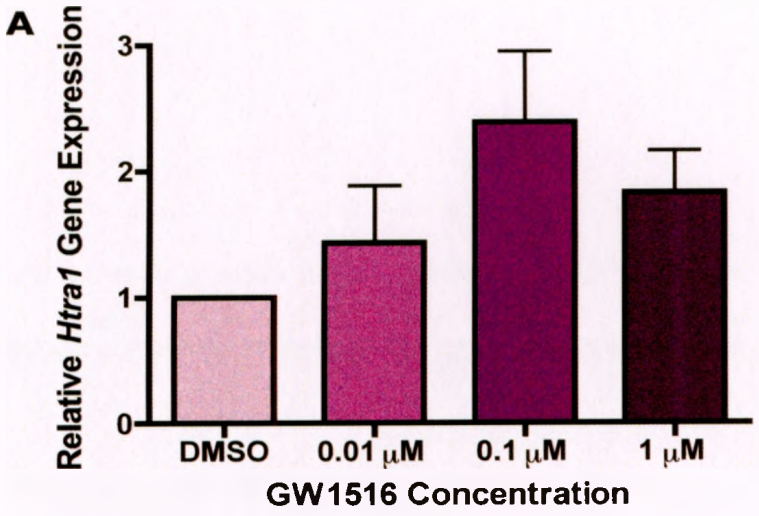
Tissue inhibitors of matrix metalloproteinases (TIMPs) are potent regulators of MMP activity in cartilage tissue. As indicated, they act by inhibiting the activity of a number of MMPs, with TIMP3 also inhibiting ADAMTS enzymes (Visse & Nagase, 2003). In order to fully analyze changes in MMPs, we also analyzed the gene expression of *Timp3* in primary chondrocytes. Results demonstrate that the relative gene expression of *Timp3* was significantly increased at 0.1 μM of GW1516 (Figure 3.7B)

3.8 Knee joint explant culture demonstrates loss of proteoglycan staining in the matrix and no change in collagen staining following GW1516 treatment

Cartilage explant cultures allow for cartilage tissue to be treated with pharmacological agents in an authentic three-dimensional context but under controlled *ex vivo* conditions. The specific effect of drug treatment on cartilage tissue can be determined by this method. Cartilage tissue treated with GW1516 displayed a trend towards decreased proteoglycan staining in the cartilage ECM when stained with Safranin O/Fast Green (Figure 3.8A). However, Picrosirius Red staining of tissue sections revealed no apparent changes in collagen content (Figure 3.8B). In

Figure 3.7 Treatment with a PPARδ agonist increases *Timp3* gene expression.

Gene expression of primary chondrocytes treated with a PPARδ agonist (GW1516) was measured using Real-time PCR. Primary chondrocytes were isolated from the long bones of embryonic day 15 mice. (A) Gene expression of *Htra1* was unaffected among the varying concentrations of treatment. (B) A significant increase in the gene expression of *Timp3* was observed at 0.1 μM of GW1516, (n=3)(*$p<0.05$).


A
Relative Htra1 Gene Expression
3
2
1
0
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

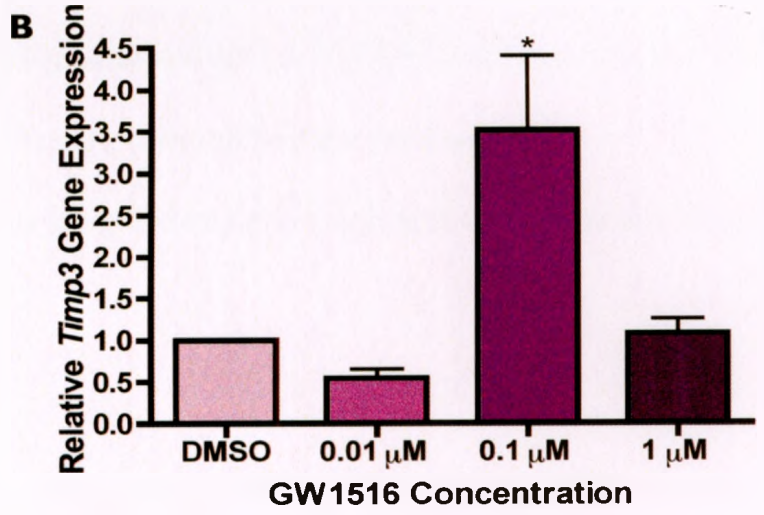
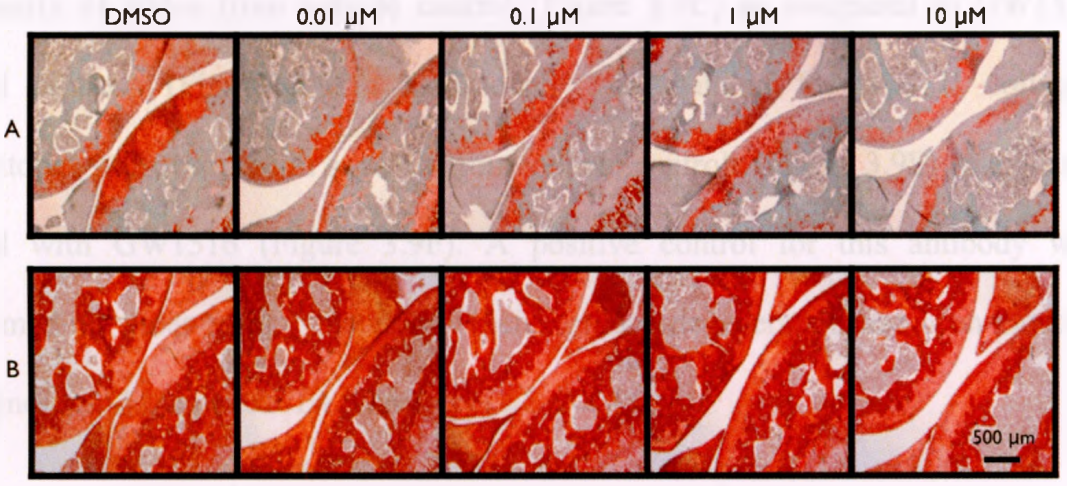

B
Relative Timp3 Gene Expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
DMSO
0.01 μM
0.1 μM
1 μM
GW1516 Concentration

Figure 3.8 Treatment with a PPARδ agonist in organ culture shows a trend towards decreased proteoglycan content in articular cartilage extracellular matrix, while no affect on collagen content becomes apparent.

Adult wild-type knee joints were dissected from mice and treated with a PPARδ agonist (GW1516) in organ culture for 1 week. Tissues were prepared for histology through standard protocols of decalcified paraffin sections. (A) Section stained with Safranin O/Fast Green for proteoglycan content displayed decreased matrix staining, most apparent at 10 μM of GW1516, (n=3). (B) Sections were additionally stained with Picrosirius Red for collagen content, with no conclusive changes being apparent, (n=3).


DMSO
0.01 µM
0.1 µM
1 µM
10 µM
A
B
500 µm

addition, immunohistochemistry using antibodies specific for cleaved fragments of aggrecan demonstrated an increase in neoepitope staining in sections from explants that had been treated with GW1516 (Figure 3.9B), as compared to vehicle control (Figure 3.9A)

To further examine the collagen component of the ECM, IHC protocols were implemented using antibodies for both type II collagen and cleaved collagen fragments. These results revealed no apparent change in type II collagen staining in the matrix of slides from vehicle control (Figure 3.9C) as compared to GW1516 treated sections (Figure 3.9D). Additionally, no staining for type II collagen neoepitopes was observed in sections from vehicle controls (Figure 3.9E) or explants treated with GW1516 (Figure 3.9F). A positive control for this antibody was performed on slides from explants in which cartilage degeneration had been induced by retinoic acid (Appendix B) (Glasson *et al.*, 2004).

3.9 Fatty acid oxidation is unaffected by AMPK agonist treatment

The AMPK pathway is a known mediator of oxidative metabolism and thus primary chondrocytes were examined for extent of fatty acid oxidation following treatment with the AMPK agonist AICAR (Viollet *et al.*, 2009). The extent of fatty acid oxidation, as measured by the amount of radioactivity in the resulting sample, was unchanged for all treatments of AICAR used for experimentation (Figure 3.10A). However, the relative gene expression of the lipid metabolism gene *Lpl* was significantly increased with a treatment of 0.01 μM of AICAR (Figure 3.10B)

Figure 3.9 PPARδ agonist treatment increases Aggrecan neopitope staining, does not affect type II collagen staining in the ECM.

Knee joints from adult wild-type mice were dissected, treated in organ culture for 1 week with a PPARδ agonist (GW1516) and prepared for histological analysis. Knee joints treated with DMSO served as controls. Sections were analyzed by standard immunohistochemistry protocols with an antibody for Aggrecan fragments, type II collagen and type II collagen fragments. An increase in staining for Aggrecan neoepitopes was apparent for sections treated with GW1516 (B) as compared to controls (A). Sections treated with GW1516 did not reveal any changes in type II collagen staining in the ECM (D) when compared to vehicle control (C). No staining for type II collagen cleavage fragments was apparent in sections treated with GW1516 (F) when compared to control sections (E), (n=3).

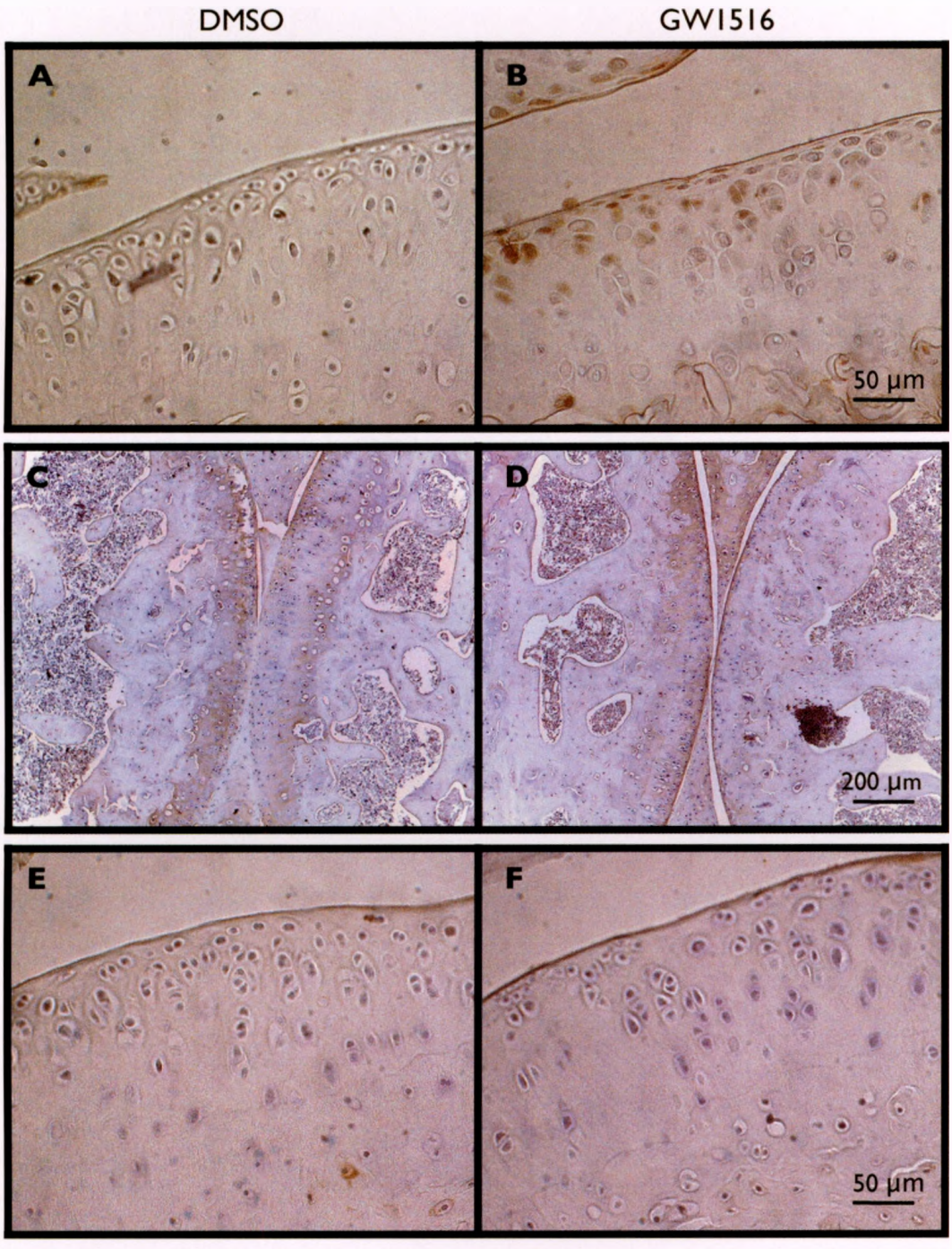
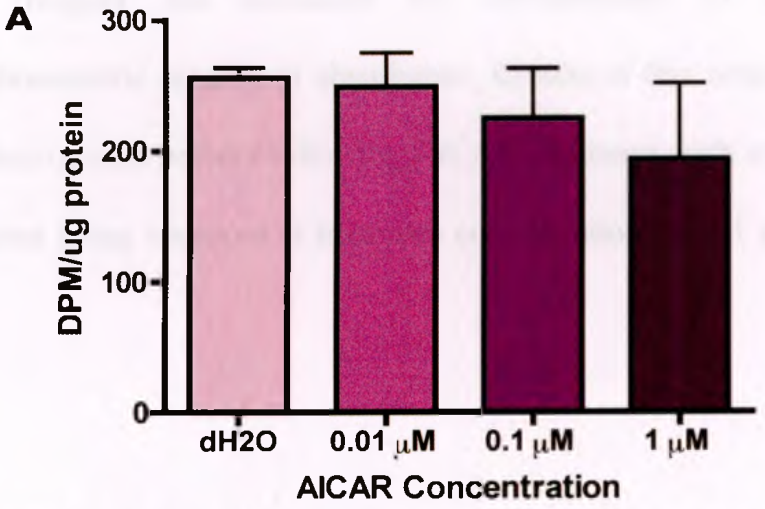
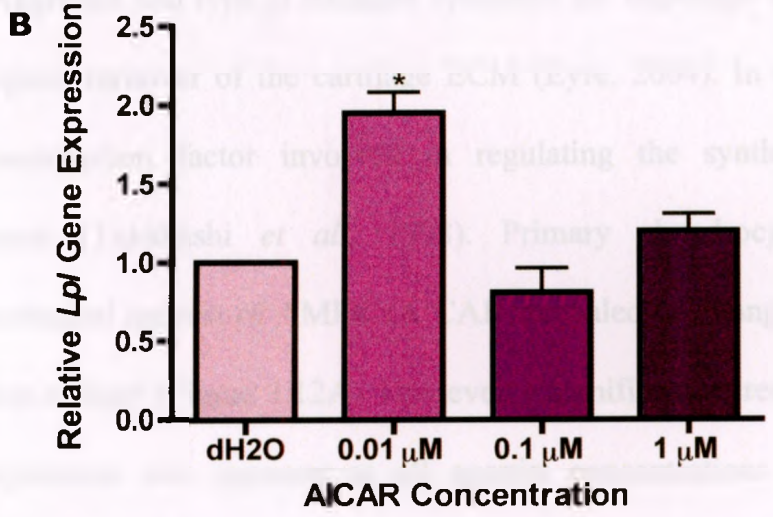

DMSO
GW1516
A
B
50 µm
C
D
200 µm
E
F
50 µm

Figure 3.10 AMPK agonist treatment does not affect fatty acid oxidation in culture.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice. Cells were treated in culture with a pharmacological agonist of AMPK, AICAR, for 48 hours. A serial dilution was performed with concentrations of 0.01 μM 0.1 μM and 1 μM. Cells treated with ddH_2O served as control. (A) The extent of fatty acid oxidation induced by these treatments was measured using a palmitate oxidation assay. Cells were incubated with [9,10, ^{3}H-palmitate for 1 hour and the extent of radioactivity was measured in each sample. Data was normalized to total protein in each sample. AICAR treatment did not affect extent of oxidation in culture at any of the experimental concentrations, (n=3). (B) Relative gene expression was measured using Real-time PCR revealing that relative *Lpl* gene expression is increased at 0.01 μM of AICAR, (n=3)(*$p<0.05$).


A
300
200
100
0
DPM/ug protein
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


B
2.5
2.0
1.5
1.0
0.5
0.0
*
Relative Lpl Gene Expression
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

3.10 AICAR treatment significantly decreases chondrocyte cell number

In order to determine the overall effect of AICAR treatment on cultured chondrocytes, an MTT assay was performed. Cells were incubated with an MTT labeling reagent and measured for incorporation of this reagent by a spectrophotometric reading of absorbance. Results of this assay revealed changes in the number of cells present following AICAR treatment, with significant decreases in cell number being observed at treatment concentrations of 0.1 μM and 1 μM (Figure 3.11).

3.11 AMPK agonist treatment does not change *Sox9* or aggrecan gene expression, but increases expression of type II collagen.

Aggrecan and type II collagen synthesis are important events in maintaining physiological turnover of the cartilage ECM (Eyre, 2004). In addition, Sox9 is the main transcription factor involved in regulating the synthesis of these ECM components (Takahashi *et al.*, 1998). Primary chondrocytes treated with a pharmacological agonist of AMPK (AICAR) revealed no change in the relative gene expression of *Sox9* (Figure 3.12A). However, a significant increase in type II collagen gene expression was apparent at all agonist concentrations (Figure 3.12B). No significant change was noted for aggrecan gene expression (Figure 3.12C).

Figure 3.11 AMPK agonist treatment causes decreased cell number in culture.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice and treated with an AMPK agonist (AICAR) for 48 hours at varying concentrations. Relative cell number in each condition was determined using an MTT reagent with absorbance being measured with a spectrophotometer at 600 nm. AICAR treatments of 0.01 μM and 1 μM significantly decreased cell number relative to vehicle controls, (n=3 in triplicate)(*$p<0.05$).

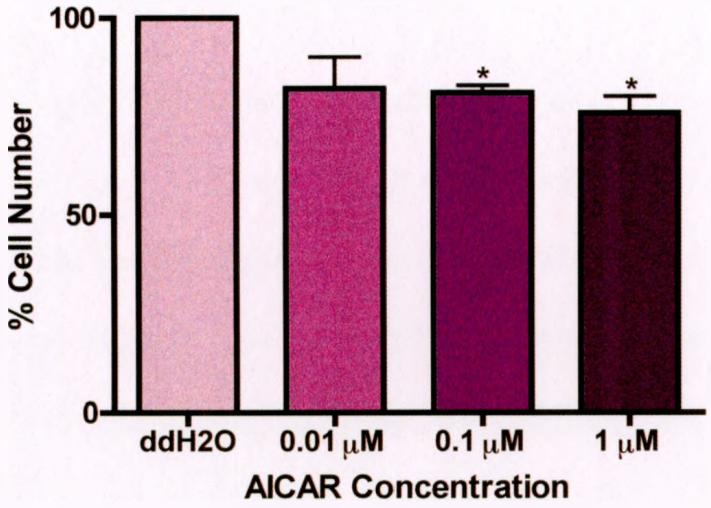
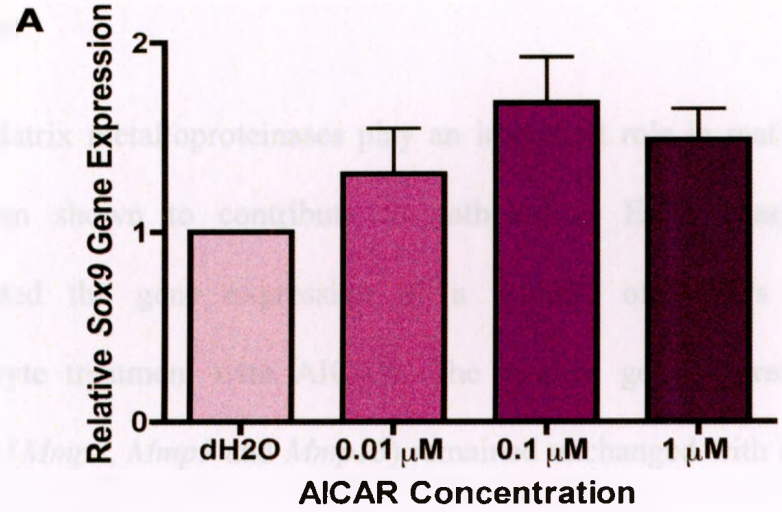
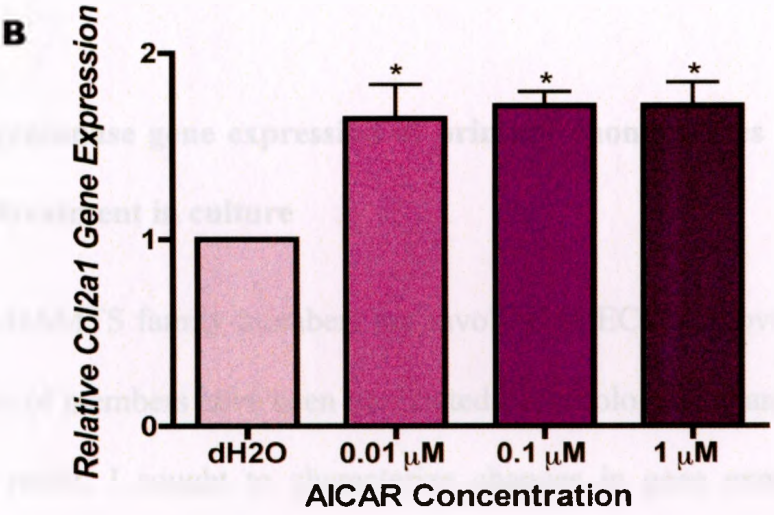
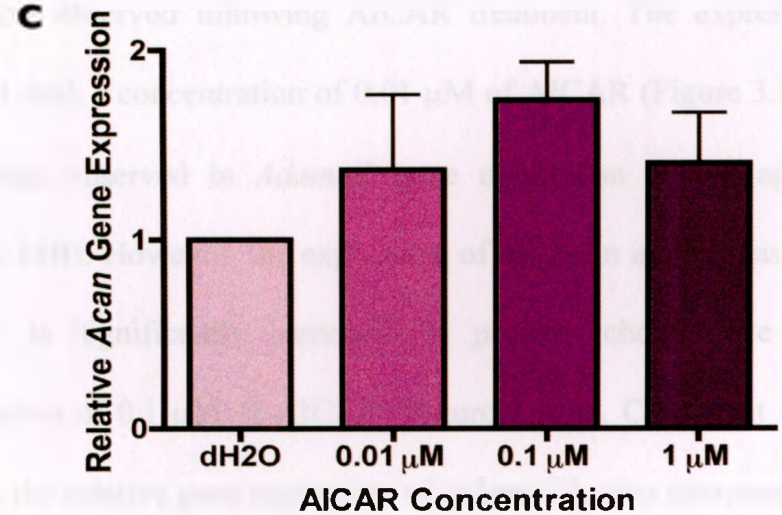

100
50
0
% Cell Number
*
*
ddH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

Figure 3.12 AMPK agonist treatment increases type II collagen gene expression.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice following which they were treated with an AMPK agonist (AICAR) at varying concentrations for a 48-hour period. Relative gene expression was quantified using Real-time PCR. (A) Relative gene expression of *Sox9* was unchanged among the varying concentrations of AICAR. (B) Type II collagen was increased at each concentration of AICAR treatment. (C) Relative gene expression of Aggrecan was unchanged among varying concentrations of AICAR, ($n=3$)($^*p<0.05$).


A
Relative Sox9 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


B
Relative Col2a1 Gene Expression
2
1
0
*
*
*
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


C
Relative Acan Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

3.12 Matrix metalloproteinase gene expression is unchanged following AICAR treatment

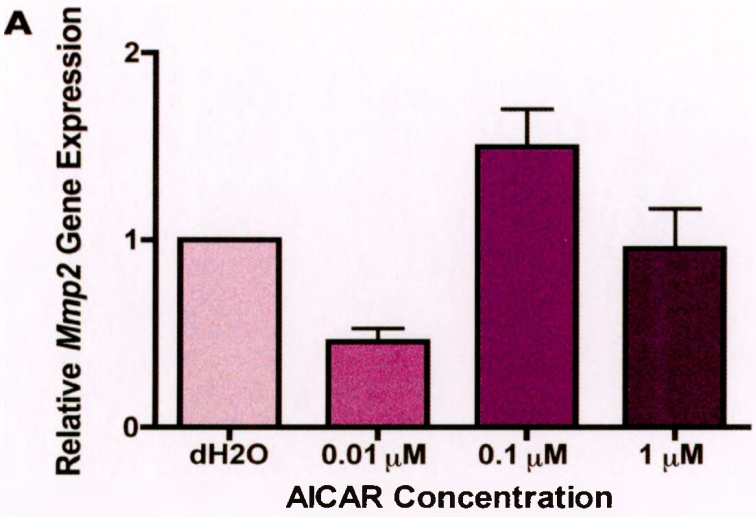
Matrix metalloproteinases play an important role in matrix homeostasis, but have been shown to contribute to pathological ECM changes during OA. I investigated the gene expression of a number of MMPs following primary chondrocyte treatment with AICAR. The relative gene expression of all MMPs analyzed (*Mmp2*, *Mmp3* and *Mmp13*) remained unchanged with all concentrations of agonist treatment (Figure 3.13A, B, C).

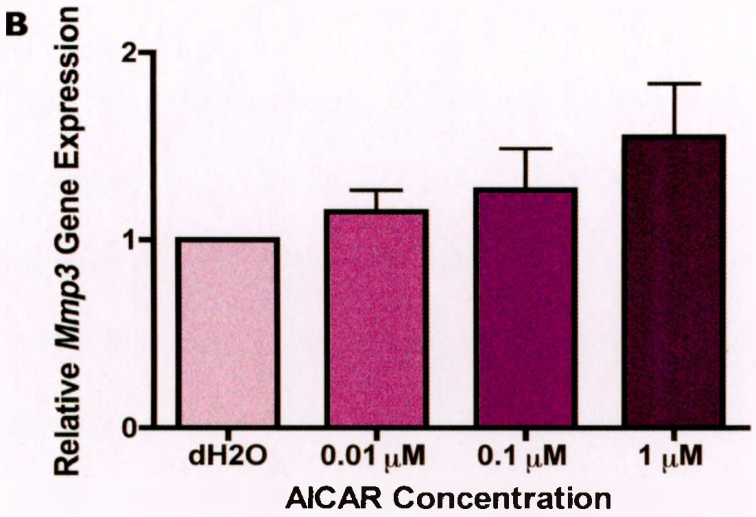
3.13 Aggrecanase gene expression of primary chondrocytes is altered following AICAR treatment in culture

ADAMTS family members are involved in ECM turnover in cartilage tissue. A number of members have been implicated in pathological changes occurring during OA. As result, I sought to characterize changes in gene expression occurring in cultures of primary chondrocytes. Changes in gene expression of a number of A*damts* genes were observed following AICAR treatment. The expression of *Adamts1* is decreased with a concentration of 0.01 μM of AICAR (Figure 3.14A). No significant change was observed in *Adamts2* gene expression at any agonist concentration (Figure 3.14B). However, the expression of the main aggrecanase in cartilage tissue, *Adamts5*, is significantly increased in primary chondrocyte cell cultures at a concentration of 0.1 μM of AICAR (Figure 3.14C). Consistent with the decrease in *Adamts1*, the relative gene expression of *Adamts7* is also decreased at an AICAR

Figure 3.13 AMPK agonist treatment does not influence matrix metalloproteinase gene expression.

Primary chondrocytes isolated from the long bones of embryonic day 15 mice were treated in culture with varying concentrations of an AMPK agonist (AICAR) for 48 hours. Real-time PCR was used to determine relative gene expression. (A), (B), (C) The gene expression of *Mmp2*, *Mmp3*, *Mmp13* was not unaffected by AICAR treatment, (n=3)(*$p<0.05$).


A
Relative Mmp2 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


B
Relative Mmp3 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

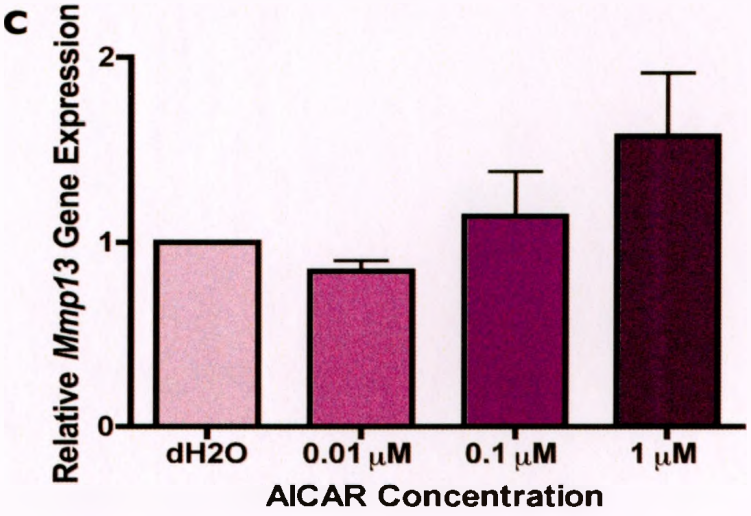
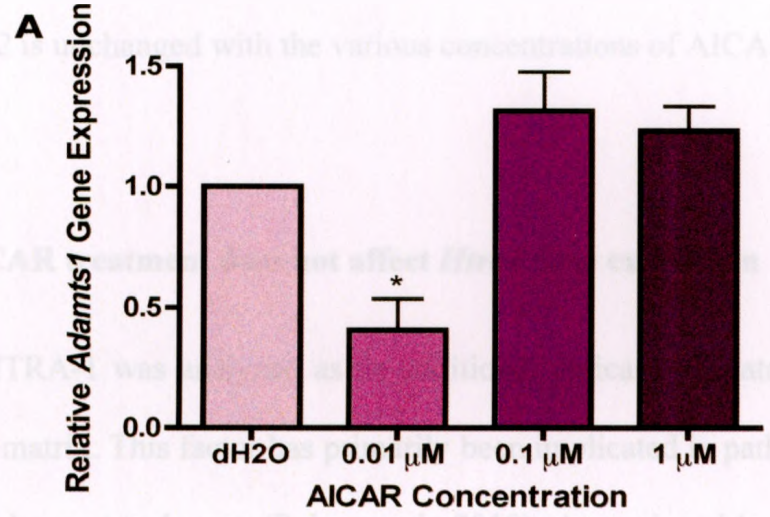
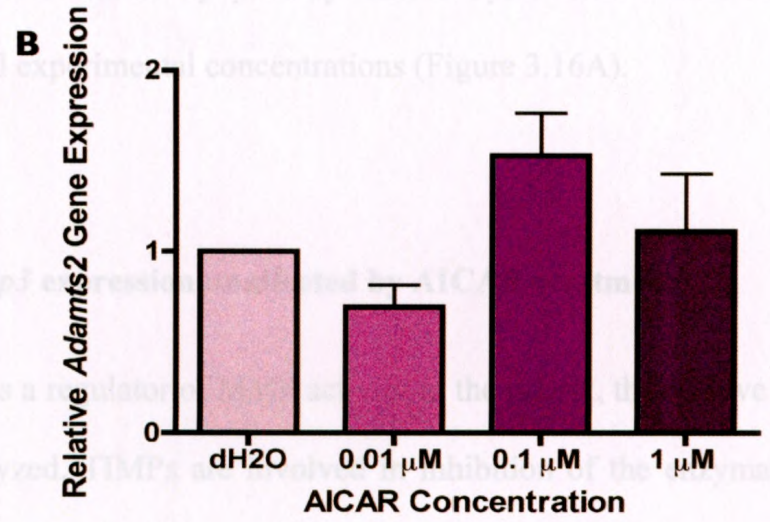
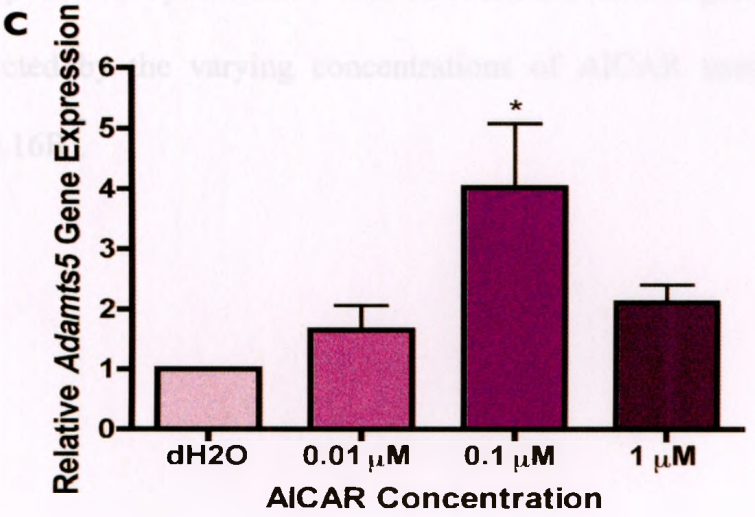

C
Relative Mmp13 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

Figure 3.14 AICAR treatment influences *Adamts1* and *Adamts5* expression.

Primary chondrocytes were isolated from the long bones of embryonic day 15 mice and treated in culture with an AMPK agonist (AICAR). Relative gene expression was quantified using Real-time PCR. (A) *Adamts1* gene expression was significantly decreased at 0.01 μM of AICAR. (B) The relative gene expression of *Adamts2* was unchanged among the various treatments of AICAR. (C) The gene expression of *Adamts5* was significantly increased at 0.1 μM treatment of AICAR, (n=3)(*$p<0.05$).


A
Relative Adamts1 Gene Expression
1.5
1.0
0.5
0.0
*
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


B
Relative Adamts2 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


C
Relative Adamts5 Gene Expression
6
5
4
3
2
1
0
*
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

treatment concentration of 0.01 μM (Figure 3.15A). Lastly, the expression of *Adamts12* is unchanged with the various concentrations of AICAR (Figure 3.15B).

3.14 AICAR treatment does not affect *Htra1* gene expression

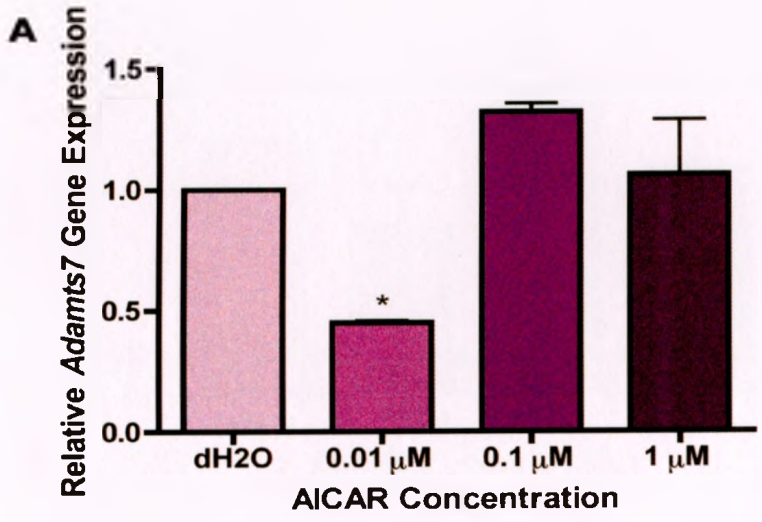
HTRA-1 was analyzed as an additional indicator of catabolic activity in the cartilage matrix. This factor has primarily been implicated in pathological cleavage of extracellular proteoglycans (Polur *et al.*, 2010). As analyzed by Real-time PCR, the expression of *Htra1* by primary chondrocytes treated with AICAR is unchanged among all experimental concentrations (Figure 3.16A).

3.15 *Timp3* expression unaffected by AICAR treatment

As a regulator of MMP activity in the matrix, the relative expression of *Timp3* was analyzed. TIMPs are involved in inhibition of the enzymatic activity of MMP and ADAMTS and thus act as a regulator of cartilage turnover (Cawston *et al.*, 1999). In primary chondrocytes treated with AICAR, the relative gene expression of *Timp3* is unaffected by the varying concentrations of AICAR used for experimentation (Figure 3.16B).

Figure 3.15 AMPK agonist treatment decreases relative gene expression of *Adamts7*.

Primary chondrocytes, isolated from the long bones of embryonic day 15 mice, and treated in culture with an AMPK agonist (AICAR). Relative gene expression was quantified using Real-time PCR. (A) *Adamts7* gene expression was significantly decreased at the 0.01 μM treatment of AICAR. (B) The relative gene expression of *Adamts12* remained unchanged among the varying concentrations of AICAR, (n=3)(*$p<0.05$).


A
Relative Adamts7 Gene Expression
1.5
1.0
0.5
0.0
*
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

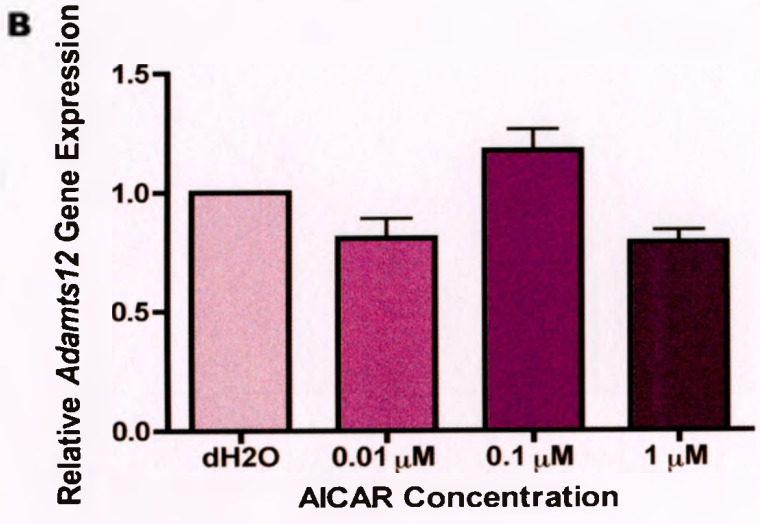
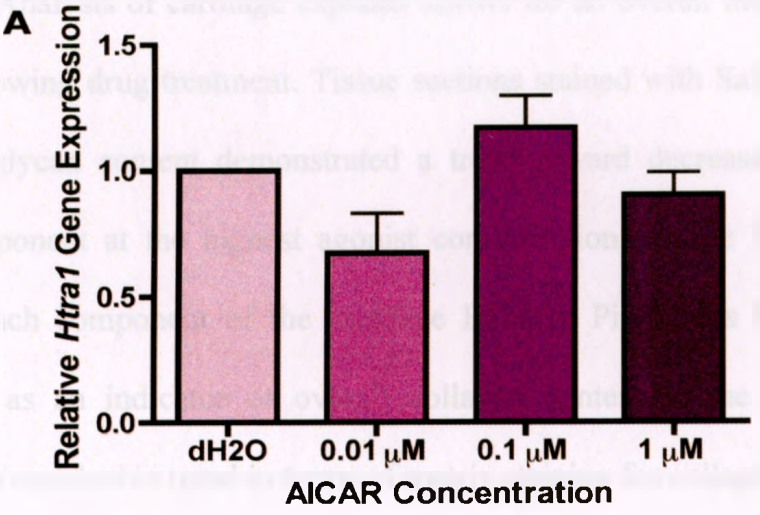
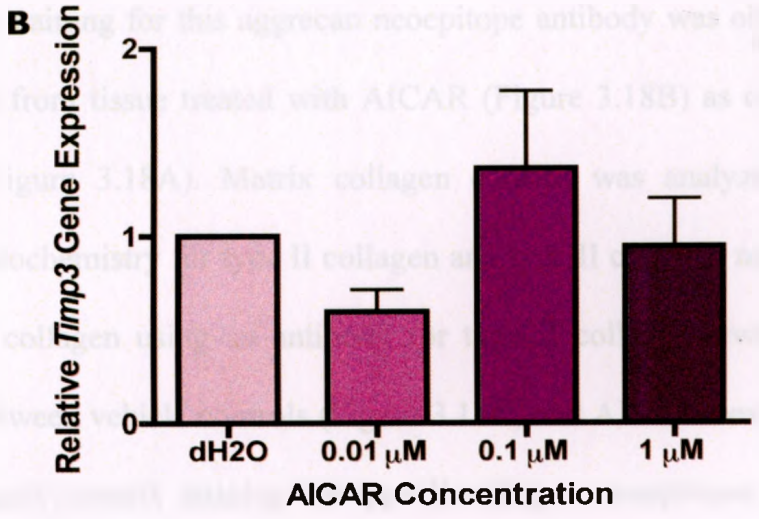

B
Relative Adamts12 Gene Expression
1.5
1.0
0.5
0.0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

Figure 3.16 Treatment with an AMPK agonist does not affect *Htra1* or *Timp3* gene expression.

Gene expression of primary chondrocytes treated with an AMPK agonist (AICAR) was measured using Real-time PCR. Primary chondrocytes were isolated from the long bones of embryonic day 15 mice. (A) Gene expression of *Htra1* was unchanged among the various concentrations. (B) The gene expression of *Timp3* was unaffected by agonist treatment, (n=3)(*$p<0.05$).


A
Relative Htra1 Gene Expression
1.5
1.0
0.5
0.0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration


B
Relative Timp3 Gene Expression
2
1
0
dH2O
0.01 μM
0.1 μM
1 μM
AICAR Concentration

3.16 Treatment of cartilage explant cultures with AICAR reveals decreased staining for proteoglycan, while no change noted in matrix collagen.

Ex vivo analysis of cartilage homeostasis was determined following AICAR treatment. Analysis of cartilage explants allows for an overall indication of cartilage health following drug treatment. Tissue sections stained with Safranin O/Fast Green for proteoglycan content demonstrated a trend toward decreased staining for this ECM component at the highest agonist concentration (Figure 3.17A). In order to examine each component of the cartilage ECM, a Picrosirius Red stain was also performed as an indicator of overall collagen content in the matrix. This stain revealed no conclusive trend in terms of matrix staining for collagen (Figure 3.17B).

To follow up with this apparent decrease in matrix staining for proteoglycans, an antibody specific for cleaved matrix aggrecan was used in an IHC protocol. An increase in staining for this aggrecan neoepitope antibody was observed in the ECM of sections from tissue treated with AICAR (Figure 3.18B) as compared to vehicle controls (Figure 3.18A). Matrix collagen content was analyzed further with an immunohistochemistry for type II collagen and type II collagen neoepitopes. Staining for matrix collagen using an antibody for type II collagen revealed no conclusive changes between vehicle controls (Figure 3.18C) and AICAR treated sections (Figure 3.18D). Lastly, matrix staining for type II collagen neoepitopes was performed by immunohistochemistry with an antibody specific to type II collagen fragment. No staining was apparent in the matrix for type II collagen neoepitopes in slides from vehicle control (Figure 3.18E) or AICAR treated sections (Figure 3.18F).

Figure 3.17 Treatment with an AMPK agonist in organ culture shows a trend towards decreased proteoglycan content in articular cartilage extracellular matrix, while matrix collagen seems to be unaffected.

Adult wild-type knee joints were dissected from mice and treated with an AMPK agonist (AICAR) in organ culture for 1 week. Tissues were prepared for histology through standard protocols of decalcified paraffin sections. (A) Sections stained with Safranin O/Fast Green for proteoglycan content display decreased staining at 10 μM compared to control, (n=3). (B) Sections stained with Picrosirius Red for collagen content displayed no apparent change in staining, (n=3).

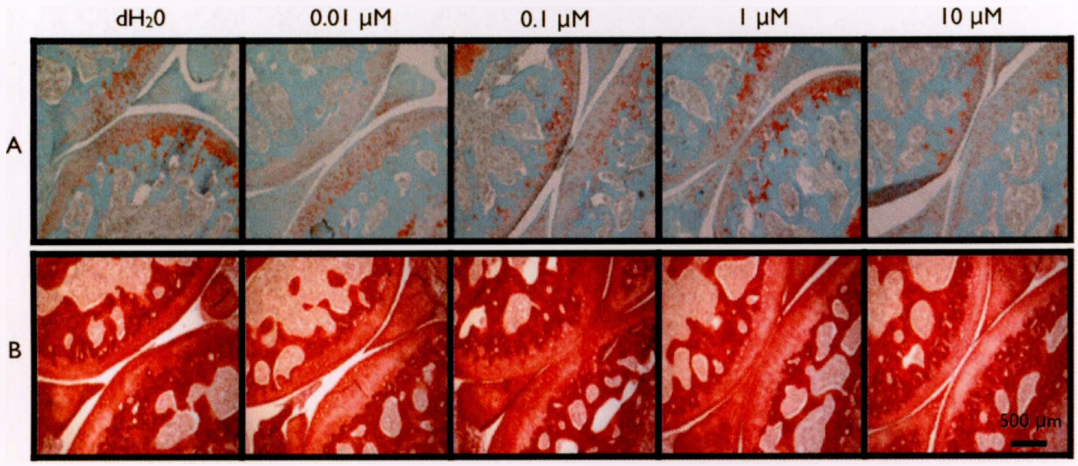
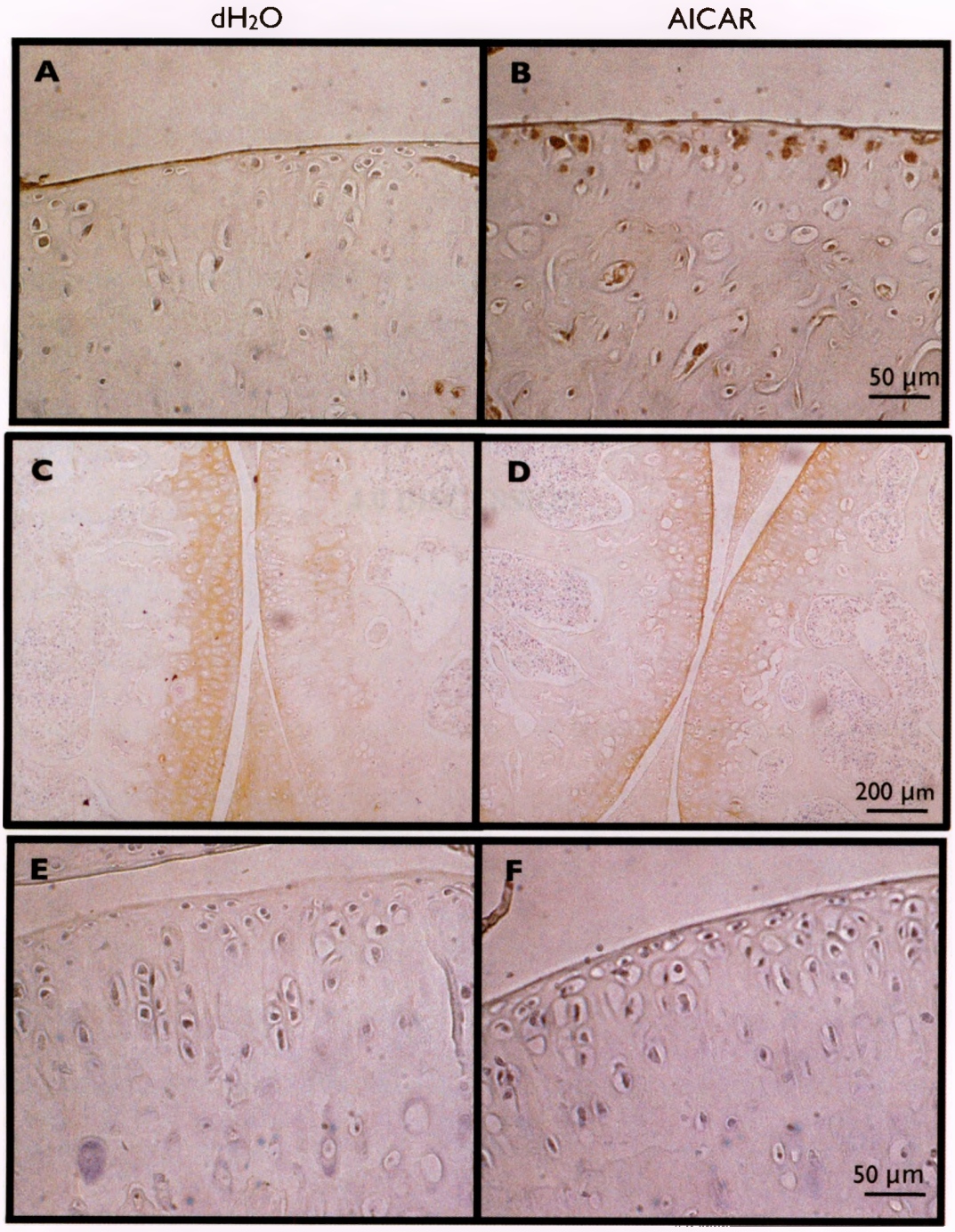

dH2O
0.01 µM
0.1 µM
1 µM
10 µM
A
B
500 µm

Figure 3.18 AMPK agonist treatment increases Aggrecan neoepitope staning, does not affect type II collagen staining in the ECM.

Knee joints from adult wild-type mice were dissected and treated in organ culture for 1 week with an AMPK agonist (AICAR) and prepared for histological analysis. Sections treated with dH_2O served as vehicle controls. Sections were analyzed by standard immunohistochemistry protocols with antibodies for Aggrecan fragments, type II collagen and type II collagen fragments. An increasing in staining for Aggrecan fragments was apparent in sections treated with AICAR (B) as compared to vehicle controls (A). No apparent change in type II collagen staining was observed in comparing AICAR treated sections (D) to controls (C). No staining for cleaved collagen fragments was apparent in sections treated with AICAR (F) as compared to vehicle controls (E), (n=3).


dH2O
AICAR
A
B
50 µm
C
D
200 µm
E
F
50 µm

4.0 DISCUSSION

4.1 Summary of Results

Extensive and premature breakdown of articular cartilage is a major characteristic of the onset and progression of OA (Aigner *et al.*, 2006). As the prevalence of OA continues to increase, it is becoming necessary to elucidate the factors involved in the disease pathology. As such, current research in this field is focused on identifying novel therapeutic targets in cartilage tissue. It is becoming increasingly evident that molecular signaling in cartilage cells plays a significant role in OA.

Though PPARδ and AMPK have both been implicated in cartilage biology, their specific roles have yet to be fully established (Shao *et al.*, 2005; Poleni *et al.*, 2007; Bohensky *et al.*, 2010; Poleni *et al.*, 2010; Terkeltaub *et al.*, 2011). Due to the limited knowledge of these pathways in cartilage biology, this study sought to clarify the role that each of these pathways play. It was hypothesized that the AMPK and PPARδ pathways each control extracellular matrix turnover in cartilage by altering chondrocyte gene expression. Experiments combined *in vitro* and *ex vivo* approaches to elucidate the roles of these pathways in cartilage tissue. Our first objective was to characterize distinct changes occurring in primary chondrocyte gene expression as a result of both PPARδ and AMPK activation by pharmacological agonists.

Primary chondrocytes treated with the PPARδ agonist GW1516 demonstrated a significant increase in the extent of fatty acid oxidation independent of agonist concentration, indicating that changes in metabolism had been fully induced at our lowest treatment concentration. Fittingly, a trend towards increased gene expression

of the lipid metabolism gene *Lpl* was demonstrated with GW1516 treatment. This increase, however, was not found to be statistically significant, indicating that within the confines of this study, initial changes in fatty acid oxidation were not paralleled by changes in this lipid metabolism gene. It is reasonable to speculate that changes in gene expression arise at a later point than the initial increase in fatty acid oxidation.

Analysis of chondrocyte gene expression following GW1516 treatment demonstrated firstly a decrease in the expression of *Sox9*. Based on the role of Sox9 in inducing synthesis of cartilage ECM components (Takahashi *et al.*, 1998), I next analyzed the relative gene expression of type II collagen and aggrecan. Interestingly, similar decreases in the expression of these matrix components were not found. It is possible that expression of type II collagen and aggrecan are altered at a later time point compared to Sox9. Additionally, matrix homeostasis is controlled by a number of factors, suggesting that type II collagen and aggrecan gene expression may be controlled by other transcription factors independent of Sox9.

Changes in gene expression of the matrix metalloproteinases *Mmp2* and *Mmp3* were noted with GW1516 treatment, while no change in *Mmp13* gene expression was observed. In analyzing the gene expression of enzymes with the potential to cleave aggrecan in the matrix, significant increases in *Adamts2*, *Adamts5*, *Adamts7* and *Adamts12* were observed. These results suggest that PPARd activation induces enzymes with the ability to cleave both the collagen and aggrecan component of the cartilage ECM, as both MMPs and ADAMTS enzymes were increased by GW1516 treatment. Surprisingly, the expression of *Timp3* was also found to be

increased with GW1516 treatment, indicating the potential for compensatory inhibition of both MMP and ADAMTS activity (Visse & Nagase, 2003).

Changes in cartilage matrix homeostasis as a result of agonist treatment were also examined. The most notable change in matrix composition following GW1516 treatment was the reduction of proteoglycan staining in the ECM. Staining for ECM collagen revealed no conclusive trend in terms of its contribution to matrix composition. Fittingly, an increase in staining for cleavage fragments of aggrecan was also observed. GW1516 is a selective PPARδ agonist and thus all observed changes can be attributed to PPARδ activity (Sznaidman *et al.*, 2003).

The fact that only the aggrecan portion of the ECM seems to be affected by treatment is not unexpected as aggrecan loss precedes collagen loss during OA (Bluteau *et al.*, 2001; Pratta *et al.*, 2003). In this regard, the collagen fibrils within the ECM matrix are more resistant to proteolytic cleavage (Lippiello *et al.*, 1977). Although MMPs are less efficient at cleaving Aggrecan than ADAMTS enzymes (mainly ADAMTS-5), they have been noted to be important contributors to pathological aggrecan cleavage during OA (Fosang *et al.*, 1996b; Durigova *et al.*, 2011). Interestingly, results of this study revealed an increase in expression of catabolic factors as well as *Timp3*. Given that Timp3 functions to inhibit both MMPs and ADAMTS enzymes, its presence may decrease proteolytic activity within the ECM to a level capable of degrading proteoglycans without affecting the stable collagen matrix. This may explain why changes in proteoglycan but not matrix collagens were observed in this study following GW1516 and AICAR treatments.

In examining the effect of AICAR treatment on primary chondrocytes, no change in the extent of fatty acid oxidation was observed following treatment. As discussed above, molecular events induced by AMPK activation are aimed at replenishing depleted levels of cellular ATP (Hardie & Pan, 2002). This increase in ATP is accomplished in part by inducing fatty acid oxidation, as well as by increasing glucose metabolism (Hardie & Pan, 2002). The fact that the confines of this study did not reveal an increase in fatty acid oxidation following AICAR treatment, yet still resulted in a number of changes in terms of chondrocyte gene expression and ECM homeostasis, could be explained in part by this additional role that AMPK plays in glucose metabolism (Holmes *et al.*, 1999). It is possible that treatment conditions did not cause a detectable change in fatty acid oxidation, while still inducing other downstream events of AMPK activation. Additionally, AICAR functions pharmacologically as an AMP mimetic and as such we cannot rule out changes occurring as a result of other physiological processes that AMP is involved in (Mu *et al.*, 2003). However, AICAR provides the most specific method available for inducing AMPK activation, as it does not involve altering levels of cellular ATP or AMP (Corton *et al.*, 1995). Since AICAR is altering how AMPK senses cellular ATP levels, it is possible that treatment concentrations used were not high enough to induce this change and thus further experimentation is needed in order to determine optimal dosing of AICAR. The specific effects induced in cartilage tissue as a result of AICAR treatment need to be determined, since previous experimentation with this drug has predominantly been performed in other tissue types, such as liver and muscle tissue (Hardie & Sakamoto, 2006; Viana *et al.*, 2006).

Further analysis by MTT assay revealed that primary chondrocytes treated with AICAR showed a significant reduction in cell number, indicating that cellular proliferation had been inhibited at higher drug concentrations. The ability of AICAR to decrease cellular proliferation has been noted for other cell types and is thought to result from changes in the cell cycle in these instances (Menze *et al.*, 2005; Guan *et al.*, 2007). Although mature articular chondrocytes display relative low levels of proliferation, any alteration in this capacity could have the potential to induce pathological changes in cartilage tissue.

Examining gene expression following AICAR treatment revealed an increase in type II collagen gene expression, while *Sox9* and aggrecan gene expression are unchanged. It is evident here that type II collagen gene expression is being induced by events independent of Sox9 transcript levels. Additionally, the gene expression of all MMPs analyzed in this study was unchanged. Interestingly, changes were observed in a number of ADAMTS enzymes, although specific to each enzyme itself. The gene expression of *Adamts1* and *Adamts2* was decreased, while expression of *Adamts5* was significantly increased upon AICAR treatment.

Changes in matrix homeostasis following AICAR treatment are similar to those observed with GW1516 treatment. A decrease in proteoglycan staining was apparent with a concomitant increase in staining for aggrecan fragments. No apparent trend in terms of collagen content was observed, which is fitting in terms of the order that loss of ECM components presents during normal OA progression (Bluteau *et al.*, 2001; Pratta *et al.*, 2003). However, it seems that in the context of AICAR treatment, ADAMTS-5 is the sole factor examined that was upregulated in cell culture, that also

has the potential to induce proteoglycan loss in the matrix, should its increased gene expression be found to result in increased enzymatic activity. All other catabolic factors examined were either decreased or remained unchanged with treatment.

4.2 Contributions to current knowledge of the roles of PPARδ and AMPK in the regulation of chondrocyte gene expression and articular cartilage homeostasis

Elucidating the mechanisms contributing to cartilage degeneration provides important evidence necessary for the development of therapeutic approaches for OA. Results of this study provide the first conclusive evidence for a role for PPARδ in cartilage breakdown. However, examining AMPK revealed a more complex relationship in terms of its role in cartilage tissue. Although results did provide substantial evidence suggesting an important role for AMPK in cartilage tissue, further experimentation is needed to determine optimal treatment concentration and to determine the events by which AMPK exerts its effects in cartilage tissue. Very little information has been provided to clarify the specific effects being induced by the PPARδ and AMPK pathways in cartilage tissue. This study was thus aimed at characterizing changes in chondrocyte gene expression occurring *in vitro*, as well as changes in the homeostasis of the cartilage ECM in an *ex vivo* model.

Here we have demonstrated for the first time that pharmacological treatment with a PPARδ agonist results in increased fatty acid oxidation by chondrocytes, suggesting that fatty acids act as a substantial energy source for chondrocytes. Fittingly, previous studies in our lab have demonstrated a link between other nuclear

receptors and lipid metabolism in chondrocytes (Woods *et al.*, 2009; Stanton *et al.*, 2010). Similar to the current study, these studies cultured growth plate chondrocytes, but focused on chondrocyte differentiation. It would be interesting to fully examine the role of lipid metabolism in chondrocytes during OA with cell culture protocols being implemented using articular chondrocytes. Additionally, results here demonstrate hitherto unrecognized changes in gene expression by PPARδ activation involving a number of catabolic factors of cartilage tissue. It is possible that changes in matrix composition observed in *ex vivo* organ cultures following GW1516 treatment could also be attributed to a decrease in the expression of *Sox9* and resultant decrease in synthesis in matrix components. Organ culture protocols are implemented for a longer period of time as compared to cell culture conditions and thus resultant changes in gene expression following a decrease in *Sox9* could be established in this context.

Although AICAR treatment did not result in the change in fatty acid oxidation that was initially suspected, important evidence for its role in cartilage biology has nonetheless been demonstrated. Future experiments should be aimed at determining optimal dosing of AICAR, as well as elucidating the exact events brought about by pathway activation in chondrocytes and thus demonstrating any cartilage-specific events that occur.

Overall, results of this study provide further evidence that the multitude of changes observed during the progression of OA can be accomplished by different molecular mechanisms independent of each other. Our results reveal decreased staining for proteoglycan in the cartilage ECM following both independent drug

treatments, while collagen staining is unaffected. This loss of proteoglycan is indicative of an earlier stage of matrix degeneration, thus suggesting that both PPARδ and AMPK are involved in early OA. This study has provided the evidence necessary to drive further experimentation aimed at both PPARδ and AMPK in cartilage tissue.

4.3 Limitations and Suggestions for Future Research

This study has provided substantial evidence for a role for both PPARδ and AMPK in cartilage tissue, which provides the basis for further studies using more physiological systems as the ones used here for our initial experiments. Primary chondrocyte cell culture was implemented in order to characterize gene expression of a number of cartilage markers. Although this model provides a context in which the action of a single pharmacological agent can be studied, it does not account for all factors that would be present *in vivo* (ie. signals provided from the synoviocytes, mechanical loading, three-dimensional tissue architecture etc). The explant cultures overcome some, but not all of these limitations. Our *in vitro* and *ex vivo* studies provide a strong impetus to study the role of these pathways using *in vivo* models of OA, using either genetic or pharmacological approaches.

In order to validate the changes in mRNA expression observed, Western blot analysis should be implemented in order to conclude that changes in transcript levels are translating into parallel changes at the protein level. An additional method by which matrix breakdown products can be quantified is enzyme-linked immunosorbent assay (ELISA) with neoepitope antibodies as used in our IHC experiments.

Additionally, assays in which enzyme activity is measured (e.g. zymography) would provide important information as to the exact enzymatic contributors to the cleavage of ECM components observed within this study. In terms of AICAR treatment, it is necessary to determine the optimal dose at which fatty acid oxidation is induced. Additionally, a time course in primary chondrocytes for both GW1516 and AICAR treatment would be useful in determining when induction of fatty acid oxidation is able to cause other changes related to lipid metabolism, such as changes in lipid metabolism genes.

The *ex vivo* organ culture model implemented in this study was useful in determining effects of agonist treatment on cartilage tissue. This model allows for cartilage tissue to be treated in a three-dimensional context representative of the *in vivo* joint structure. However, although this model allows for the specific effect of drug treatment to be determined, it does not account for all factors that contribute to changes in cartilage tissue during OA, for example biomechanical loading. Additionally, the histological stains implemented in this study have provided useful information concerning the overall structural composition of cartilage tissue. Due to the apparent decrease in matrix proteoglycan staining and the increase in aggrecan neoepitope staining with both GW1516 and AICAR treatment, it would be reasonable to implement Western blot analysis in order to examine matrix breakdown products released into culture media, thus providing a semi-quantitative measure of the matrix changes observed.

A limitation of the present study exists as the neoepitope antibody used in our experimentation recognizes aggrecan fragments generated by MMP cleavage. Our

results from cell culture suggest a potential for both MMP and ADAMTS cleavage events. In order to fully establish the mechanism by which aggrecan is being cleaved, future experiments need to include IHC with a primary antibody recognizing ADAMTS cleavage fragments. These antibodies were not available to us during this study, but through a collaboration with Dr. A Fosang (Melbourne) we will be provided with them for future experiments.

It is important to note that although the studies outlined here provide novel insight into the roles of both PPARδ and AMPK in cartilage tissue, results are representative of *in vitro* and *ex vivo* conditions. As such, *in vivo* validation is needed for the role of these pathways to be effectively established. Established animal models of OA exist and specific surgical models are currently used in our lab. Destabilization of the medial meniscus (DMM surgery) is a well-established surgical model of OA (Glasson *et al.*, 2007) and has been extensively implemented as an experimental protocol in our lab. In terms of investigating the role of PPARδ in OA, PPARδ$^{fl/fl}$ mice have been generated and would provide the ideal mouse model for this study. A cartilage-specific knockout for PPARδ could be generated with cre-recombinase mice already available in our lab (Wang *et al.*, 2007; Solomon *et al.*, 2009; Gillespie *et al.*, 2011). Additionally, to study the role of AMPK in an *in vivo* model, DMM surgery could be used in whole body knockouts for both the α1 and α2 catalytic subunits. Despite the fact that this mouse model is not as ideal as a cartilage-specific knockout, it would nevertheless provide useful information concerning the role of AMPK in OA.

The current study implemented a 'gain of function' approach to analyze the role of PPARδ and AMPK. Gain of function studies are crucial in providing evidence about the events resulting from activation of a specific pathway, with the caveat that we don't know to what degree such activation occurs in a specific disease such as OA. The genetic models suggested above would provide the complementary 'loss of function' approach to examining each of these pathways. Loss of function studies allow to us to answer more specific questions about the activities a specific gene is involved in. In order to fully establish a role of these pathways in OA and to assess their suitability as therapeutic targets in cartilage tissue, loss of function studies must first be implemented.

Contrary to the findings of the current study, Terkeltaub et al demonstrated that following AICAR treatment, matrix degradation induced by IL-1β and TNFα was suppressed (Terkeltaub *et al.*, 2011). The difference in results between the Terkeltaub study and the current study are surprising, although a number of factors could account for these differences. IL-1β and TNFα represent only two of the multitude of catabolic factors in cartilage tissue that exist. It is possible that in the context of IL-1β and TNFα induction of matrix degeneration, AMPK functions to decrease catabolic activities, while in the context of our study, AMPK promotes ECM degeneration. Additionally, a noteworthy difference is the use of human articular chondrocytes and human cartilage samples, as compared to the mouse models used in the current study. Nevertheless, the differences between the two studies are surprising and further experimentation is required to resolve these discrepancies.

4.4 Significance

Currently, no suitable treatment options are available for OA, as the disease pathology remains largely unknown. This study sought to characterize the role that the PPARδ and AMPK pathways play in cartilage tissue and subsequently aid in revealing a potential role in OA pathology. Ultimately, the studies outlined here provide evidence for a role of both pathways in early changes in cartilage tissue indicative of OA. These pathways thus provide novel targets for prevention of OA given that cartilage tissue has been shown to possess the potential for repair following aggrecanase-mediated cleavage, a feature that is lost following later MMP-mediated cleavage events (Bluteau *et al.*, 2001; Karsdal *et al.*, 2008). Although the therapeutic potential of these pathways has yet to be completely determined, the targeting of these pathways for potential therapeutic approaches to OA is not an unreasonable goal. However, in order for this to occur, additional insights into the mechanisms by which PPARδ and AMPK exert their effects in chondrocytes and cartilage tissue, especially *in vivo*, are required.

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APPENDIX A

Animal Use Protocol

Western

AUP Number: 2007-045-06
PI Name: Beier, Frank
AUP Title: Regulation Of Endochondral Bone Growth By Hormones

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Regulation Of Endochondral Bone Growth By Hormones" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2007-045-06::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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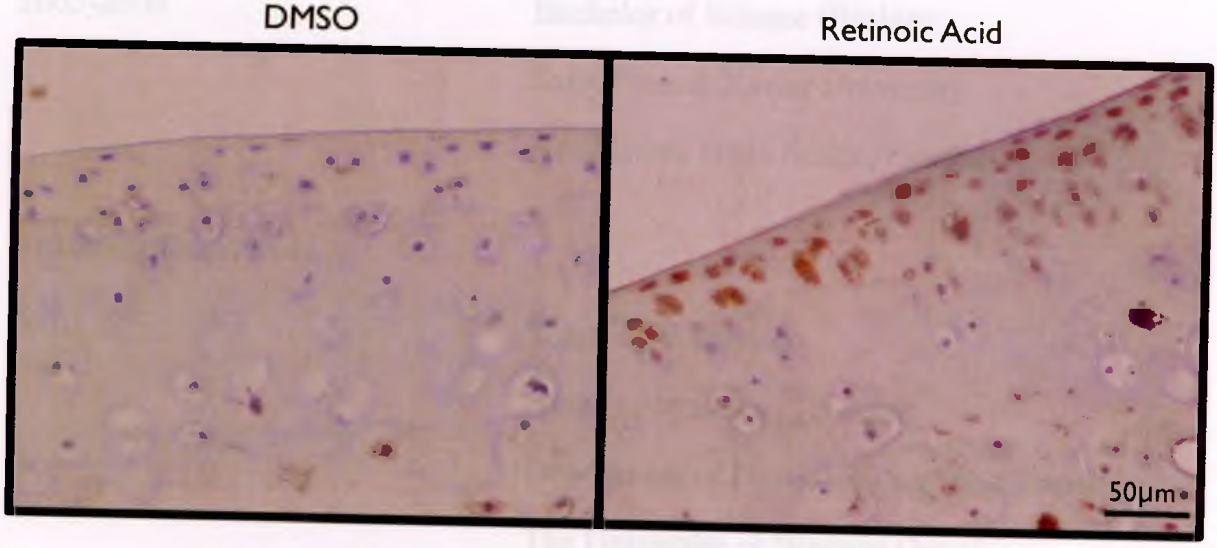
APPENDIX B

Supplementary Figures

Supplementary Figure.

Adult wild-type knee joints were dissected from adult wild-type mice and treated with 10μM retinoic acid in order to induce cartilage ECM breakdown. Knee joints treated with DMSO served as vehicle controls. Tissues were prepared for histology through standard protocols of decalcified paraffin sections. Sections were stained with a primary antibody for type II collagen neoepitopes and served as positive control for this antibody.


DMSO
Retinoic Acid
50µm