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Peroxisome Proliferator Activated Receptor δ in Osteoarthritis

Anusha Ratneswaran
The University of Western Ontario

Supervisor
Dr. Frank Beier
The University of Western Ontario

Graduate Program in Physiology
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Abstract

Osteoarthritis (OA) affects 1 in 10 Canadians and is a leading cause of mobility disability worldwide. This condition is characterized by cartilage degeneration, subchondral bone damage and inflammation of the synovium, resulting in pain and joint failure. No treatments exist to stop the progression of this disease, and its underlying molecular mechanisms remain largely unknown. We previously identified the peroxisome proliferator activated receptor (PPAR) nuclear receptor pathway as altered in OA cartilage. In-vitro studies identified PPAR δ as a promoter of catabolic activity in chondrocytes, providing the foundation for my overarching hypothesis that PPAR δ inhibition is protective in OA.

I commenced my thesis by generating *Ppard* cartilage-specific knockout mice to investigate the role of this gene in skeletal development. I evaluated the anatomy, morphology, and cellular organization of the skeleton, long bones and growth plate through histological techniques and concluded that there were no congenital abnormalities predisposing these mice to OA. I next compared the progression of disease severity between *Ppard* KO mice and WT controls after destabilization of medial meniscus surgery to induce post-traumatic osteoarthritis (PTOA). After histopathological assessment, I found that mice lacking PPAR δ were significantly protected from cartilage damage and displayed decreased cartilage matrix breakdown in lesioned areas.

Subsequently, I evaluated pharmacological inhibition of PPAR δ in PTOA in rats. I discovered that PPAR δ inhibitors prevent behavioural modifications associated with OA development and pain. However, their effects on structural progression of OA remains inconclusive and more stringent quantitative methods are needed to assess these differences.

Lastly, I examined global gene expression through microarray analysis of chondrocytes treated with a PPAR δ agonist. I discovered that genes induced were primarily involved in lipid metabolism, which translated into functional changes in lipid metabolism, such as significantly decreased cellular triglycerides. Mediators of oxidative stress were also identified, and *Txnip*, an inhibitor of anti-oxidant thioredoxin, was significantly elevated in response to PPAR δ activation. Immunohistochemistry revealed increased TXNIP staining in OA cartilage, but substantially less in cartilage of *Ppard* KO mice.

Overall, these data demonstrate a novel role for PPAR δ in Osteoarthritis. My data support my hypothesis that PPAR δ inhibition is protective in OA.

Keywords: Peroxisome Proliferator Activated Receptor delta, Osteoarthritis, Articular Cartilage, Joint Homeostasis, Transgenic Mice, Skeleton, Synovial Joint

Co-Authorship Statement

Chapter 2 is adapted from: Ratneswaran A, LeBlanc EA, Walser E, Welch I, Mort JS, Borradaile N, and Beier F. Peroxisome Proliferator Activated Receptor δ promotes the progression of post-traumatic osteoarthritis in a mouse model. *Arthritis Rheumatol.* 67(2):454-64, 2015. A.R. performed most experiments, contributed to study design, and wrote the manuscript. E.A.L. performed in-vitro experiments (Fig 2.1, 2.2 a,b,d). E.W. analyzed long bone lengths for supplementary data. I.W. performed mouse surgery. J.S.M. is a collaborator who provided neopeptide antibodies and contributed to editing the manuscript. N.B. is a collaborator who facilitated the beta-oxidation experiment and contributed to manuscript editing. F.B. contributed to study design and editing of the manuscript. All authors read and approved the submitted version of the manuscript.

Chapter 3 A.R. performed most of the experiments, contributed to study design and wrote the manuscript. M.A.P assisted with dissections, sectioning and scoring. C.H. collected behavioural data. H.D. assisted with dissections and scoring. V.P. performed surgeries. F.B. contributed to study design and manuscript editing.

Chapter 4 is adapted from: Ratneswaran A, Sun MMG, Dupuis H, Sawyez, C, Borradaile N, and Beier F. Nuclear receptors regulate lipid metabolism in chondrocytes. Submitted to *Arthritis Rheumatol.* A.R, M.M.G and H.D. performed most experiments. A.R. performed analysis, contributed to study design and wrote the manuscript. C.S. performed cellular lipid mass experiments. N.B. is a collaborator, contributed to study design and manuscript editing. F.B. contributed to study design and manuscript editing. All authors read and approved the submitted version of the manuscript.

Epigraph

*I am among those that think science has great beauty. A scientist in [her] laboratory is not only a technician, [she] is also a child confronting natural phenomena that will impress [her] as if they were fairy tales.” -
Marie Curie*

Acknowledgments

For those of you who made it past the epigraph, you are probably a bit surprised. You may have been expecting something like “it is more fun to talk with someone who doesn’t use long, difficult words but rather short, easy words like ‘what about lunch’” by A.A. Milne, or the equally appropriate Mark Twain epigraph “Notice: Persons attempting to find a motive in this narrative will be prosecuted; persons attempting to find a moral in it will be banished; persons attempting to find a plot in it will be shot.” On second thought that fits quite well! For those of you who were surprised, we probably haven’t met. Perhaps you are an impressionable young grad student looking for answers in all the wrong places... I’m serious- the real answers start near chapter 2!

Over the past five years, the lab and London have become my home (begrudgingly?), and I owe my first thank you to my supervisor, Dr. Frank Beier. Speaking *frankly*, I could not have imagined a better mentor. In 2011, you took a chance on a crazy kid who had a passion for research, but didn’t know a genetically modified mouse from Mickey Mouse. Since then, you have never run out of patience and wisdom. Most mentors would think in order to improve skills they would need to ‘fix’ or ‘change’ them, but you enhance our existing abilities. For instance, many might believe that better time management would mean taking more time to accomplish a given task. Written out that seems almost paradoxical...you taught me using less time can be just as efficient (and now I put the pro- in procrastination). Speaking of time- you somehow make time for all of us students despite your hectic schedule. You have always encouraged me to pursue every available opportunity to learn more, to experience more and

to become a better scientist. You have challenged me to challenge myself. Frank, you are a brilliant scientist and a great person. I can only endeavor to be more like you.

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List of Abbreviations

ACAN	Aggrecan
COL2	Collagen 2
DMMB	Dimethylmethylene Blue
DZ	Deep Zone
ECM	Extracellular Matrix
GAG	Glycosaminoglycan
GLUT	Glucose Transporter
IHC	Immunohistochemistry
IL 1	Interleukin-1
LXR	Liver X Receptor
MZ	Mid Zone
NGF	Nerve Growth Factor
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PTOA	Post-traumatic Osteoarthritis
PPAR	Peroxisome Proliferator Activated Receptor
RXR	Retinoid X Receptor
TNF α	Tumor Necrosis Factor alpha
SZ	Superficial Zone

Chapter 1

1 Introduction

The severity and impact of musculoskeletal disease is tremendous. As the number one cause of ‘years lost to disability’ worldwide, they detrimentally influence both the morbidity and mortality of those afflicted[1]. Osteoarthritis (OA) is the most prevalent joint disease worldwide, affecting 10% of men and 18% of women over the age of 60[2]. Although commonly thought of as a disease of aging, approximately half of all individuals diagnosed with arthritis are working age adults[3]. OA places a substantial economic burden on countries, annually accounting for approximately \$190 billion in North American direct and indirect health care costs or between 1.0 and 2.5% of the gross domestic product in developed countries[2, 4]. However, we currently have no effective treatment to alter the course of disease progression. This condition can affect one or more synovial joints but most commonly the knees, hips, hands and facet joints of the spine, and the principal symptom of this disease is pain[5, 6]. In this chapter we examine the synovial joint, with a special focus on articular cartilage and the crucial link between form and function in joint health and the pathogenesis of OA.

1.1 The Synovial Joint

Joints facilitate movement and flexibility through interaction between two or more skeletal elements[7]. Synovial joints are comprised of two opposing long bones, the ends of which are covered in articular cartilage. The bones provide structure and strength while the cartilage is responsible for receiving and dissipating loads associated with

weight-bearing. The joint is enclosed by a fibrous capsule, the joint capsule, which is lined by synovium. Synovium is the defining feature of the literally monikered synovial joint; it produces synovial fluid which enables frictionless movement, and also helps deliver nutrients and remove waste from the cartilage[8, 9]. Supporting structures include ligaments which confer stability and maintain proper alignment, and muscles which enable movement. Additionally, some joints have menisci, fibrocartilaginous pads, which further contribute to shock absorption[10]. Together these tissues act synchronously to stabilize the joint, distribute loads and permit frictionless movement. An example of a healthy synovial joint is shown in Figure 1-1.

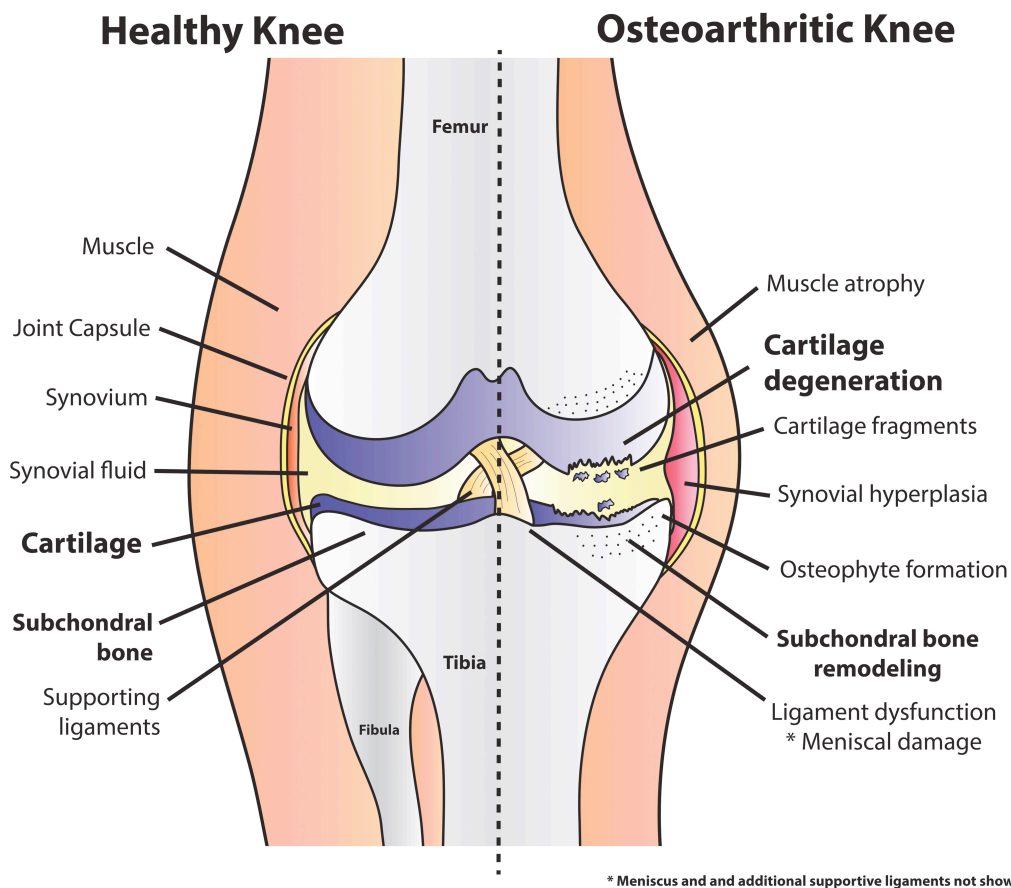


Figure 1-1 Synovial joint in health and OA

The synovial joint is comprised of opposing long bones, the ends of which are covered in articular cartilage. The cartilage and bone act in concert to receive and dissipate loads associated with movement and weight bearing. The joint is enclosed by the joint capsule and lined with a semipermeable membrane; the synovium. The synovium produces synovial fluid which fills the joint cavity, lubricating the joint. Ligaments and musculature confer stability, and ensure joint alignment. Figure courtesy of Dr. M. Pest.

1.2 Cartilage

Cartilage is a dense connective tissue, whose function is varied and dependent on its composition, mechanical and biochemical environment[11]. The relative proportion and organization of collagen fibers that form its extracellular matrix determine whether it is elastic, hyaline or fibrocartilage. Elastic cartilage is characterized by collagen 1 and, as the name suggests, elastin fibers. This type of cartilage is commonly found in the ear, and epiglottis. Fibrocartilage is also predominantly composed of collagen 1, and this type of cartilage is located in the intervertebral discs of the spine as well as in the menisci of many synovial joints[12]. Comparatively, hyaline cartilage is principally composed of collagen 2 and is found in growth plate and articulating surfaces of long bones[13].

1.2.1 Articular Cartilage Composition

Articular cartilage is a type of hyaline cartilage located at the articulating surfaces of long bones in synovial joints. It is both avascular and aneural, and functions to provide a frictionless, shock-absorbing surface to aid mobility and load bearing[14-16].

Chondrocytes are the sole cell type of hyaline cartilage, originating from mesenchymal stem cells. They vary in size and shape and distribution depending on the region of cartilage they are located in. Although they make up only 2% of cartilage in human articular cartilage, they function to produce and maintain the health of cartilage by regulating extracellular matrix (ECM) turnover[11, 17]. The ECM of cartilage is comprised of a fluid and a macromolecular component. A specialized matrix comprised predominantly of collagen and of aggregate proteoglycans forms the framework responsible for the structural integrity of the tissue. Other molecules such as lipids,

phospholipids, glycoproteins and non-collagen ECM proteins are present in smaller amounts. The fluid component of the ECM consists of water, cations and gases and small electrolytes. In fact, 70-80% of the mass of human articular cartilage is water[18-20].

Aggrecan is the principal proteoglycan in cartilage, and is made up of a core protein with glycosaminoglycan (GAG) side-chains. In aggrecan, these sidechains are chondroitin sulfate (majority) and keratan sulfate (minority). Perlecan, decorin and biglycan are additional proteoglycans found in the cartilage matrix[21]. The GAG sidechains are negatively charged, and this attribute is what enables the most distinguishing characteristic of cartilage; its unique ability to attract and trap water[22]. In articular cartilage, aggrecan, hyaluronic acid (HA) and link protein form non-covalently associated large aggregate complexes which exponentially enhance this attribute[19, 23]. Collagen 2 is the predominant collagen in cartilage and forms the filamentous ultrastructure of the ECM. The organization and distribution of Collagen 2 throughout the matrix is responsible for the tensile stiffness and for restricting tissue deformity when swelling proteoglycan aggregates trap water. Other collagens in articular cartilage include type IX and type XI which are associated with collagen II fibrils [24, 25].

1.2.2 Zonal and Regional Organization of Cartilage

Articular cartilage can be divided into four zones. Each of the zones has differentially organized cells, proteoglycans and collagen fibrils that influence its specific function. The zones of cartilage include: 1) superficial zone; 2) middle zone; 3) deep zone; 4) calcified cartilage, as depicted in Figure 1-2. Chondrocytes of the superficial zone (SZ) that borders the synovial space are flattened, with tightly packed collagen fibrils that are

oriented tangential to the surface. As the thinnest zone, the SZ has the lowest concentration of proteoglycans, and the highest concentration of water[11, 16]. Mid Zone (MZ) chondrocytes are more spherical, and as depth progresses from the mid zone (MZ) to the deep zone (DZ) the concentration of proteoglycan increases. Cartilage cells in the deep zone synthesize the most proteoglycans and are arranged in columns[26]. Collagen fibrils in the MZ are uniformly dispersed and randomly oriented, and become thicker and perpendicularly arranged to the joint surface in the DZ[27]. The tidemark, a histologically visible line, forms the boundary between the uncalcified and calcified cartilage. It is here that collagen bundles form an interlocking network that strongly anchors the overlying uncalcified cartilage to the calcified cartilage below[19, 28].

Articular cartilage ECM is also regionally organized. While zones are stratified based on depth from surface, regional organization is determined by distance from the chondrocyte. The region in closest proximity to the chondrocyte is the pericellular matrix, which fully envelopes the chondrocyte forming a unit called a chondron. The chondron is very dense in proteoglycans, contains non-collagenous proteins and contributes to signal transduction in response to mechanical stimulation[11, 16, 17]. The territorial matrix surrounds individual chondrons, clusters or columns of chondrons, forming a basketlike network of fibrils, and is thought to help protect chondrocytes from deformations associated with loading. Lastly, the interterritorial matrix is the largest and farthest away from the chondrocyte and consists of a network of collagen fibrils that confers tensile strength to the cartilage[29].

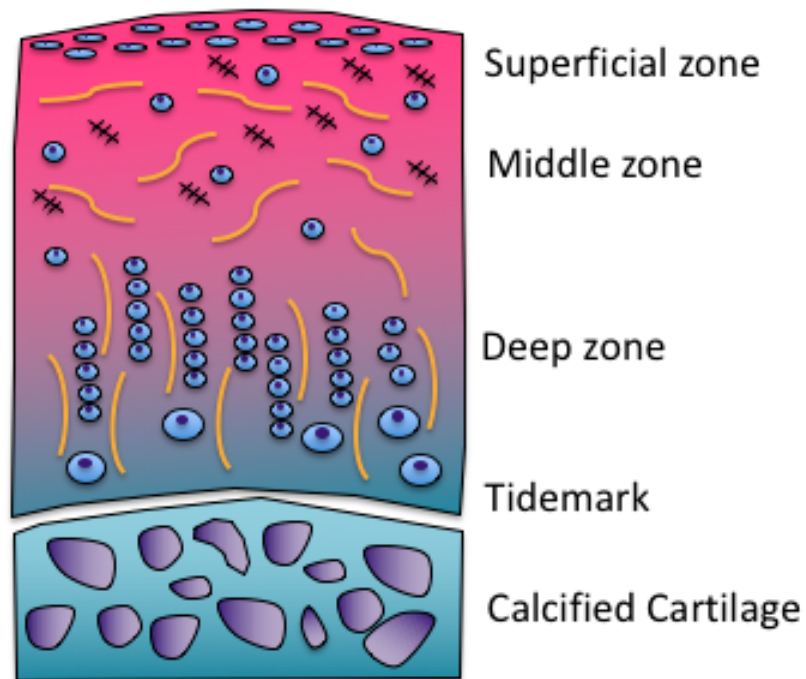


Figure 1-2 Zonal organization of articular cartilage

Articular cartilage is organized into four zones with distinct organization, composition and function. 1) The SZ possesses the greatest water content and collagen 2 fibril density. Collagen fibrils are oriented tangentially, and proteoglycan density is lowest. Chondrocytes are small and flattened. 2) The MZ has less water content than the superficial zone and collagen 2, but has greater proteoglycan density than the SZ. Collagen fibers are heterogeneously dispersed and randomly oriented. Chondrocytes are spherical. 3) The DZ has greatest proteoglycan content, but the lowest water concentration and collagen content. Collagen fibers are radially oriented and are thickest here. Chondrocytes are oriented in clusters, and have larger volumes. 4) The calcified cartilage is closest to the bone and separated from the DZ by the tidemark.

1.2.3 Biomechanical Properties of Cartilage

It has been established that each chondrocyte produces the matrix surrounding itself, and thus creates its own microenvironment. In turn, the cell is trapped by the matrix; the very environment it has established[17]. This can be beneficial as the surrounding matrix shields the cell from forces which act to deform the shape of the cartilage during weight-bearing and movement (as discussed above). Consequentially, there is very little direct cell to cell communication in healthy articular cartilage. On the other hand, chondrocytes are extremely interactive with their microenvironment and respond to growth factors, mechanical loads, piezoelectric forces, hydrostatic pressure and, as we will discuss later, lipids[30].

Although cartilage is 70-85% water, the distribution of this water content varies according to the depth of cartilage. The fluid content is the greatest for the upper 25% of cartilage and drops as depth increases[31]. The composition and zonal organization of the cartilage is essential to its role distributing stresses associated with load bearing. When a force is applied, cartilage on opposing sides of the joint are pushed towards each other and this initial force causes a rapid and immediate increase in fluid pressure in the joint[32]. The increase in fluid pressure squeezes the fluid out of the contact area in the solid matrix[33]. However, frictional resistance against this flow is very high and permeability of the cartilage is low. As fluid is forced out of the matrix, it becomes less porous, thereby decreasing permeability and preventing water leaving from deeper regions of the cartilage[11, 34]. Additionally, areas of cartilage adjacent to the contact stress are also pressurized, while the subchondral bone underneath the cartilage is

impervious to fluid flow[31, 35]. The combination of these properties limit the extent of deformation of the cartilage and is the reason why cartilage is capable of withstanding substantial loads.

1.2.4 Biochemical and Metabolic Properties of Cartilage

The composition and porous structure of articular cartilage is not only important for loading, but also necessary to facilitate nutrient transport because cartilage lacks innervation from blood and lymphatic vessels. Thus chondrocytes must either receive nutrients from the synovial fluid or from diffusion from the subchondral bone below[36, 37]. Although adult articular cartilage is relatively metabolically inactive and the turnover rate for matrix components is quite low, matrix synthesis is an energy intensive process and chondrocytes rely heavily on energy stores and ATP production[38, 39]. Anaerobic glycolysis is primarily used by the cell since oxygen levels are low due to the lack of blood supply, and up to 80% of glucose is metabolized to lactate[40]. The chondrocyte expresses both GLUT1 (Glucose transporter 1) and GLUT3 (Glucose transporter 3) transporters to facilitate the movement of glucose into the cell, and lactic acid transporters MCT4 (monocarboxylate 4) and MCT 1 (monocarboxylate 1) to efflux lactic acid from the cell[41-43]. There is evidence that the TCA cycle is used, and that oxidative phosphorylation occurs, but this accounts for less than 10% of normal chondrocyte energy metabolism[40, 43].

1.2.5 Molecular Regulation of Extracellular Matrix Turnover

In healthy cartilage, a balance between ECM synthesis and degradation maintains homeostasis. The half life of aggrecan is approximately 24 years while the half life of collagen II is more than 100 years in articular cartilage; thus, turnover of matrix is exceptionally slow[44]. Genes encoding matrix proteins such Aggrecan (*Acan*) and Collagen 2 (*Col2a1*) are directly regulated by the cartilage ‘master’ transcription factor Sex- determining- region-Y Box 9 (*Sox9*). Other cartilage matrix components such as biglycan, decorin, cartilage oligomatrix protein, and matrilins also exhibit very low turnover. Genes encoding proteases that degrade the matrix such as Matrix Metalloproteinases (*Mmps*), as well as A Disintegrin and Metalloproteinase with Thrombospondin Motifs 4, 5 (*Adamts4*, *Adamts5*), are active at low levels to break down damaged collagen II and aggrecan, respectively[45].

1.3 Bone

One might say it is humerus that the bone performs so many functions including the protection of vital organs, locomotion through transmission of loads and serving as an anchor for muscle attachment, the maintenance of systemic calcium and phosphate mineral homeostasis, as well as a providing the environment for hematopoietic and mesenchymal stem cells[46, 47]. Bone cells, like cartilage cells, synthesize an ECM whose organization is paramount to its function. It can be divided into organic and inorganic components. Collagen 1 is the principal component of the organic portion, and it is organized in parallel layers which confer both tensile strength and flexibility. The collagen fibrils act as a framework for the hydroxyapatite crystals $[Ca_{10}(PO_4)_6(OH)_2]$,

that form the inorganic portion of bone and pack tightly around this framework to provide structural support against compressive loading[48].

ECM maintenance in bone is regulated by osteoblasts and osteoclasts. Osteoblasts, or bone forming cells, are responsible for the synthesis of matrix constituents such as Collagen 1, non-collagenous proteins (such as osteocalcin, biglycan, bone sialoprotein etc.), and the osteoid that eventually becomes mineralized to form the calcified bone matrix[49, 50]. Like cartilage cells they are derived from mesenchymal stem cells, and differentiation to this lineage is regulated by runt-related transcription factor, *Runx2*. A portion of the osteoblasts become trapped within the osteoid, and are then called osteocytes. These cells still function to produce necessary proteins and signal to recruit osteoclasts when the matrix is damaged, or bone remodeling is required[51]. Osteoclasts are cells that resorb bone matrix and subsequently recruit osteoblasts to ensure structural integrity of the structure. They are derived from mononuclear cells. Unlike cartilage, bone matrix has a higher turnover rate[52].

1.3.1 The role of subchondral bone in the synovial joint

The primary roles of subchondral bone within the joint is to dissipate the load and distribute the strain associated with weight bearing, thus protecting articular cartilage against damage caused by excessive loading. It also functions to provide nutrients to the overlying cartilage by diffusion through the calcified cartilage matrix. The subchondral bone region is highly vascularized and innervated, and helps supply cartilage with oxygen, water and glucose[53].

The articular cartilage, which is zonally organized, becomes more stiff as the uncalcified cartilage transitions to calcified cartilage and then subchondral bone. While many collagen 2 fibrils lock the uncalcified cartilage to the calcified layer beneath, there are no such connections between the calcified cartilage and underlying bone[54]. Therefore, to adequately transmit shear forces, the calcified cartilage has a jagged boundary in which it fits into the subchondral bone like a “jigsaw puzzle” in order to lock in into place. In fact, the majority of force transmitted through the joint is absorbed by the subchondral bone, even though healthy articular cartilage can withstand 2.5-5 times the peak deformation caused by walking (which is roughly equal to several times one’s body weight)[53, 55, 56]. Accordingly, bone and cartilage must work in unison to enable frictionless movement and transmit loads effectively.

1.4 Synovium and Synovial Fluid

The joint capsule acts as a barrier from the rest of the body by encapsulating the joint. The interior of the joint capsule is lined with synovium; a selectively permeable membrane that filters blood plasma to form an ultra-filtrate[57]. This filtrate is combined with lubricating molecules such as lubricin and hyaluronan to form synovial fluid (SF). Superficial zone chondrocytes produce lubricin, while synoviocytes (highly metabolically active cells lining the synovium) produce both lubricin and hyaluronan[15, 58]. SF is a viscous fluid, and its primary role is as a lubricant in the joint to enable frictionless movement with minimal wear to other joint tissues[9, 36, 57-59]. It also facilitates the exchange of nutrients and wastes between the cartilage, synovium and plasma, and

contains soluble molecules such as growth factors, and cytokines to mediate communication between different cell populations within the joint[59].

1.5 Supporting Structures of the Joint

Muscle and ligaments are also integral components of the joint and serve to confer stability and maintain joint alignment during ambulation. Appropriate skeletal muscle contraction and nerve control is necessary to perform accurate and controlled movement[60, 61]. Ligaments are responsible for joint alignment which ensures forces are distributed correctly over the joint surface[62]. Some joints have menisci, fibrocartilaginous pads composed of meniscal cells, an ECM which is mostly type I collagen, and water which makes up 70% of the structure by weight. Menisci play a crucial role in shock absorption within the knee, wrist, jaw and collar bone joints[63].

1.6 Etiology of Osteoarthritis

OA is a heterogenous, multifactorial condition that can affect multiple joints, and the severity of pain and loss of function are variable between patients[64, 65]. It is the leading cause of mobility disability worldwide[66, 67]. It is characterized by the progressive breakdown of articular cartilage, subchondral bone changes, and synovial hyperplasia. OA primarily targets weight-bearing joints and can make even routine self-care tasks extremely difficult. The molecular mechanisms responsible for this condition are not well understood and therefore no treatments exist to halt or delay the progression of the disease. The exact etiology of OA is unknown, but factors which promote the progression of this condition have been and continue to be extensively studied.

1.6.1 Classification

Osteoarthritis is conventionally classified as primary or secondary OA. Primary OA is idiopathic, and stems from no discernible cause. It is commonly referred to as age associated OA, and rarely occurs in individuals less than 40 years of age[68]. Secondary OA develops is attributable to a specific cause. This includes joint trauma, hereditary, metabolic, mechanical reasons as well as diseases that affect joint alignment or biochemical tissue composition[69]. In many cases, individuals with primary OA have some genetic susceptibility or predisposition to it, and there is no clear distinction between the two[70].

1.6.2 Risk Factors

Risk factors that increase the probability of developing OA are described below and shown in Figure 1-3. It should be noted that these risk factors are not mutually exclusive, and that individuals can possess more than one risk factor. It has also been demonstrated that the progression of OA varies significantly between individuals; some joints remain radiographically stable while others deteriorate rapidly.

1.6.2.1 Age and Gender

Age is the strongest, most predictive risk factor for OA affecting the hip, knee, and hands[71-74]. The effects of other factors such as obesity, ligament laxity, sarcopenia, and impaired proprioception are compounded with age, and may contribute to increased load or abnormal loading of the joint[75]. Aging decreases reparative responses in the

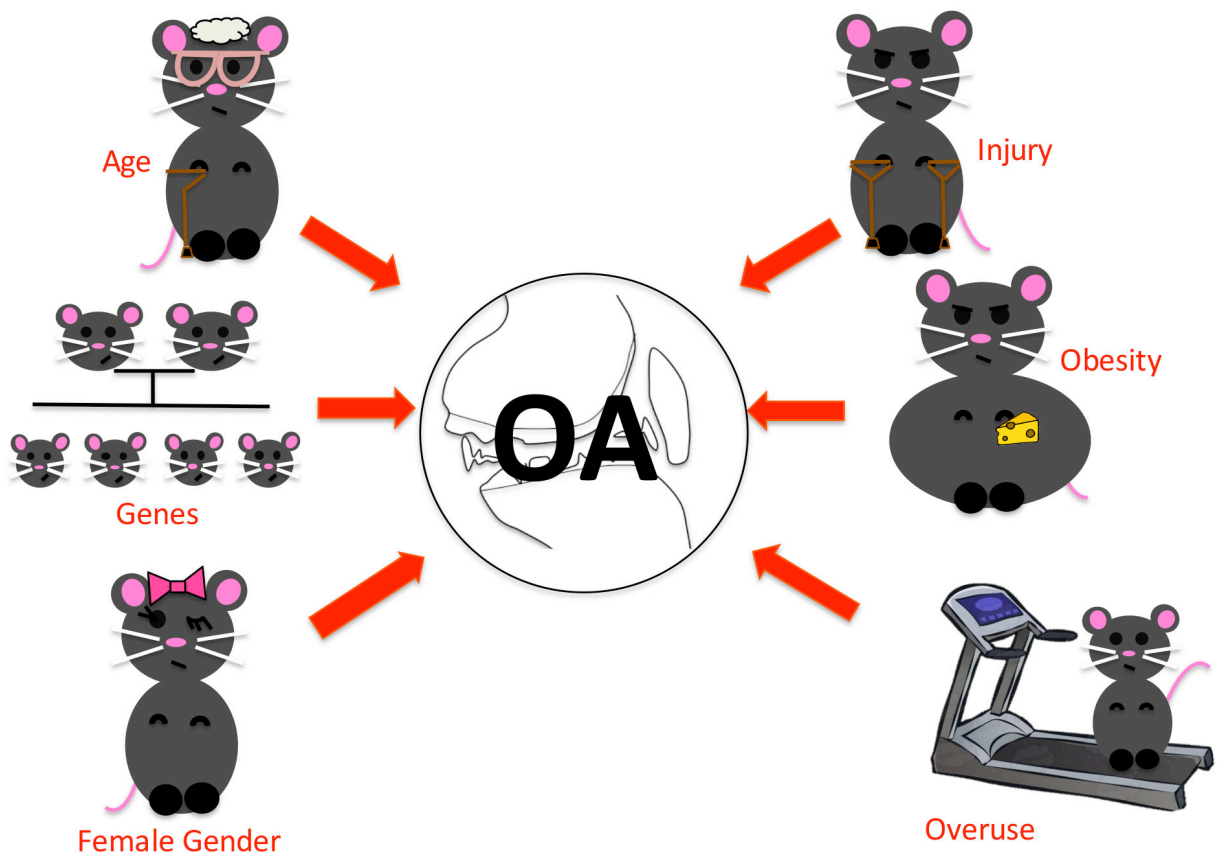


Figure 1-3 Risk factors for osteoarthritis

Risk factors which increase individuals' susceptibility to OA are shown above. Age, female gender, hereditary factors, joint injury, obesity (metabolic factors), and overuse (mechanical factors) all increase likelihood of OA development.

joint, and joint injury can be especially detrimental with older patients demonstrating progressive changes more quickly[76, 77].

The incidence of polyarticular, hand and knee OA is increased in females, and so are symptoms of OA such as pain, and decreased physical function[75, 78, 79]. Interestingly, women also have increased rates of cartilage loss and structural OA progression though reasons for this remain inconclusive[80].

1.6.2.2 Hereditary Factors

Hereditary factors represent another unmodifiable risk factor for OA progression. There are multiple genes that have been implicated in OA susceptibility, and their predisposition in combination with environmental factors cannot be ignored. Twin studies have shown that heritability of hand, knee and hip OA to be 40-60% in women[81, 82]. Mutations in genes associated with rare skeletal malformation disorders contribute to the pathogenesis of OA, through anatomical changes in joint shape or tissue integrity which influence joint mechanics[83]. Current studies suggest that because OA is genetically heterogeneous, each individual common gene variant only contributes modestly to the risk of developing OA[84]. The most consistent and reproducible genetic association with OA development has been *Gdf5* (growth and differentiation factor 5), a member of the TGF- β superfamily[85-87]. This gene is involved in ECM signaling, and plays an essential role in development, maintenance and repair of bone, and cartilage[88].

1.6.2.3 Metabolic Factors

Metabolic OA, has recently been defined as a type of secondary OA. It was long believed that obesity contributed to the progression of OA *only* through mechanical overload in the joint. This concept has since been poignantly opposed through stringently peer-reviewed articles such as “Metabolic factors in osteoarthritis: obese people do not walk on their hands”[89]. Presently, it is the second most frequent subtype of OA, and can arise from several systemic changes contributing to the pathogenesis of this disease[90, 91]. Epidemiologically, obesity and adiposity are associated with increased rate of knee cartilage volume loss, and the risk of primary knee and hip joint replacement due to OA is correlated with fat mass[92, 93]. Adipokines such as leptin are upregulated in osteoarthritic cartilage and osteophytes from OA patients[94]. Increased levels of products of lipid peroxidation have also been found in joints of OA patients, while levels of total fatty acids and arachidonic acid are significantly elevated in OA and correlated with histological severity of disease[95-97]. Several prominent studies suggest that weight loss, specifically fat loss, greatly improves symptoms in patients[98, 99].

1.6.2.4 Anatomical and Mechanical Factors

Morphology affects how the tissues of the joint accept and distribute load. Consequently, a loss of joint congruity can influence susceptibility to OA[100]. Impingement of the hip joint, malalignment of the femur and tibia of the knee resulting in ‘bow-legged’ or ‘knock-kneed’ phenotypes (varus and valgus, respectively), and differential lower limb lengths can all increase OA susceptibility[101-104]. Mechanical factors influencing the likelihood of OA development include excessive use of the joint, and overloading of the

joint, while unloading the joint (such as bed rest) can also lead to atrophy of joint tissues[105-107].

1.6.2.5 Joint Trauma

Post-traumatic OA affects 12% of North American OA patients[108], and arises from an initial insult to the joint, such as sports injuries, falls, or any condition that destabilizes the joint architecture[83, 109, 110]. Most often this involves torn or damaged ligaments or menisci; 40% of men, and 50% of women under the age of 40 who had an ACL injury developed post-traumatic OA[62, 111]. Other injuries include intraarticular fractures and micro-fractures of the subchondral bone[77]. Joint trauma not only induces deleterious changes through altered mechanics, but also increased inflammation, and inappropriate reparative responses which can lead to further joint damage[77].

1.7 Diagnosis

Pain is most often the reason why individuals seek medical attention, and the combination of pain frequency (days of the month) with structural changes assessed through radiography are most commonly used to diagnose OA[112-114]. Radiographic structural pathology includes joint space narrowing, presence of osteophytes, subchondral bone cysts and abnormal joint congruity[115]. Patients may also present with joint stiffness, ligament laxity, and synovial inflammation or distention[116]. More recently, MRI has been used in research settings due to its ability to clearly distinguish between cartilage, menisci and ligaments in contrast to radiographs which cannot differentiate between non-ossified tissues[117]. It enables a three dimensional comprehensive view of

the joint, and the patient is not subjected to ionizing radiation. However, the clinician may perform scoring through WORMS (whole-organ MRI scoring), a comprehensive semi-quantitative system that assigns pathological scores to each of the joint tissues[118]. While this presents a good alternative to traditional radiography, further validation and refinement of the scoring system is needed. Currently, there are no established, validated biomarkers predictive of OA but biomarkers indicative of ECM breakdown such as (CTX-II, Col2-1, C2C and C2M) may be used as objective tools to evaluate the efficacy of interventions[119].

1.8 Treatment

There are no current treatments available to ‘cure’ osteoarthritis, or alter the course of disease progression. Current therapy modalities treat symptoms such as pain and inflammation; they can be classified as life-style centric, medical or surgical. Lifestyle interventions include patient education, diet, weight-loss and physical therapy[120]. Formerly recommended therapies such as glucosamine and chondroitin sulfate supplements, arthroscopies and debridement are advised against as clinical trials have not demonstrated benefits[120]. Medical symptomatic relief options include viscosupplementation, steroids, analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) but this is often not enough, and chronic pain is one of the reasons that most patients choose to undergo end-stage joint replacement surgery[5, 121]. End-stage OA is treated through prosthetic joint replacement surgery that carries inherent medical risks, and patients may need multiple joint replacements and subsequent rehabilitation over the

course of their lifetime[122-127]. Moreover, all these approaches are directed at symptom management and do not address underlying causes.

1.9 Pathophysiology of Osteoarthritis

Osteoarthritis develops when tissue integrity of one or more joint structures is compromised. Each of these structures is integral to the whole joint, and a failure of one will result in failure of the entire joint (Figure 1-1).

1.9.1 Pain

Pain can be categorized as either physiological or pathological pain. Physiological pain is acute, presents a normal response to a stimulus (mechanical, thermal, chemical), and occurs at a threshold where the stimulus can become physiologically harmful[128]. For example; eliciting a withdrawal response for touching a hot stove. Pathological pain, commonly referred to as chronic pain, stems from damage to the nervous system or as an inflammatory response subsequent to a significant tissue injury. This type of pain can occur in the absence of any stimulus, can be amplified in severity or duration, and the threshold for eliciting pain is decreased to the point where normally non-painful stimuli elicit a pain response (allodynia)[128]. OA pain can be characterized as pathologic pain. It is important to note that a significant proportion of individuals that have radiographic joint damage do not experience pain and are considered asymptomatic[129]. Although cartilage breakdown may be one of the first steps in OA progression, this tissue is both aneural and incapable of generating pain directly[130]. The joint capsule, synovium, ligaments, subchondral bone and surrounding muscle are all richly innervated; in addition

to post-ganglionic sympathetic efferent fibers, they also have a number of sensory afferent fibers (proprioceptive and nociceptive)[131]. Thus, nociceptive input from the joint can be processed through central and peripheral pathways.

Under pathological conditions such as OA, inflammatory mediators are released into the joint and sensitize the nociceptors to mechanical stimuli so they are more likely to respond to any stimulus[132]. Cytokines including IL-1 β are expressed in peripheral blood leukocytes and are associated with increased pain and OA progression[132]. Persistent inflammation can also trigger hyper-excitability, an exaggerated response to previously innocuous stimuli. Joint neurons can also exhibit primary and secondary hyperalgesia, increased responses to stimuli applied to regions close to the joint (for example periarticular muscle) and at a distance from the joint (such as in the lower limb muscles)[133, 134]. Other cytokines such as TNF- α and IL-6 are elevated during these stages and work as pro-nociceptive mediators by directly acting on nociceptive neurons that express receptors for these cytokines[135]. Imaging studies of the knee joint have shown a correlation between pain, inflammation in the synovium and changes in subchondral bone in symptomatic OA, indicating these tissues are reactive and responsive in pain pathogenesis[136, 137].

Continued pathological neuronal input such as in advanced OA, can lead to central sensitization, changes within the brain and spinal cord that affect the properties of sensory neurons wherein they elicit a pain response for non-provoking stimuli. These patients report pain widespread from the site of OA, and demonstrate lower pain

thresholds for the entire leg[138, 139].

Nerve growth factor (NGF) is a neuropeptide necessary for the development and continued functionality of nociceptors[140]. At sites of inflammation several cell types including the chondrocytes and synovial cells produce NGF that has been shown to induce hyperalgesia in vivo[141-143]. In humans with moderate to late stage OA, injections of Tenazumab (a monoclonal antibody against NGF), provided prolonged pain relief and improved function. However, some patients presented with osteonecrosis and required total joint replacements[144].

1.9.2 Supporting Structures

Muscle weakness can act as both a risk factor for osteoarthritis (described earlier) but also as a consequence of OA. From a pathological perspective, OA can affect muscles through hyperalgesia due to central sensitization[145]. Disuse atrophy is common in many joints including the hips, knees and ankles[146-150]. It can reduce motor neuron excitability, decreasing voluntary muscle activation and proprioception. This reduces stability around the joint as well as ability to execute movement[151].

Menisci from OA patients display morphological changes such as calcium deposition, fibrillations, tears and scar tissue[152]. Macroscopic changes have been investigated in lapine menisci where the ECM has fine fibrillations, cyst like cavities, and irregular proteoglycan staining, as well as a loss of cellular organization, with clusters of cells is common [153, 154]. Advanced stages of meniscus degeneration stain intensely and positively for the aggrecan breakdown product NITGE[155].

1.9.3 Synovium

Synovitis is a common feature throughout OA with different types of synovitis associated with disease progression. Hyperplasia of the synovial lining is characteristic of early OA, while later stages are mainly characterized by capsular fibrosis and accumulation of breakdown products of bone and cartilage in the SF[156]. Later stages of radiographic OA are also associated with a greater presence of synovitis, with 83% of patients with late stage OA presenting with synovitis, versus 38% of those with early OA[157]. OA synoviocytes produce less lubricin, and hyaluronan so the composition and viscosity of SF is affected along with the joints ability to execute frictionless movement[158, 159]. Inflammation and hyperplasia also adversely affect the permeability of the synovial membrane which then permits hyaluronan to escape the joint[160].

Injury to the joint, increased permeability, and damage to the cartilage ECM also activate the complement system that responds by formation of the membrane attack complex (MAC) on chondrocytes. MAC is comprised of complement effectors C5b-9, and formation can result in chondrocyte death or production of matrix degrading enzymes[161]. Elevated levels of complement proteins are found in synovial fluid of OA patients[162]. ECM damage also increases the expression of TLRs (toll-like receptors) in the synovium. Toll-like receptors can stimulate NFkB activation which in turn stimulates the production of cytokines and chemokines[163]. These inflammatory mediators then perpetuate the feedback loop with cartilage and synovium.

1.9.4 Subchondral Bone

During the course of OA, the composition and structure of the cortical subchondral bone plate and the underlying trabecular bone changes, directly affecting the ability of the bone to support load and indirectly affecting the overlying articular cartilage[164]. Trabecular bone volume increases by approximately 20%, and there is an increase in bone turnover. However, the newly formed bone is hypomineralized so it does not confer the same stiffness[165-167].

In concert with changes in composition, bony spurs called osteophytes are formed at joint margins. The osteophytes are a hallmark feature of OA and can even be observed prior to joint space narrowing radiographically[168]. Osteophytes are derived from mesenchymal stem cells in the periosteum or the synovial membrane[168]. TGF- β and BMP2 (Bone Morphogenetic Protein 2) are anabolic factors believed to be key mediators in the formation of osteophytes; under their influence, precursor cells first differentiate into chondrocytes, deposit an ECM, undergo hypertrophy and orchestrate bone formation[169]. Functionally, osteophytes contribute to pain and limit joint mobility[170].

The tidemark, known as the demarcation between uncalcified and calcified cartilage (Figure 1-2), advances during OA and moves further into the uncalcified cartilage[171]. This thins the overlying articular cartilage. It is believed that proangiogenic factors are released from DZ chondrocytes undergoing hypertrophy and recruit factors to initiate local remodeling.[171, 172]. This contributes to increased stiffness in the articular cartilage, and decreases its ability to deform and distribute load[173]. Microfractures can

also occur in the zone of calcified cartilage or subchondral trabeculae. The cracks formed by these fractures enables the influx of synovial fluid into the zone of calcified cartilage and subchondral bone and formation of bone cysts[174]. Bone marrow lesions can also form during the pathogenesis of OA, and the location of these lesions corresponds to focal articular cartilage defects[175]. When the articular cartilage is completely eroded, a loss of joint congruity occurs concurrently with fracturing of the subchondral bone plate and sclerosis which in turn can cause the bone to collapse, further offsetting congruity[176].

1.10 Cartilage in Osteoarthritis

Adult articular cartilage is a relatively quiescent tissue maintaining ECM homeostasis, but in OA this balance is disrupted with a shift towards tissue catabolism[177]. During the pathogenesis of OA, cartilage becomes progressively fibrillated and eroded, destroying its ability to receive and dissipate loads and rendering it functionally inept.

Although the etiology of what initiates these events remains unknown, initial responses to OA involve upregulation of genes involved in matrix breakdown by chondrocytes, likely in order to remodel the damaged ECM. IL-1 β and TNF- α are upregulated and increase the synthesis of matrix metalloproteinases (1,3 and 13) which are primarily collagenases, but also capable of cleaving aggrecan[64, 178]. These inflammatory mediators also act to decrease the expression of TIMP (tissue inhibitor of metalloproteinases) proteins. On the other hand, upregulation of anabolic genes such as those encoding TGF- β , BMPs, IGF-1 (Insulin like growth factor 1), and FGFs (Fibroblast Growth Factor) promotes synthesis of extracellular matrix. However, there is greater tissue catabolism than anabolism, and

this changes the properties of the ECM. The chondrocytes are sensitive to their microenvironment. In response to this environment, they can differentiate to a hypertrophic phenotype, dedifferentiate to a fibroblast-like cell type, and undergo apoptosis[179]. Cellular disorganization is also observed with chondrocyte clustering as a result of cell proliferation[65, 180].

Possible mechanisms to initiate these changes in chondrocytes include increases in ROS (reactive oxidative species) in chondrocytes which can result in mitochondrial dysfunction and reduced autophagy [181]. Additionally, the accumulation of cartilage matrix proteins that have been modified by oxidative stress in the endoplasmic reticulum and golgi apparatus can also decreased ECM synthesis, and cell death[18, 182, 183].

Oxidative stress and ageing can also increase the accumulation of advanced glycation end-products (AGEs) in cartilage. Crosslinking of Collagen 2 by these products can increase the stiffness of the cartilage, changing its functional properties[184].

Furthermore, elevated levels of ROS and ageing can trigger cellular senescence which can trigger a DNA damage response, influencing the cell to stop proliferating and increase expression of proinflammatory cytokines and degradative enzymes[185, 186].

Phenotypically, the upregulation of anabolic factors can result in the formation of osteophytes as the cartilage incorrectly attempts to repair itself. The increase in degradative enzymes, particularly ADAM-TS 4,5 and MMP-13, results in the breakdown of aggrecan and Collagen 2 and degeneration of the ECM[187-189]. Aggrecan breakdown precedes Collagen 2 breakdown in OA, and further exacerbates it[190].

Decreased levels of functional aggrecan results in failure of the remaining cartilage to accept loads properly, making it even more vulnerable to structural damage. Cartilage

ECM fragments, such as those of fibronectin, can induce the expression of MMPs in chondrocytes, further perpetuating cartilage degradation[191]. In addition, cartilage detritus can activate TLRs and the complement system in the synovium which, causing the release of more inflammatory mediators and degradative enzymes[68, 156]. TLRs identified in chondrocyte can also be activated by endogenous products of cellular stress or matrix breakdown, and it has been observed that TLR-2 and 4 signals can mediate catabolic responses by increasing the expression of MMPs in mouse cartilage[161, 192]. In particular, aggrecan breakdown product 32-mer, can act as a ligand for TLR-2.

However, despite accumulation of knowledge of specific molecular and cellular events involved in OA, we still lack many parts of the complete picture, for example on gene regulator mechanisms connecting external stimuli to chondrocyte responses. The nuclear receptor family could play a crucial role in this context.

1.11 Nuclear Receptors

Nuclear receptors are transcription factors that are regulated by lipophilic small molecule ligands, where upon ligand binding they regulate the transcription of their target genes[193]. They are comprised of an NH₂ terminal domain which houses the ligand independent transcriptional activator, AF-1 (Activating Function 1). The DNA binding domain (DBD) which contains two highly conserved zinc-finger motifs that recognize and bind to specific sequences of DNA termed hormone response elements. The hinge region connects the DBD with the LBD (ligand binding domain), and the LBD consists of folded alpha helices that contain AF-2 (Activating Function-2) which is responsible for ligand dependent transcriptional effects. When a ligand binds to the LBD it induces a conformational change which results in the recruitment of co-activators, dissociation of

co-repressors promoting transcription of target genes. Nuclear receptors can be divided into 4 classes[194]. Class I nuclear receptors exist as homodimers and are usually found in the cytosol bound to heat shock proteins. Upon ligand binding they dissociate from these proteins and translocate to the nucleus where they bind to the hormone response elements that are inverted repeat sequences. Class I nuclear receptors are typically steroid hormones such the estrogen, androgen, glucocorticoid and progesterone receptors. Class II receptors are typically found in the nucleus bound to the DNA, they usually exist as heterodimers (with RXR, Retinoid X Receptor) and bind to direct repeat sequences such as the Peroxisome Proliferator Activated Receptors (PPARs), Liver X Receptor (LXR), Farnesoid X Receptor (FXR), Retinoic Acid Receptor(RAR), and Vitamin D Receptor(VDR). Class III nuclear receptors also exist as homodimers but bind direct repeat sequences such as RXR, and Class IV exist as monomers that bind half sites such as Steroidogenic Factor-1 Receptor (SF1R)[195].

Many of the Class II receptors are responsible for mediating lipid homeostasis including metabolism, storage, transport and elimination of lipids. They are activated by ligands such as fatty acids (PPARs), oxysterols (LXR), bile acids (FXR) and xenobiotics (SXR/PXR and CAR). Activation of these receptors often results in a feedforward positive regulation loop, since it occurs from low-affinity binding that happens at physiological concentrations influenced by dietary intake[196].

Currently 49 nuclear receptors have been identified in the human genome, and 31 of these are expressed in articular cartilage. Further, 23 of these are dysregulated in osteoarthritic cartilage[197]. However, only a few of these have been studied.

1.11.1 PPARs

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor super family, with fatty acids and fatty acid-derived molecules functioning as naturally occurring ligands[198]. This is of particular interest to us due to emerging evidence linking obesity to osteoarthritis, in a subtype of primary OA known as *metabolic OA*[90]. Subchondral bone ischemia, compromised nutrient exchange, and oxidative stress have all been implicated as mechanisms for the initiation and progression of this subtype of OA. Furthermore, synthetic agonists and antagonists specific for PPARs have been developed for therapeutic reasons, and nuclear receptors have been indicated as a strong potential target for OA therapy[199]. PPARs heterodimerize with the Retinoid X Receptor (RXR) allowing for recruitment of co-activators or co-repressors to occur[200], and ligand binding to PPARs can either activate or repress target gene transcription, depending on the specific target gene and cellular context. PPARs also have a low level of basal or constitutive activity (Figure 1-4). Of the three subtypes [alpha, delta, gamma], PPAR δ (also historically known as PPAR β) has the broadest expression pattern and functions in lipid and glucose metabolism, cell differentiation, proliferation, apoptosis and immune regulation. In skeletal muscle, it specifically activates pathways involved in remodeling with response to exercise[201]. While PPAR α and PPAR γ act as anti-inflammatory factors, and could thereby confer protection from OA, PPAR δ does not seem to share these anti-inflammatory attributes[202-205] Current studies on PPAR γ indicate abnormal skeletal development in cartilage-specific knockout mice, as well as spontaneous osteoarthritis development in adult cartilage-specific knockout mice, indicating a role for PPAR proteins in controlling chondrocyte behavior[203, 206].

Additionally, several studies on lipid metabolism illustrate how PPAR subtypes can have antagonistic and complementary action[207-210]. Thus a role of PPAR δ in OA, and in particular in promotion of OA, is plausible, and supported by our own studies described below.

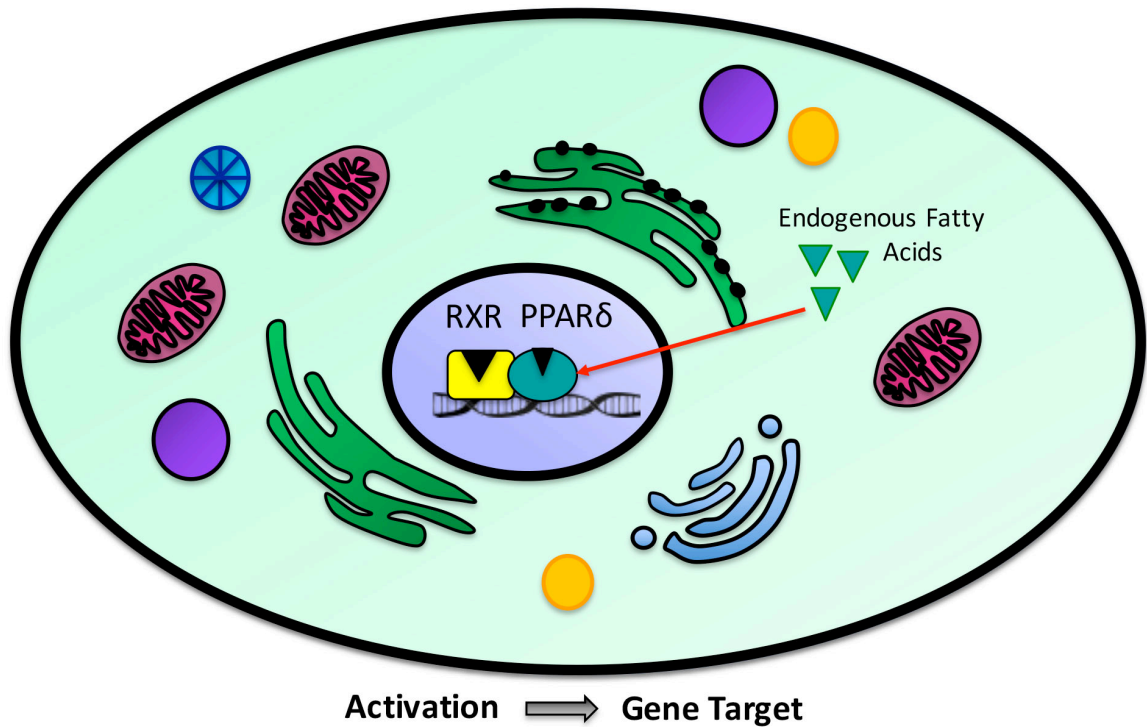


Figure 1-4 Nuclear receptor PPAR δ

In chondrocytes, endogenous fatty acids function as a ligand to activate PPAR δ . In its inactive state, it remains bound to the nucleus with RXR. Upon ligand binding, it changes conformation, to its active form thereby enabling transcription of its gene targets.

1.12 Overall Objectives and Hypothesis

Although OA affects all joint tissues, cartilage breakdown is a hallmark feature of OA and an irreversible process. PPAR δ was initially identified as a possible target for OA therapy in several of our genome-wide gene expression studies[211-213]. Previously, we have shown that chondrocytes treated with the PPAR δ agonist GW501516 respond with an increase in gene expression of several proteases involved in OA, including several ADAMTS and MMP genes[214]. Known target genes of PPAR δ such as *Lpl*, *Angpl4*, and *Pdk4* were also induced, confirming functionality of both PPAR δ and GW501516 in chondrocytes.

These data suggest that activation of PPAR δ in chondrocytes promotes ECM degradation through increased expression of catabolic proteases. We have also demonstrated that PPAR δ promotes fatty acid oxidation in chondrocytes[214], and there is potential that the dysregulation of lipid metabolism plays an important role in the initiation and progression of cartilage degeneration in OA[95]. Based on this data indicating that PPAR δ promotes cartilage breakdown, the overarching hypothesis for my thesis is that inhibiting PPAR δ will stop or delay cartilage breakdown in OA.

1.12.1 Objective #1

To characterize the role of PPAR δ in Osteoarthritis, in-vivo, by generating cartilage-specific *Ppard* knockout mice and examining them in a surgical model of OA.

1.12.1.1 Rationale #1

Based on our in vitro data demonstrating catabolic effects of PPAR δ activation, we asked whether inactivation of the *Ppard* gene would protect from cartilage degeneration in osteoarthritis. Mouse models are commonly used to study OA because they allow for genetic manipulations, and provide an efficient timeline using surgical models, enabling greater sample sizes[215]. Destabilization of the medial meniscus (DMM) is the most accepted surgical OA model in mice and use of genetically modified mice avoids potential complications from non-specific effects of pharmacological compounds[208, 216, 217].

1.12.1.2 Hypothesis #1

Cartilage specific depletion of PPAR δ will slow progression of post-traumatic OA.

1.12.2 Objective #2

To determine the effects of pharmacological PPAR δ inhibition in post-traumatic OA.

1.12.2.1 Rationale #2

The PPAR δ agonist GW501516 induces protease expression and proteoglycan loss in vitro, and cartilage specific depletion of PPAR δ in mice results in delayed progression of OA when compared to wild-type mice in a surgical model[214]. Yet, the effect of pharmacological inhibition of PPAR δ in mediating response to injury (post-traumatic OA) has yet to be elucidated. While the genetic model has provided a strong foundation of information to characterize this gene's role in the progression of OA, a

pharmacological approach is necessary to investigate whether the inhibition of PPAR δ could be a feasible therapeutic target for OA in human patients.

1.12.2.2 Hypothesis #2

Pharmacological inhibition of PPAR δ will protect against post-traumatic Osteoarthritis.

1.12.3 Objective #3

To elucidate novel target genes for PPAR δ in chondrocytes.

1.12.3.1 Rationale #3

Our preliminary results suggest that PPAR δ is important in the promotion of OA, but the underlying mechanisms remain unknown. Since PPAR δ is a transcription factor, it most likely works by regulating the expression of target genes. To understand PPAR δ 's mode of action, and identify additional targets for OA therapy, it is imperative that we first identify its target genes.

1.12.3.2 Hypothesis #3

PPAR δ alters chondrocyte behavior by regulating gene expression.

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Chapter 2

2 PPAR δ promotes the progression of post-traumatic osteoarthritis in a mouse model

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2.1 Abstract

Objective: Osteoarthritis (OA) is a serious and common disease of the entire joint, characterized by the degeneration of articular cartilage, subchondral bone changes, osteophyte formation and synovial hyperplasia. Currently, there are no pharmaceutical treatments to slow, stop or reverse disease progression, resulting in greatly reduced quality of life for OA patients and the need for joint replacement surgeries in many cases. The lack of available treatments for OA is partially due to our incomplete understanding of the molecular mechanisms promoting disease initiation and progression. Here we identify the nuclear receptor PPAR δ as a promoter of cartilage degeneration in a mouse model of post-traumatic (e.g. injury-induced) OA.

Methods: Mouse primary chondrocytes and knee explants were treated with pharmacological agonist of PPAR δ (GW501516) to evaluate changes in gene expression (qPCR), histology (Safranin-O, immunohistochemistry), and matrix glycosaminoglycan breakdown (DMMB assay) consistent with OA, and potential recovery. In-vivo, PPAR δ was specifically deleted in the cartilage of mice. Mutant and control mice aged 20 weeks were compared 8 weeks after a destabilization of medial meniscus (DMM) surgery.

Histopathological scoring (OARSI) and immunohistochemistry for known markers of OA were performed.

Results: In vitro, PPAR δ activation by the agonist GW501516 results in increased expression of several proteases implicated in cartilage matrix breakdown. GW501516 also induces aggrecan degradation and release in a knee joint explant culture system. In vivo, cartilage specific *Ppard* knockout mice do not display any abnormalities of skeletal growth or development, but show marked protection in the DMM model of post-traumatic OA (compared to control littermates). OARSI scoring and immunohistochemistry for cartilage matrix breakdown products confirm strong protection of the mutant mice from DMM-induced cartilage degeneration.

Conclusion: These data demonstrate a catabolic role of endogenous PPAR δ in post-traumatic OA and suggest that pharmacological inhibition of PPAR δ is a promising therapeutic strategy.

Keywords: *PPAR δ , cartilage, osteoarthritis, knock-out mouse, destabilization of medial meniscus*

2.2 Introduction

Osteoarthritis (OA) affects more than 150 million individuals worldwide and is predicted to rise in prevalence due to increasing life expectancies. OA severely influences the independence and quality of life of those afflicted; 14% of OA patients experience pain severe enough to significantly limit activities[1, 2]. Economically, OA accounts for

upwards of 190 billion dollar in North American direct and indirect health care costs annually, yet we have no current treatment modalities to prevent or delay its progression[3, 4].

In recent years, the view of OA has shifted from what was fundamentally seen as a disease of “wear and tear” to the realization that OA is an active process marked by cartilage attrition, catalyzed by the induction of proteases targeting principal extracellular matrix components of cartilage. The responsible proteases, in particular metalloproteinases and ADAM-TS enzymes, target the major components of the cartilage extracellular matrix, type II collagen and aggrecan, respectively[5, 6]. Dysregulation of cartilage homeostasis results in chondrocyte proliferation, hypertrophy, and cell death, concurrent with articular cartilage fibrillation, erosion, subchondral bone thickening and a loss of joint congruity[7]. Ensuing functional consequences include joint pain, stiffness and loss of mobility[8]. However, previously published studies using human tissue obtained from end-stage OA have yielded little insight into the etiology of this disease[9-11]. Thus it is essential to understand the molecular mechanisms underlying this condition throughout the disease process, in order to develop novel treatment approaches targeting core processes involved in cartilage remodeling and destruction.

Another recent development has been the classification of *metabolic* Osteoarthritis, which encompasses the contributing roles of hypertension, dyslipidemia, hyperglycemia, and obesity in the development of OA[12]. Subchondral bone ischemia, compromised nutrient exchange, and oxidative stress have all been implicated as mechanisms for the initiation and progression of this subtype of OA. In fact, a significant proportion of proteins related to lipid metabolism have been identified in proteomic analyses of

osteoarthritic cartilage and isolated chondrocytes[13]. Among these proteins are PPARs (Peroxisome Proliferator Activated Receptors), members of the nuclear receptor super family that are activated by lipid ligands. Of the three subtypes (alpha, gamma, delta), PPAR δ is most widely expressed and functions in lipid and glucose catabolism, cell proliferation, apoptosis and immune regulation. In skeletal muscle, it specifically activates pathways involved in remodeling with response to exercise[14]. PPAR δ has been detected in growth plate chondrocytes, and has been shown to be activated by the cytosolic Vitamin A metabolite retinoic acid[15, 16]. Vitamin A derivatives have been associated with the development of OA[17], suggesting that inhibition of PPAR δ could constitute a novel approach for treatment of OA. In this study, we examined the effects of PPAR δ activation in chondrocytes in vitro and the consequences of specifically deleting the encoding gene (*Ppard*) in cartilage of mice. Our data show that PPAR δ activation promotes catabolic processes in cartilage and that its inhibition indeed protects from post-traumatic OA in mice.

2.3 Methods

2.3.1 Primary Cell Culture and Isolation

Chondrocytes were isolated from embryonic day 15.5 CD1 mice (Charles River Laboratories) as described[18] and placed in culture medium with 1% FBS at a density of 1×10^5 per well. Cells were treated with the PPAR δ agonist GW501516 at concentrations of 0.01 μ M - 1 μ M for 48 hours. Dimethyl sulfoxide (DMSO) treated cells served as vehicle control.

2.3.2 Palmitate-Oxidation Assay

Primary chondrocytes were treated as above. Palmitate oxidation was assessed as previously described[19]. Radioactivity in the aqueous fraction of media and cell lysates was measured using a Beckman Coulter LS6500 Multipurpose Scintillation Counter. Total aqueous radioactivity, representing conversion of ^3H -palmitate to $^3\text{H}_2\text{O}$, was normalized to total protein.

2.3.3 MTT assay

Primary mouse chondrocytes plated at a density of 10,000 cells per well (96 well Falcon plate) were treated as above for 48 hours. Cell numbers were examined via MTT Assay as described[20], using a Cell Proliferation Kit (Roche Applied Science).

2.3.4 RNA extraction and Real-Time Polymerase Chain Reaction

Total RNA was isolated from cells using RNeasy kit (Quiagen). Real-Time Polymerase Chain Reaction (PCR) was performed using One-Step RT qPCR Master Mix kit and TaqMan Gene Expression Assays (Applied Biosystems) with 40 cycles on the ABI Prism 7900 HT sequence detector (PrismElmer Life Sciences). Probes were purchased from Life Technologies for *Mmp2* (Mm00439498_m1), *Mmp3* (Mm00440295_m1), *Mmp13* (Mm00439491_m1), *Adamts2* (Mm00805170_m1), *Adamts5* (Mm00478620_m1), *Adamts7* (Mm01239067_m1), *Adamts12* (Mm00615603_m1), *Acan* (Mm00545794_m1), *Col2a1* (Mm01309565_m1), *Sox9* (Mm00448840_m1), *Gapdh* (Mm99999915_g1). Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Relative gene expression was calculated using the delta-delta Ct method[21], as

described[22, 23].

2.3.5 Explant Culture

Knee joints from 10-week-old male CD1 wild-type mice (Charles River Laboratories) were isolated and placed in organ culture medium overnight. Knee joints were treated every 48 hours for 6 days with GW501516 (0.01 μM - 10 μM) or DMSO (10 μM) as vehicle control. Following organ culture, explants were fixed in 4% paraformaldehyde for 24 hours.

2.3.6 Dye Binding Assay

Dimethylmethylene blue (DMMB) dye binding assay was performed on media collected from knee joint explant cultures. Knee joints were cultured with 1 μM of GW501516 or vehicle control DMSO (see above). After culture, epiphyses were isolated and cartilage was extracted and digested overnight according to[24] DMMB assay was performed with chondroitin sulfate as a standard, and absorbance was measured on a Tecan Safire Fluorescence, Absorbance and Luminescence Reader at a wavelength of 595nm with a reference wavelength of 655nm. Aggrecan released into the medium was normalized to total aggrecan present in each sample.[24].

2.3.7 Tibia organ culture

Tibias from E15.5 wild-type CD1 mice were treated every 48 hours for 6 days with DMSO (control, 1 μM), GW501516 (0.01 μM - 10 μM), GSK3787 (1 μM), or combination treatment (1 μM GW501516 + 1 μM GSK3787). Tibias were measured at the beginning and end of culture using an eyepiece in a Leica EC3 stereomicroscope.

Following organ culture, tibias were fixed in 95% paraformaldehyde for 24 hours, followed by paraffin embedding and sectioning[25, 26].

2.3.8 Animals and Surgery

All animal experiments were approved by the Animal Use Subcommittee at The University of Western Ontario and conducted in accordance with the guidelines from the Canadian Council on Animal Care. Cartilage-specific *Ppard* KO mice were generated by breeding mice carrying the Cre-Recombinase gene under the control of the collagen II promoter[22, 23, 25, 26] to *Ppard*^{fl/fl} mice (obtained from The Jackson Laboratory)[27]. Male mice were used for all of the subsequent experiments. Mice were harvested for analysis of skeletal development by staining whole mouse skeletons with Alcian Blue/Alizarian Red, as described[28, 29], or for preparation of paraffin sections. DMM or Sham surgery was performed by Dr. Ian Welch on 20 week old cartilage specific *Ppard* knockout mice and control littermates, as described[30, 31]. DMM- and sham-operated mice were harvested 8 weeks post-surgery for analysis.

2.3.9 Histopathology of the Knee

Knees were dissected, fixed in 4% Paraformaldehyde, decalcified in Ethylenediaminetetraacetic_acid (EDTA) and embedded in paraffin. Frontal sections were cut at the Molecular Pathology Facility[28] and stained with Safranin-O/Fast Green as previously described[32]. Serial sections were graded by three blinded observers according to the OARSI histopathological scale[33], on the four quadrants of the knee.

2.3.10 Immunohistochemistry (IHC)

IHC was performed on frontal sections of paraffin embedded knees, slides with no primary antibody added were used as controls. Collagen 2 goat anti-human antibody was purchased from Santa Cruz Biotechnology (sc-774). Rabbit anti-human aggrecan (peptide CGGVDIPEN ovalbumin conjugate MMP cleavage site), Rabbit anti-human aggrecan (peptide CGGNITEGE ovalbumin conjugate ADAMTS 4/5 cleavage site) and Rabbit anti-cleaved collagen II (CGP-Hyp-GPQG ovalbumin conjugate human collagenase C1, 2C) were used as primary antibodies[34-36].

2.4 Results

2.4.1 PPAR δ induces the expression of enzymes involved in proteoglycan breakdown

GW501516 is a very specific, synthetic agonist of PPAR δ [37]. Since PPAR δ is an important regulatory gene in lipid metabolism in a number of tissues[38], and dysregulation of lipid metabolism has been implicated in OA, we first examined whether chondrocytes can respond to GW501516 by changes in lipid metabolism. To examine the extent of fatty-acid oxidation in primary mouse chondrocytes in response to GW501516 treatment, a palmitate-oxidation assay was performed. A significant increase in fatty acid oxidation was observed upon GW501516 treatment, independent of agonist concentration (Figure 1A). This effect implies that chondrocytes express functional PPAR δ and that oxidative lipid metabolism was fully induced at even the lowest concentration of drug treatment. MTT assays demonstrated that PPAR δ activation did not alter cell number and therefore was not toxic to chondrocytes, even at the highest concentration (Fig. 2-1B).

In order to determine the effects GW501516 on gene expression in chondrocytes, quantitative RT-PCR (qPCR) was performed on RNA isolated from primary mouse cartilage cells treated with vehicle or various concentrations of GW501516. We first examined the effects of GW501516 on markers of extracellular matrix (ECM) synthesis and breakdown. PPAR δ activation significantly increased the expression of genes encoding Matrix Metalloproteinases 2 and 3 (*Mmp2*, *Mmp3*) as well as A Disintegrin and Metalloproteinase with Thrombospondin Motifs 2, 5, 7, and 12 (*Adamts2*, 5, 7, 12) (Figure 2-1C-F and Suppl. Fig. 1A,B). This indicates that PPAR δ induces expression of enzymes with the ability to cleave both the collagen and aggrecan components of the ECM. In contrast, no significant changes were observed for transcript levels of *Mmp13*, aggrecan and collagen II (Suppl. Fig. 1 C-E). Interestingly, the expression of *Sox9*, the key transcriptional regulator of collagen II and aggrecan genes, was slightly but significantly decreased with increasing concentrations of agonist (Suppl. Fig. 1F).

We therefore investigated ECM turnover ex vivo using cultured knee explants. Cultures were treated with GW501516 at 0.01, 0.1, 1, and 10 μ M concentrations and stained with Safranin-O/Fast-Green. Loss of ECM glycosaminoglycans was observed with increasing concentrations of agonist treatment, consistent with aggrecanase activity (Figure 2-2A). Immunohistochemistry staining with neo-epitope antibodies confirmed degradation of aggrecan in the ECM (Fig. 2-2B) with 1 μ M agonist treatment compared to DMSO controls. Dimethylmethylene blue (DMMB) dye binding assays were also performed on knee explants cultured with GW501516 (1 μ M) or vehicle control (DMSO). Sulfated glycosaminoglycan released into the media was significantly greater for treated compared to untreated controls (Fig 2-2C). In contrast, staining with antibodies for collagen II or

collagen II breakdown products showed no effect of PPAR δ activation (Suppl. Fig. 2A,B, 7). In agreement with the absence of detectable collagen II degradation, Picrosirius red staining for fibrillar collagen did not show major differences between treatments (Fig. 2-2D). This is not unexpected as aggrecan loss precedes collagen loss during OA[39, 40]. Furthermore, the mechanical stimulation required for the breakdown of fibrillar collagen was not present in our culture system[41].

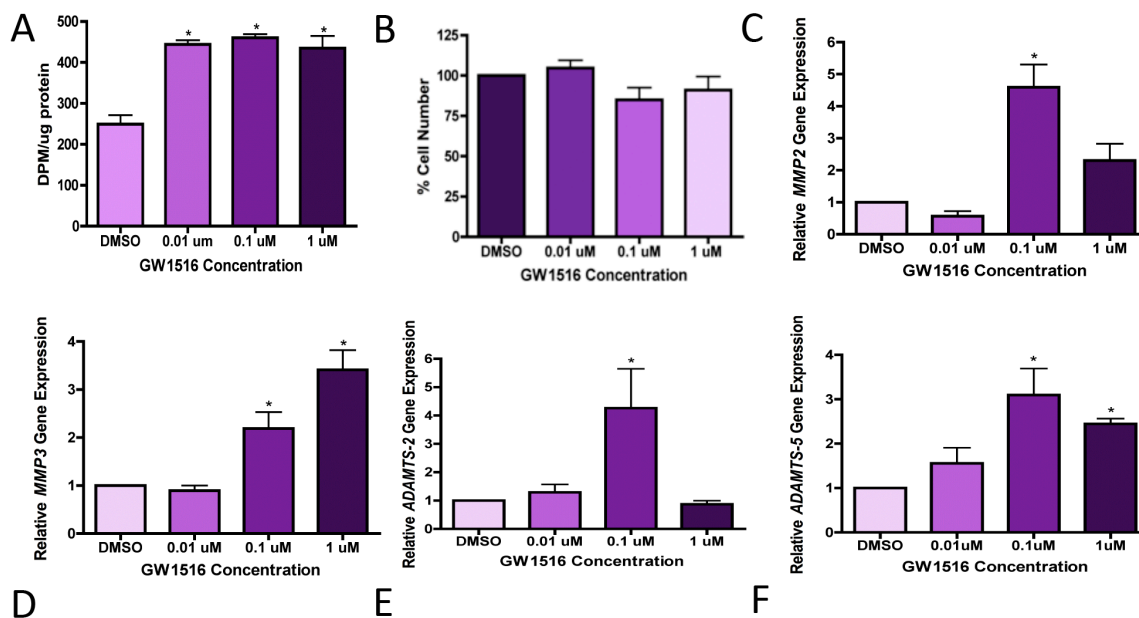


Figure 2-1 PPAR δ activation affects metabolism and gene expression in chondrocytes

Primary mouse chondrocytes were incubated with DMSO or various doses of the PPAR δ agonist GW501516 for 48 hours. A) PPAR δ agonist treatment increased fatty acid oxidation in culture. Significant increases in β -oxidation were observed at all treatment concentrations. B) MTT assays demonstrate that GW501516 does not alter cell number, indicating greater concentrations are not toxic and observed results are not due to cell death. C) Relative *Mmp2* gene expression is significantly increased at 0.1 μ M concentration of GW501516. D) *Mmp3* gene expression is significantly upregulated at 0.1 μ M and 1 μ M concentrations of PPAR δ agonist treatment. E) *Adamts2* relative gene expression is increased at 0.1 μ M of GW501516 treatment. F) PPAR δ agonist treatment significantly elevates *Adamts5* gene expression at 0.1 μ M and 1 μ M. (N \geq 3, *p \leq 0.05, data represented are mean \pm SEM)

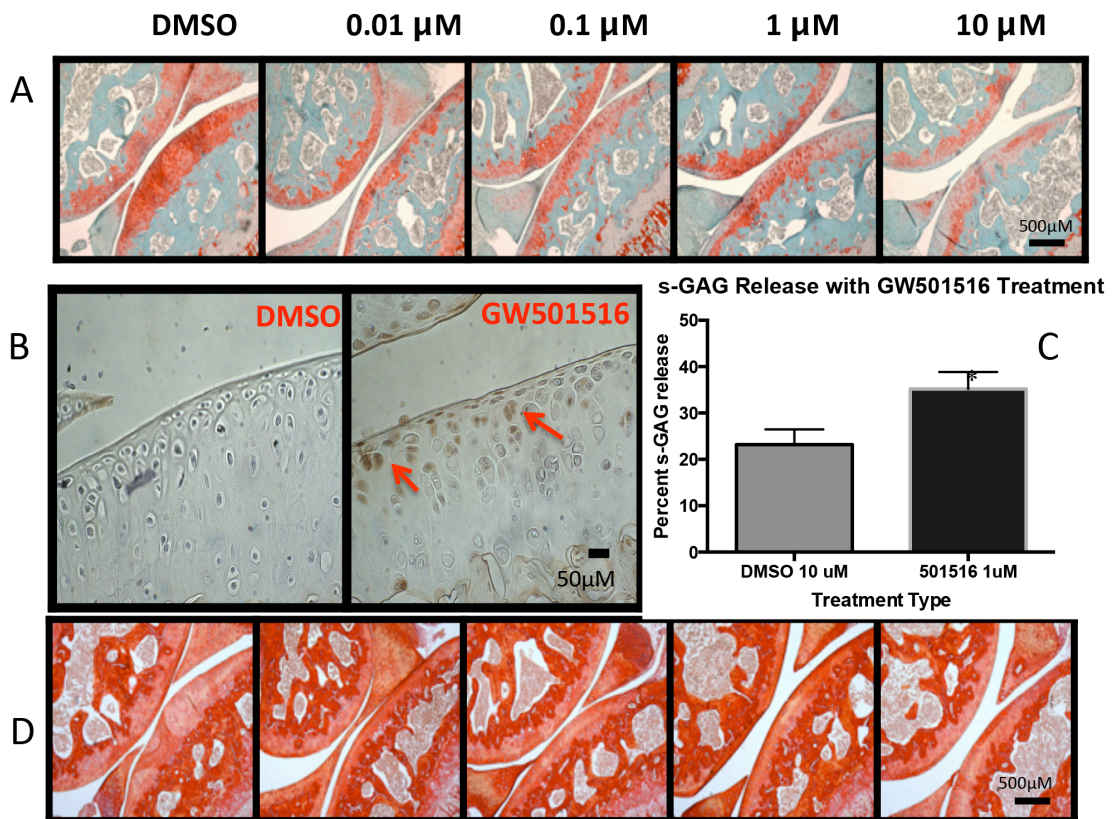


Figure 2-2 Induction of proteoglycan degradation by PPAR δ agonism

Adult mouse knee joints were incubated with DMSO (vehicle) or increasing concentrations of the PPAR δ agonist GW501516 for 6 days (N = 3). A) Paraffin sections were stained with Safranin O (for cartilage glycosaminoglycans) or a neo-epitope antibody for MMP13-cleaved aggrecan; B. Immunohistochemistry for cartilage explants used 1 μ M of GW501516 and DMSO vehicle control. C) A DMMB assay was performed to quantify sulfated glycosaminoglycan release into the media, (N=5). Picrosirius Red was also performed (fibrillar collagen; D). Safranin O staining (red) is reduced upon GW501516 treatment, while aggrecan neo-epitope staining (brown; indicated by red arrows) is increased. DMMB assay confirmed a significant difference between 1 μ M GW501516 treatment and DMSO controls. These results indicate increased glycosaminoglycan and proteoglycan breakdown in response to PPAR δ activation. In contrast, Picrosirius Red staining (red) does not change dramatically. (N \geq 3)

2.4.2 Cartilage-specific PPAR δ knockout mice display no developmental phenotype

In light of these promising in vitro results, we initiated in vivo studies in order to determine whether PPAR δ inhibition could be a valuable approach for OA therapy. We crossed *Pppard*^{f/f} mice[27] with a strain expressing Cre recombinase under control of the collagen II promoter[28, 29, 42, 43]. We first assessed the developmental phenotype of cartilage-specific *Ppard* KO mice. Alcian Blue/Alizarin Red staining performed on skeletal preparations of postnatal day 10 (P10) and P21 control and *Ppard* knockout littermates (Figure 2-3A,D) demonstrated normal skeletal growth and morphology in mutant mice. Safranin-O staining was performed on paraffin sections on femurs from P10 and P21 KO mice and wild-type littermates (without Cre) in order to assess growth plate development (Figure 2-3B,C,E,F). Measurement of the length of the proliferative zone, hypertrophic zone and total growth plate indicated normal phenotypes of mutant growth plates compared to wild-type mice. This was confirmed by normal morphology and tissue architecture of the mutant growth plates.

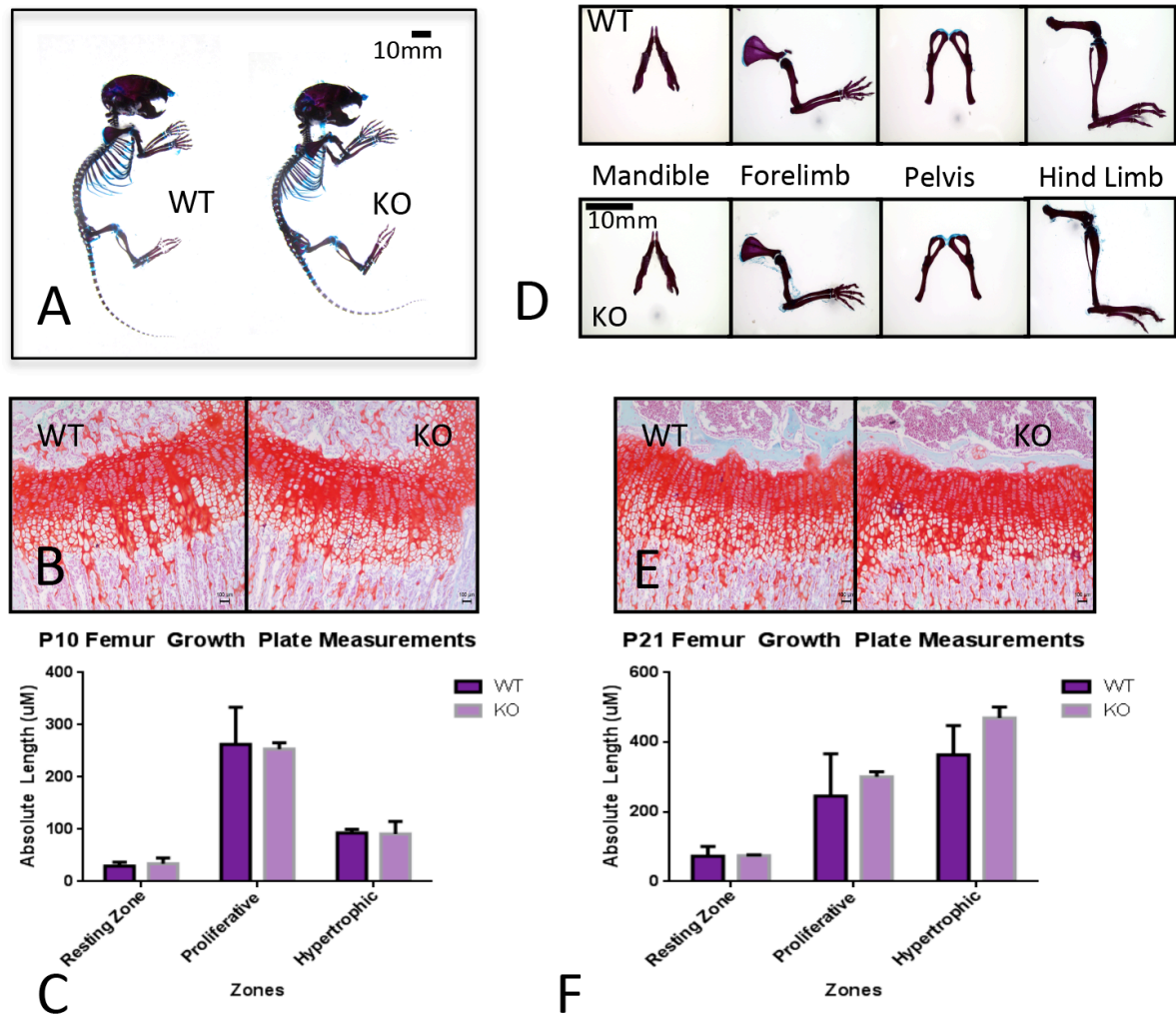


Figure 2-3 Cartilage-specific Ppard KO mice show no developmental phenotype

A) Alcian Blue/ Alizarin Red Stains of P10 control and KO mice reveal no differences in skeletal size or morphology. B) Safranin-O staining of p10 proximal femoral growth plates reveal no differences in morphology in the resting, proliferative or hypertrophic zones. C) Measurements of these zones indicate similar skeletal development between genotypes at P10. D) Alcian Blue/ Alizarin Red staining of the mandible, arm, pelvic girdle and lower leg reveal no differences in growth or mineralization between genotypes at age P21. E) Safranin-O staining of P21 proximal femoral growth plates reveals no differences between WT and control mice. F) Measurements of absolute length indicate similar absolute length of resting, proliferative and hypertrophic zones at age P21. (N≥5)

In addition, to test the effects of GW501516 on skeletal growth, embryonic day 15.5 (E15.5) tibias were isolated from wild-type CD1-mice and treated with the same concentrations of GW501516 as above for six days. There was no difference between treated and non-treated bones, as documented by measurement of tibia growth over the culture period and by Alcian Blue/Alizarin Red staining (Suppl. Fig. 3). Finally, isolated wild-type E15.5 tibias were cultured in the same system with the PPAR δ inhibitor GSK3787 or both the agonist and inhibitor together, in order to evaluate how PPAR δ inhibition affects skeletal development. Similar to the *in vivo* results, there was no difference between treated, non-treated, or combination treated bones in mineralized bone length, absolute bone length (Suppl. Figure 3), or the length of growth plate zones and the total growth plate (Suppl. Figure 4). Thus, there is no obvious developmental phenotype in our mutant mice that would interfere with the analyses of OA pathology, and inhibiting PPAR δ pharmacologically did not interfere with regular bone growth.

2.4.3 PPAR δ mutant mice display chondroprotection after surgical induction of Osteoarthritis

At 20 weeks of age, cartilage-specific *Ppard* mutant mice and wild-type control littermates underwent a destabilization of medial meniscus (DMM) surgery [30] to induce OA, and sham surgery as control. Studies of baseline mice (without surgery) at this age show no difference in articular cartilage health and morphology between genotypes (Suppl. Fig. 5). 8 weeks post-surgery, mice were harvested and histopathological analyses were performed to determine the effects of *Ppard* inactivation on OA development and progression. Eight serial frontal knee sections per mouse were assessed semi-

quantitatively by three blinded scorers according to OARSI recommendations[33]. The OARSI method uses a combination of severity, breadth and depth of damaged surface to assign a score ranging from 0-6. The four quadrants of the knee were assessed both separately and cumulatively. Wild-type mice showed significant damage after DMM surgery, as expected, in particular on the medial side of the knee (in both femoral condyle and tibial plateau; Figure 2-4 A,B,C). Remarkably, mutant mice showed a much lower OARSI score after DMM surgery than their wild-type control littermates (Figure 2-4 A,B,C). Damage in the KO DMM mice was restricted to focal glycosaminoglycan loss (Fig.2-4C), and OARSI scores were similar to those observed in sham-operated mice, as well as baseline control (no-surgery) mice. Cartilage degeneration was also assessed per animal (cumulative joint score) where results indicate a significant difference between WT DMM mice on one hand and KO DMM, sham-operated and control mice on the other hand (Figure 2-4D,E). As expected, lateral compartments showed very little damage and no significant differences between all groups (Suppl. Fig. 6). These results clearly demonstrate the chondroprotective role of *Ppard* inactivation in the DMM model and suggests that inhibition of PPAR δ is a potential therapeutic strategy in OA. Immunohistochemistry was performed to elucidate downstream mechanisms of PPAR δ inhibition (Fig. 2-5), with no-primary antibody controls (Suppl. Fig 7) Known markers of cartilage breakdown, e.g. MMP-cleaved aggrecan, ADAM-TS 4/5-cleaved Aggrecan, and

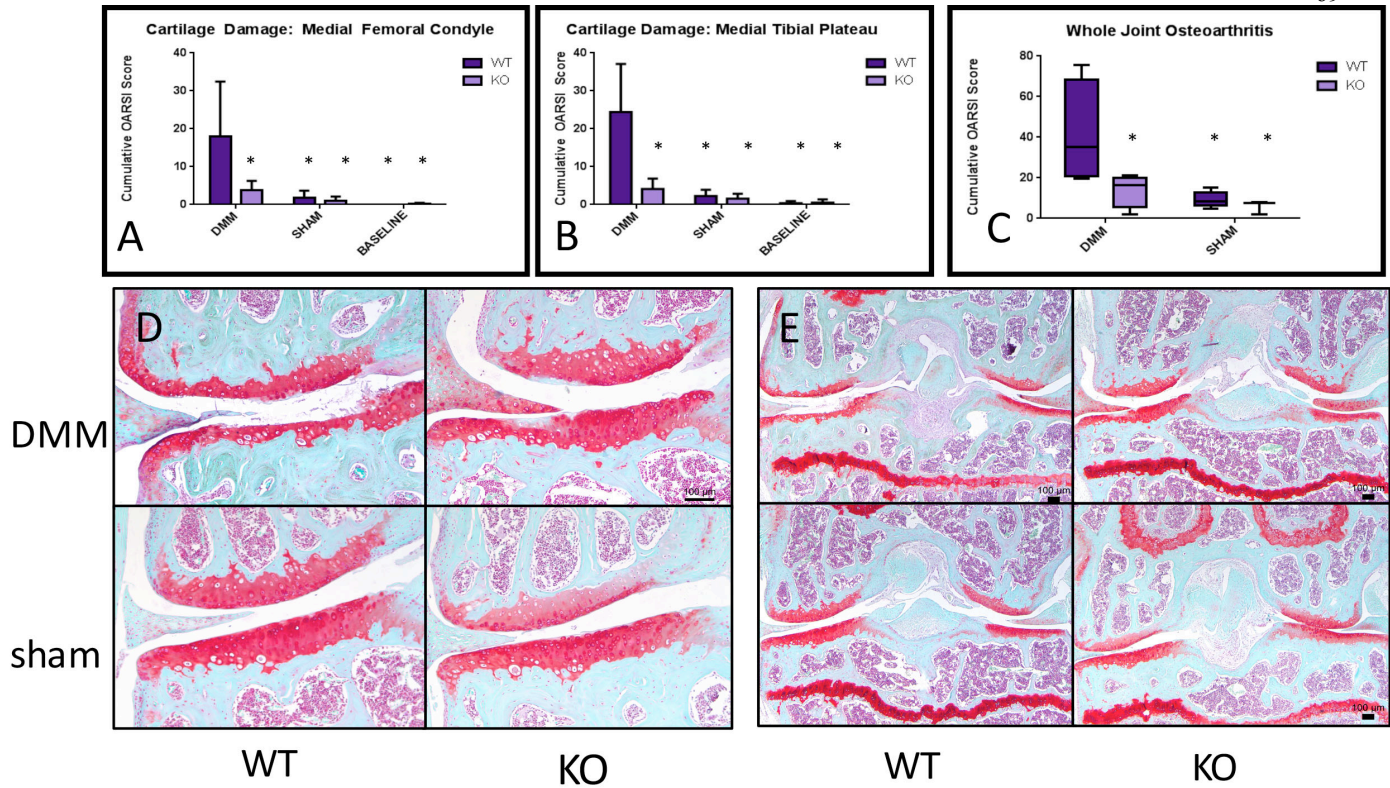


Figure 2-4 Cartilage-specific *Ppard* KO mice are protected from DMM-induced OA

Wild-type (WT) and littermate control cartilage-specific *Ppard* KO mice underwent DMM or sham surgery. 8 weeks after surgery, paraffin sections of joints were examined by Safranin O and OARSI scoring. Cartilage Damage in the Medial Femoral Condyle (MFC) was assessed by three blinded observers according to the OARSI recommendations. A) The MFC of WT DMM mice had significantly more damage than those of KO DMM, or sham-operated mice. B) Cartilage Damage in the Medial Tibial Plateau (MTP) was significantly greater for WT DMM mice than any other group. The MTP was greatest affected quadrant of the knee. *Ppard* KO mice were protected from surgical induction of OA. C) Whole Joint OA was assessed from the cumulative scores of each quadrant. KO DMM and both sham-operated groups presented with minimal damage, whereas WT DMM mice developed the most damage. D) Representative Safranin O-stained sections from the medial compartment show cartilage degeneration in WT mice after DMM surgery, while KO mice only show focal loss of Safranin O staining. E) Representative Safranin O-stained sections from the total knee joint. (N=5 DMM/sham, * $p \leq 0.05$)

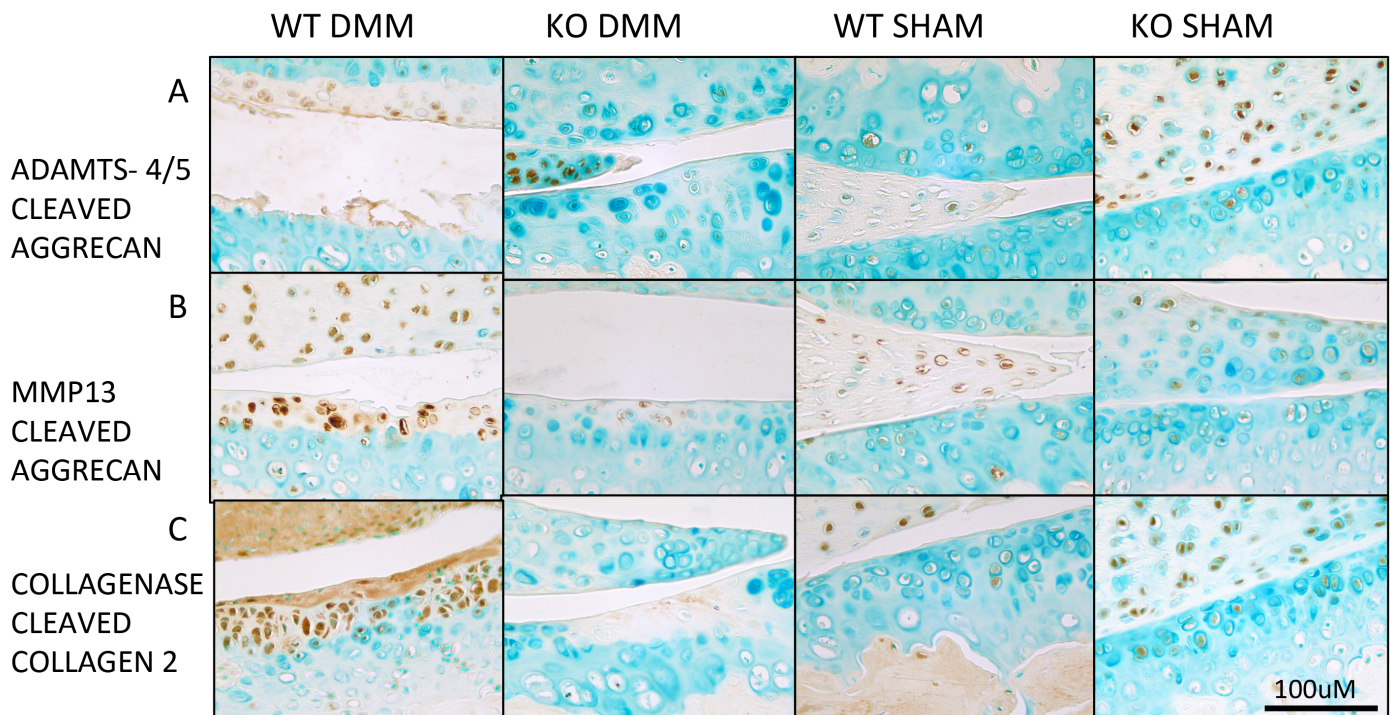


Figure 2-5 Cartilage-specific *Ppard* KO mice are protected from DMM-induced ECM degradation

WT and cartilage-specific *Ppard* KO mice underwent DMM or sham surgery. 8 weeks after surgery, mice were sacrificed, and joints were examined by IHC staining of paraffin sections. IHC indicates a strong presence of aggrecan and collagen breakdown products after DMM surgery. WT DMM mice display more intense staining for ADAMTS4/5 and MMP13-cleaved aggrecan and collagen II in cartilage than all other groups. (N=5 per group)

MMP-cleaved collagen II were assessed using staining with neopeptide antibodies. WT DMM stained sections demonstrated intense staining for all three of these neopeptides relative to sham-operated controls. In agreement with the OARSI scores, KO DMM mice showed much less neopeptide staining in cartilage. We can infer from these results that cartilage-specific *Ppard* KO mice are protected from ECM degradation in the DMM model of post-traumatic OA.

2.4.4 Discussion

Our study provides the first examination of the role of PPAR δ in the pathogenesis of OA and provides insights to the underlying molecular mechanisms that regulate this disease. Through our in-vitro, ex-vivo and in-vivo models we have demonstrated the degenerative properties of PPAR δ agonism in cartilage.

Our in-vitro studies have established that PPAR δ signaling in primary murine chondrocytes causes upregulation of genes capable of proteolytic activity on the cartilage ECM. Our ex-vivo experiments have validated these findings, with explants displaying glycosaminoglycan and aggrecan loss after treatment with GW501516. In addition, explants treated with the same agonist demonstrated significant glycosaminoglycan release into the media. This further illustrates the detrimental activity of PPAR δ agonism on the cartilage ECM, especially glycosaminoglycans and proteoglycans. Alternatively, the PPAR δ inverse agonist GSK3787 does not adversely affect skeletal growth or development in cultured embryonic tibiae. While these data suggest that PPAR δ agonism

contributes to the pathological processes that drive OA, they also identify PPAR δ inhibition as a potential therapeutic approach.

Our in-vivo study demonstrates that inactivation of PPAR δ specifically in cartilage confers significant protection from the progression of cartilage degeneration in the DMM model of post-traumatic OA. Our study also demonstrates that inactivation of PPAR δ results in decreased matrix breakdown products as the cartilage matrix remains relatively intact despite surgical induction of OA. Interestingly, our in vitro qPCR data did not show any induction of *Mmp13* transcript levels by GW501516, but we observed strong reduction in MMP-generated cleavage products in our mutant mice after DMM surgery. One possible reason for this apparent discrepancy is that MMP13 activity is regulated at multiple levels, not only transcriptionally. For example, MMP3 (which is induced at the mRNA level by GW501516) activates MMP13 through proteolytic cleavage[44], possibly resulting in greater MMP13 activity despite constant *Mmp13* mRNA levels. Additionally, increased collagen II neopeptide staining could result from MMP-2 cleavage. MMP-2 is a collagenase, and was shown to be significantly increased after treatment with GW501516, indicating a potential alternative pathway for collagen II breakdown. Our in-vitro data for qPCR shows a decrease in gene expression for certain catabolic markers (*Mmp2*, *Adamts2*, *Adamts5*, *Adamts7*, *Adamts12*) at 1 μ M relative to the 0.1 μ M treatment with GW501516. We cannot completely exclude that this is due to off-target effects of the drug at higher concentrations, but these effects could also reflect different sensitivity of different PPAR δ target genes to the agonist. For example, only high doses of agonist suppress *Sox9* mRNA levels; it is possible that these high doses are required for induction of specific target genes responsible for repressing the transcription

of *Sox9* and possibly some of the protease genes. Different levels of sensitivity to the agonist are also suggested by comparisons of the responses of metabolic target genes to the protease genes (discussed below).

PPAR δ is an orphan nuclear receptor that heterodimerizes with Retinoid X Receptor (RXR) inside the nucleus[45]. While all of the PPARs have lipids functioning as activating ligands, the same ligand can exert opposite effects on different nuclear receptors. For example, retinoic acid in the cytosol of keratinocytes has been shown to bind to both the Retinoic Acid Receptor and/or PPAR δ , depending on which cytoplasmic transporter that it commits to. This determines whether the cell ultimately undergoes growth arrest or proliferation[16, 31]. Our recent studies on another PPAR subtype, PPAR γ , indicate abnormal skeletal development in cartilage-specific knockout mice, as well as spontaneous osteoarthritis development in adult cartilage-specific knockout mice[46, 47]. Thus, PPAR γ and δ potentially have opposing roles in OA development, with PPAR γ acting in a protective and PPAR δ in a degenerative manner. Several studies conducted in lipid metabolism illustrate how PPAR subtypes can have antagonistic and complementary actions[48-51], similar to our observations.

We have also demonstrated that PPAR δ promotes fatty acid oxidation in chondrocytes, and there is potential that the dysregulation of lipid metabolism plays an important role in the initiation and progression of cartilage degeneration in OA[52, 53]. In our study we noted that the concentration of PPAR δ agonist needed to stimulate fatty acid oxidation is ten times lower than the concentration needed to promote catabolic enzyme activity. This suggests that PPAR protease genes are less sensitive to low levels of PPAR δ activation

than lipid metabolism genes. As described earlier, our findings remain consistent with other studies,[48, 54] suggesting a significant amount of fatty acid oxidation upon PPAR δ agonist treatment. In kidney cortical tubules and bovine articular endothelial cells, increased presence and activity of fatty acid oxidation enzymes is associated with greater oxidative phosphorylation and net reactive oxygen species (ROS) production[55, 56]. Additionally, ROS have been implicated in the development of OA[57-59]. Consequently, this ability of PPAR δ to induce ROS production could explain, at least in part, the opposite roles of PPAR δ and PPAR γ in osteoarthritis. It will be interesting to determine in the future whether altered lipid metabolism contributes to the function of PPAR δ in OA, or whether direct induction of protease expression in response to robust PPAR δ activation is required for cartilage destruction. The multi-factorial nature of OA and the different contributors to its development – such as mechanics, metabolism, age, and gender - give rise to a multitude of genes that may influence its progression and networks of molecules that determine its pathogenesis[9].

OA has extensive physiological, psychological and functional ramifications, and decreases mobility and increases the dependency of affected patients[60]. Our results indicating that cartilage-specific *Ppard* knockout mice are protected from OA may have tremendous potential for the treatment of OA through the identification of PPAR δ as a new therapeutic target.

2.5 Acknowledgements

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2.6 Supplementary Figures

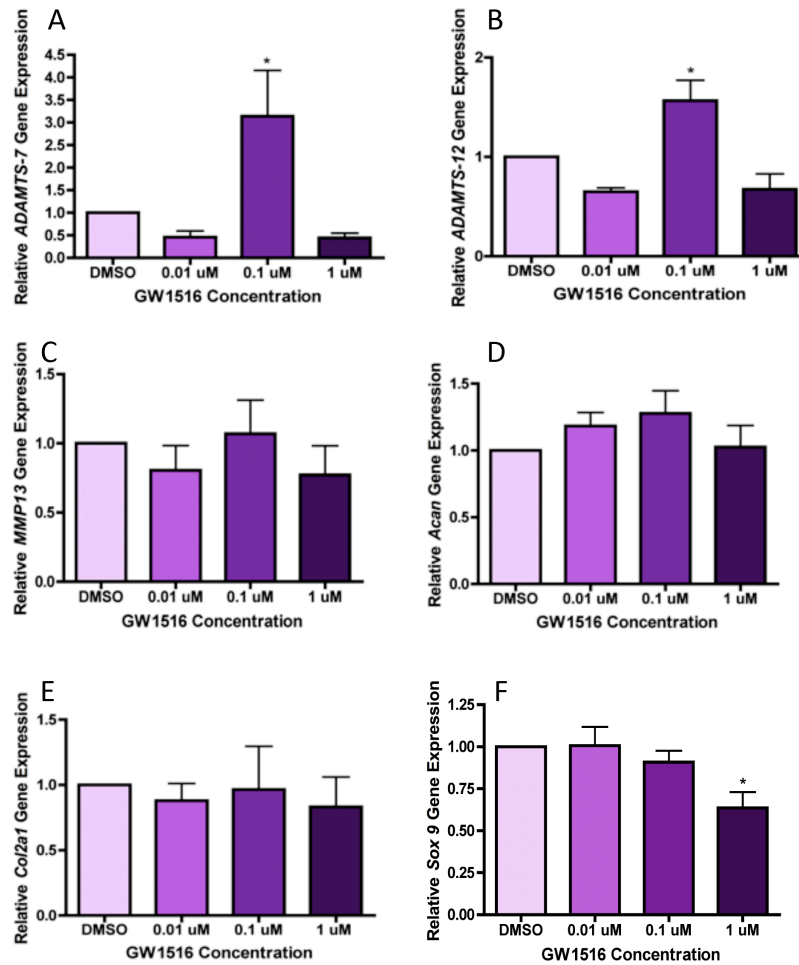


Figure 2-6, Supplementary Figure 1: PPAR δ agonism induces catabolic gene expression

A,B) Relative expression of ADAMTS-7 and ADAMTS-12 is significantly increased at 0.1uM treatment with GW501516. C) Relative Expression of MMP13 remains unchanged. D,E) Gene regulation of principal ECM components Aggrecan, and Collagen II is not significantly altered after treatment. E) qPCR analyses demonstrates that *Sox9* gene expression is slightly yet significantly decreased by GW501516 treatment (N=3, *p<0.05)

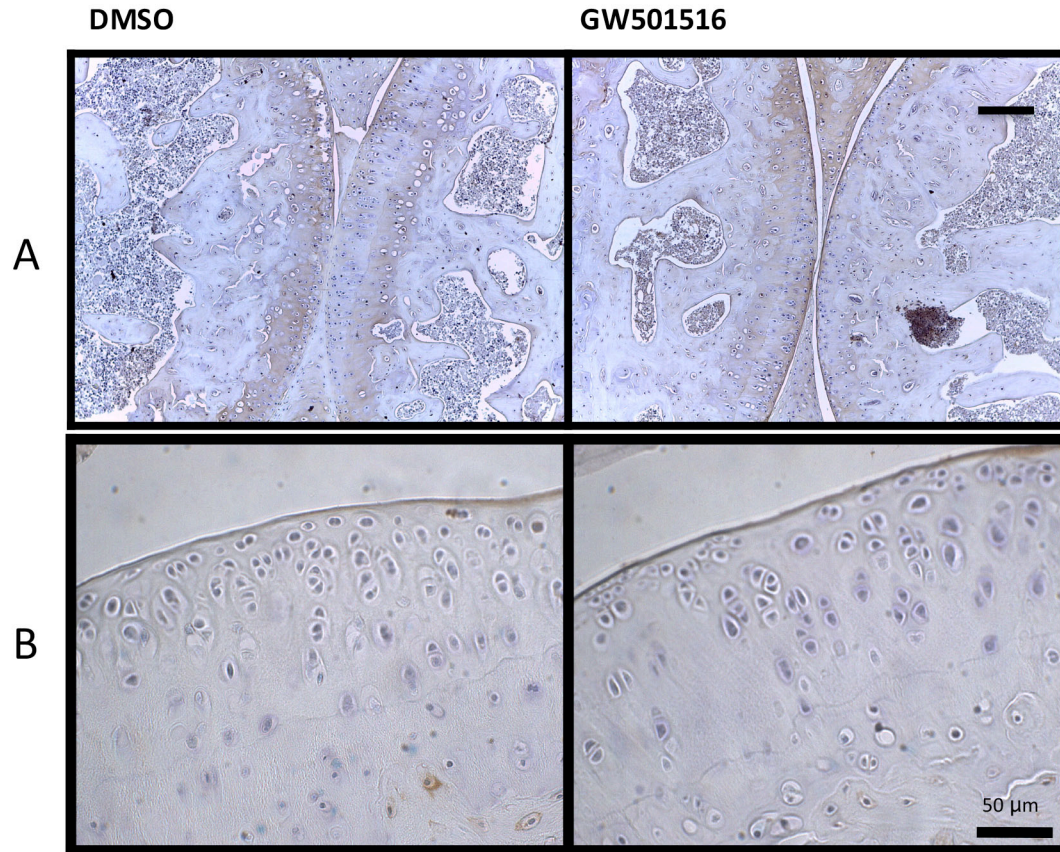


Figure 2-7, Supplementary Figure 2: No changes in Collagen 2 content of joint explants treated with PPAR δ agonist

Immunohistochemistry (IHC) for Collagen 2 on 10 week knee organ culture explants treated for 6 days with PPAR δ agonist GW501516 indicate no change in Collagen 2 or Collagen 2 breakdown product staining. A) IHC reveals no staining difference between vehicle controls (DMSO) or agonist treated explants for type-2 Collagen. B) IHC staining reveals no difference between controls or treated Collagen-2 neopeptides in the matrix of cultured knee explants.

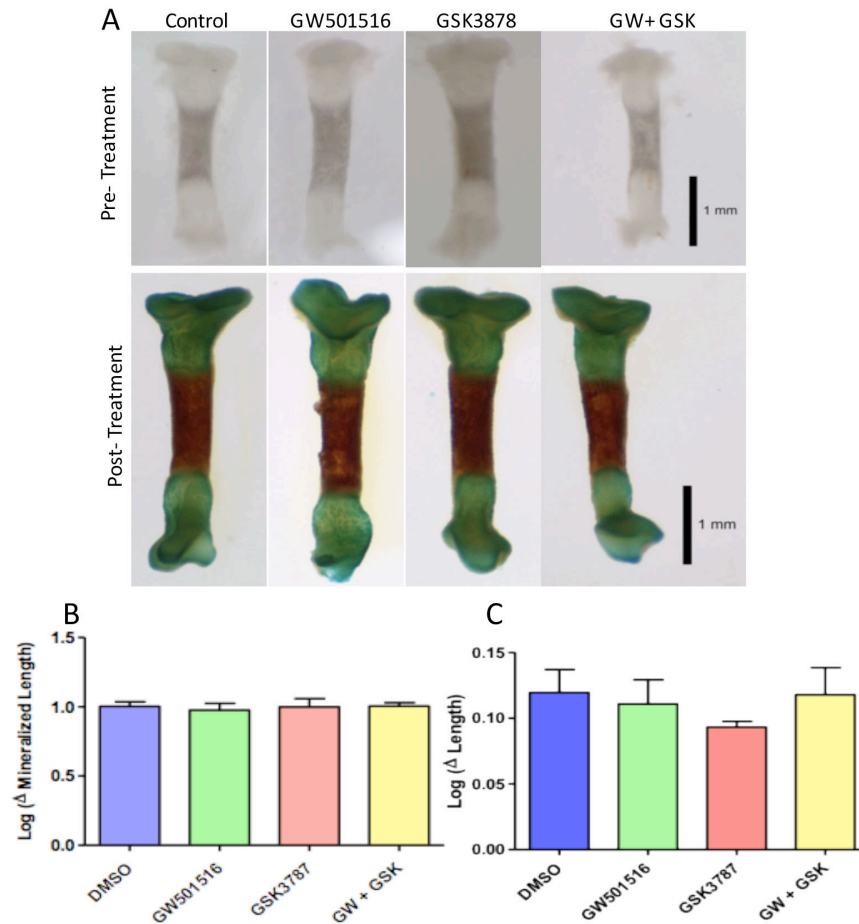


Figure 2-8, Supplementary Figure 3: Bone length and mineralization remains constant after PPAR δ agonist and inhibitor treatment

E15.5 tibias isolated from wild-type CD1 mice were cultured for 6 days with GW501516, GSK3787 (PPAR δ inhibitor), GW501516+GSK3787, or DMSO at 1 μ M concentrations. Measurements were taken immediately after dissection and before fixation. A) Alcian blue (cartilage)/Alizarin Red (bone) staining of tibias revealed no morphological differences between treatments. B) There was no change in length of the mineralized section of bones between treatments. C) No change in absolute length of bones was observed after treatment. N=3

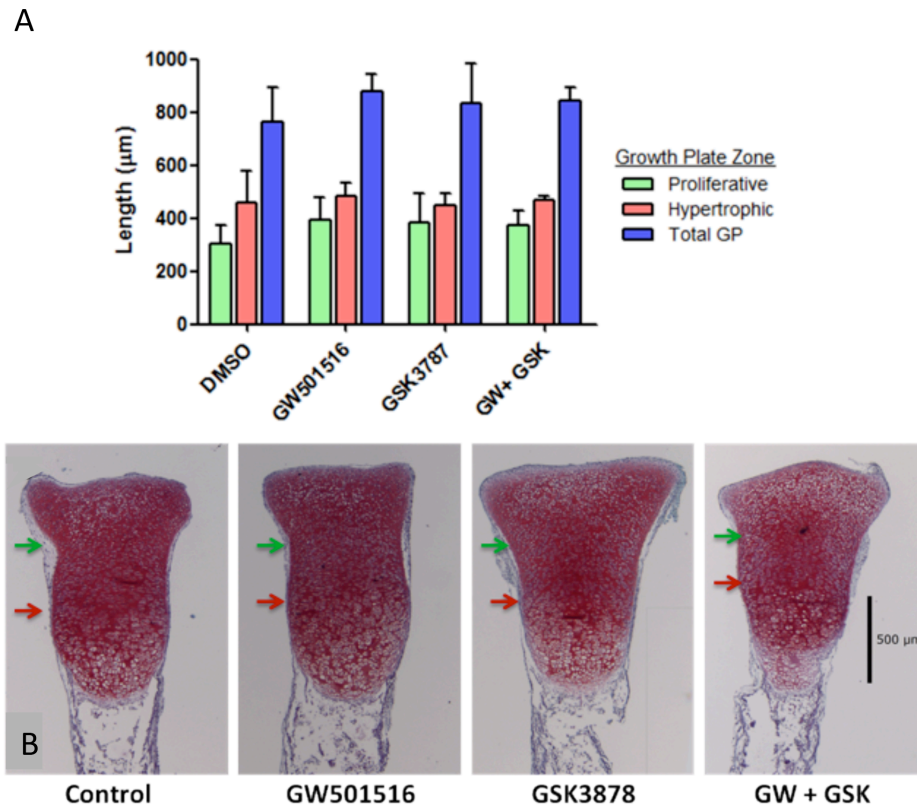


Figure 2-9, Supplementary Figure 4: E15.5 mouse tibia organ cultures reveal no effects of pharmacological PPAR δ manipulation

E15.5 mouse tibias were isolated and cultured with 1 μ M agonist (GW501516), inhibitor (GSK3787), both, or vehicle (DMSO). A) Measurements of the proliferative, hypertrophic, and total growth plate of the proximal tibia indicate no difference in zone length between treatments. B) Safranin-O/Fast Green Staining of tibias demonstrate normal growth plate morphology. The resting-proliferative borders are indicated with green arrows, while the proliferative- hypertrophic borders are indicated with red arrows. (N \geq 3)

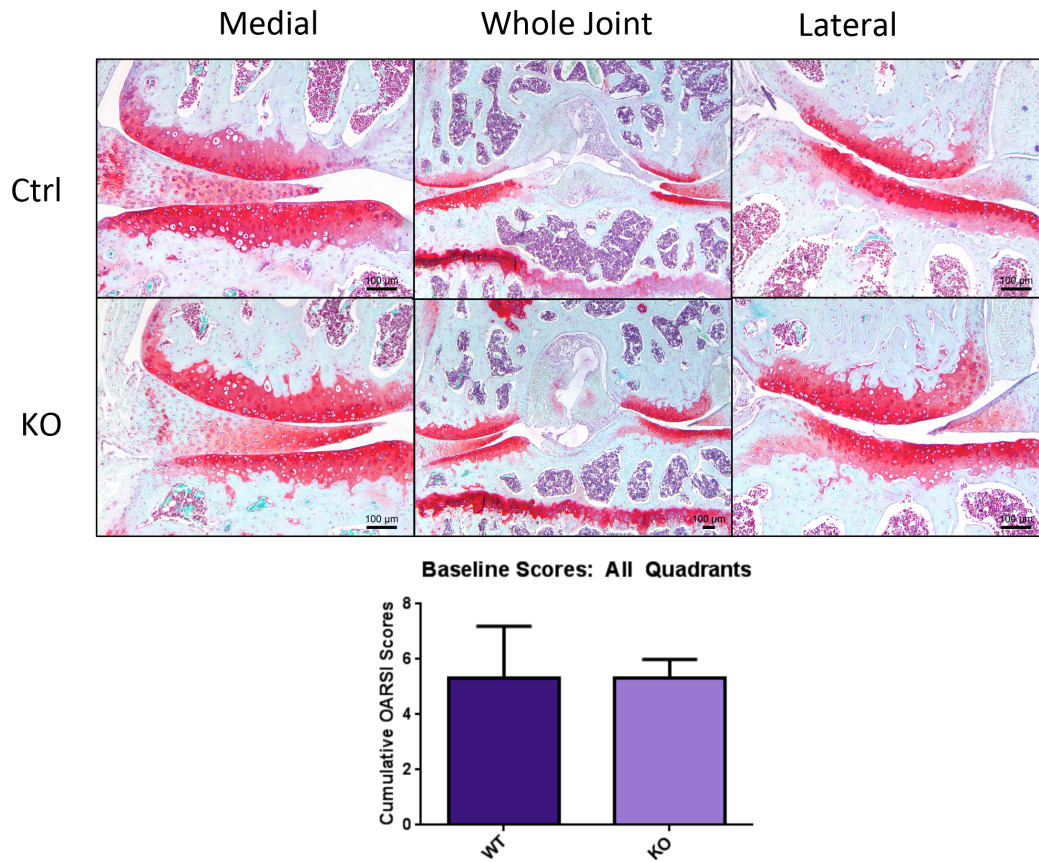


Figure 2-10, Supplementary Figure 5: Assessment of knee cartilage at baseline

Representative Safranin-O images of medial, lateral and whole-joints from 20 week-old base-line naïve mice show healthy articular cartilage with regular proteoglycan staining. (N≥3) Cumulative OARSI scores from all four quadrants indicate no difference in cartilage health between WT and PPAR δ KO mice.

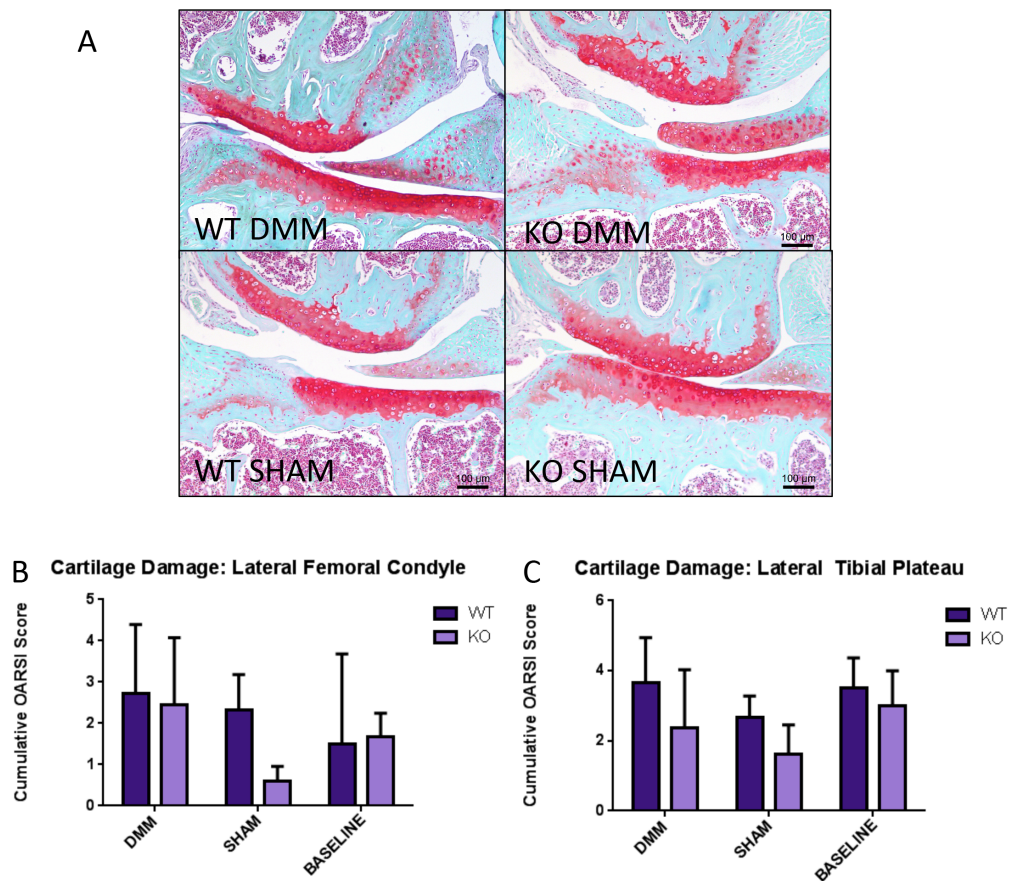


Figure 2-11, Supplementary Figure 6: OARSI scoring does not indicate differences in lateral compartments of the knee after DMM surgery

A) Safranin-O stained joints reveal little proteoglycan loss or articular cartilage damage across genotypes and surgical conditions in the lateral femoral condyle and lateral tibial plateau 8 weeks post-surgery. B,C) Cumulative OARSI scores of the Lateral Femoral Condyle and the Lateral Tibial Plateau indicate minimal damage to the articular surfaces of the lateral knee. (N=5 DMM/sham)

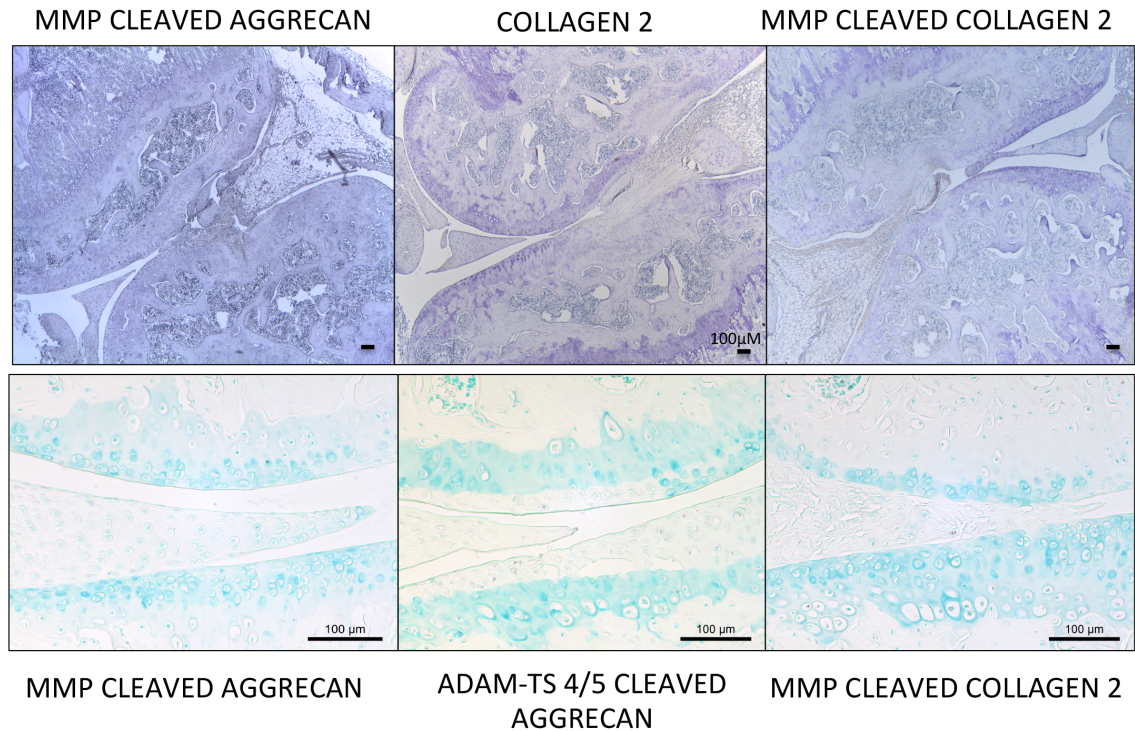


Figure 2-12, Supplementary Figure 7: No-primary antibody controls for cartilage knee explants and surgical trial mice

No primary controls for Immunohistochemical staining displays no staining for MMP cleaved aggrecan, collagen 2, MMP cleaved collagen 2 with hematoxylin counterstain in explant cultures (above). No primary antibody staining for MMP cleaved aggrecan, ADAM-TS 4/5 cleaved aggrecan, and MMP cleaved collagen 2 displays no staining with methyl green counterstain in mice after surgery (below).

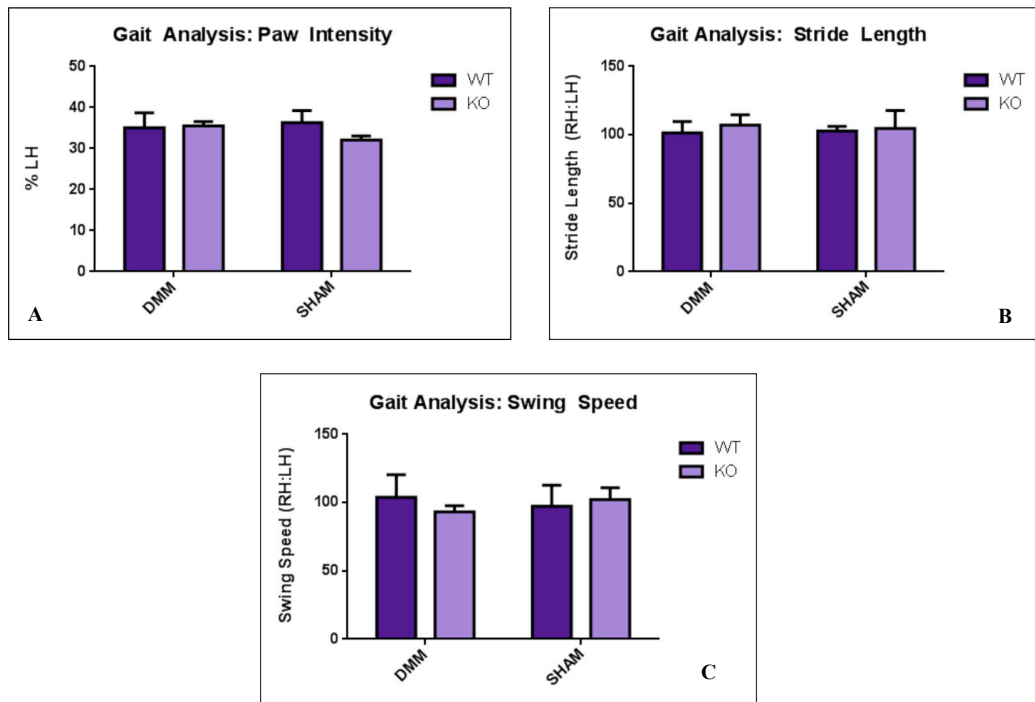


Figure 2-13, Supplementary Figure 13: Gait Analysis on mice post DMM surgery reveals no changes between groups

8 weeks post-DMM surgery, both PPAR δ and WT control littermates underwent gait analysis on the Noldus CatWalk™. Measurements were calculated as an average of 5 runs, and normalized relative to the ipsilateral limb. There were no differences between either group in A) Paw Intensity, B) Stride Length, or C) Swing Speed.

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Chapter 3

3 Effects of pharmacological administration of PPAR δ inhibitors on post-traumatic osteoarthritis

These data are currently unpublished but will contribute to an original first author research paper to be submitted for publication.

Ratneswaran A, Pest MA, Hamilton C, Dupuis H, Pitelka V, Chesworth B, and Beier F. Effects of pharmacological administration of PPAR δ inhibitors on post-traumatic osteoarthritis. To be submitted to Arthritis and Rheumatology, 2016.

3.1 Abstract

Objective: Currently there are no effective drugs to alter the course of disease progression in Osteoarthritis (OA). Post-traumatic osteoarthritis (PTOA) is a subtype of OA that is initiated by joint trauma. We have previously demonstrated that genetic ablation of the nuclear receptor PPAR δ results in protection from cartilage damage after induction of PTOA in a murine model. We aim to characterize whether pharmacological inhibition of PPAR δ is a feasible treatment to stop or delay the progression of osteoarthritis.

Methods: PTOA was induced in male rats via anterior cruciate ligament transection (ACLT) with partial medial meniscectomy (PMMx) surgery, with SHAM surgery as control. PPAR δ inhibitors (GSK0660, GSK3787) or vehicle control (DMSO) were administered daily for four weeks (6 days on, 1 day off) via subcutaneous injection at a dose of 1mg/kg. Behavioural outcomes were assessed through Open Field Testing and

Incapacitance testing. Structural progression of OA was evaluated using Toluidine Blue staining of paraffin sections with histopathological scoring, and polarized picosirius red.

Results: Rats undergoing ACLT/PMMx surgery exhibited behavioral changes (less movement and vertical activity) that were blocked by either PPAR δ inhibitor. However, PPAR δ inhibition did not significantly protect from progression of cartilage and subchondral bone damage.

Conclusions: PPAR δ inhibition positively affects functional outcomes after PTOA induction in rats, but our current data do not indicate efficacy to alter disease progression.

3.2 Introduction

Osteoarthritis (OA) is a musculoskeletal disorder that culminates with joint failure. It may affect one or more joints, and can stem from systemic or local factors that initiate a cascade of inflammatory and degradative events in one or more joint tissues, leading to impaired function and joint pain[1-3]. While primary age-associated OA predominantly affects individuals over the age of 60, post-traumatic OA (PTOA) affects younger individuals - usually starting under the age of 40[4]. Primary OA is characterized by the gradual deterioration of articular cartilage, synovitis and remodeling and hypomineralization of subchondral bone[5, 6]. PTOA shares many of these characteristics in its later stages but its initial and acute stages are markedly different.

PTOA develops after joint trauma, particularly mechanical overload from ligament or meniscus injuries and results in 900,000 new cases annually in the U.S[6]. Initial impact from injury can cause destruction to the collagen 2 framework of the cartilage ECM, as

well as glycosaminoglycan loss and chondrocyte death[7, 8]. Cartilage can shear from the subchondral bone plate, and fractures of the articular cartilage and underlying bone can occur. Bone marrow edema, and hemarthrosis are also common features of PTOA[9, 10].

In the months after the injury, there is sustained systemic inflammation through increased expression of inflammatory markers such as IL-6 and TNF- α in synoviocytes, and IL-1 in both synoviocytes and chondrocytes[11]. These inflammatory mediators can act on the synovium and cartilage to induce expression of matrix degrading enzymes such as the matrix metalloproteinases (MMPs)[12]. As a consequence of hemarthrosis and inflammatory cell invasion, the synovial fluid has decreased concentrations of both hyaluronic acid and lubricin[13, 14]. Eventually, the inflammatory response subsides but 20-50% of any joint trauma still leads to the progression of OA[15]. PTOA can develop even after reconstructive surgery to repair ligaments, joint congruity and mechanics. Unfortunately, the age demographic of PTOA combined with the limited lifespan of implants renders joint replacement surgery a poor option[4, 16]. Currently, there are no treatments to stop the structural progression of OA and no effective chronic pain medications to ameliorate the burden of disability for patients experiencing this condition[17].

Nuclear receptors have been suggested as attractive pharmacological targets due to their ability to bind ligands and regulate transcriptional activity[18]. Our previous studies have shown that the nuclear receptor PPAR δ promotes induction of proteases and destruction of articular cartilage in-vitro. We have also demonstrated that inhibition of PPAR δ , through cartilage specific inactivation of the *Ppard* gene protects against

cartilage degeneration after surgical induction of OA through a destabilization of medial meniscus (DMM surgery)[19]. However, to determine whether inactivation of PPAR δ could be a feasible treatment option for humans with PTOA, we needed to characterize if pharmacological inactivation of PPAR δ protects against the progression of PTOA.

In the present study, we examined the effects of pharmacological PPAR δ inhibition on OA progression, after surgical induction of OA through anterior cruciate ligament transection (ACLT) and partial medial meniscectomy (PMMx). Our data showed that PPAR δ inhibition results in improved functional outcomes 4 weeks after surgery, yet unaltered structural pathology.

3.3 Methods

3.3.1 Animals and Surgery

All animal experiments were approved by the Animal Use Subcommittee at The University of Western Ontario and were conducted in accordance with the guidelines from the Canadian Council on Animal Care. Rats were group housed (2 rats per cage) preceding surgery, on a standard 12h light/dark cycle with free access to standard rat chow and water. Rats were separated to single housing post-surgery.

Anterior cruciate ligament transection (ACLT) with partial medial meniscectomy (PMMx) surgery or SHAM surgery was performed on the right knees of male rats weighing 300-350g (Charles River Laboratories, Quebec, Canada) at baseline, as described [20, 21]. Isoflurane (5% induction, 2% maintenance) was used as surgical anesthetic. Saline (9g/L NaCl, 5 mL total) was administered postoperatively for hydration. Buprenorphine (0.1 mg/kg x2) was administered intramuscularly as a post-

operative analgesic, and ampicillin (10mg/kg x 2) was administered subcutaneously as a prophylactic antibiotic. Rats were randomly allocated to one of four groups, ACLTx/PMMx with vehicle control (DMSO), PPAR δ inhibitor GSK3787, PPAR δ inhibitor GSK0660, or SHAM operation (with DMSO). Rats were injected subcutaneously with one of the above specified treatments for 6 days a week for 4 weeks. Weight was measured weekly. Rats were euthanized at 4 weeks post surgery for preparation of paraffin sections, blood glucose and liver weight measurements, and subsequent histological analysis.

3.3.2 Behavioural Testing

Exploratory behaviour in rats was assessed by measuring spontaneous locomotor activity. Rats were placed in an Open Field Tester (Omnitech Electronics Inc., Columbus Ohio) for 30 minutes, 1 day per week in the dark. Rest, movement, ambulation and vertical activity time and incidence were measured as a proxy for OA pain, as described [22].

Incapacitance testing was conducted to measure load distribution per limb. Rats were placed on an incapacitance tester (Linton Instruments, Norfolk, UK) and ipsilateral: contralateral limb load was calculated to measure change in load distribution or compensation after surgery as per [22].

3.3.3 Histopathology and Scoring

Right knees were dissected, fixed in 4% paraformaldehyde, decalcified in Formical-4TM (StatLab, Baltimore, MD), bisected along their coronal plane, embedded in paraffin and sectioned frontally. Serial sections of 6 μ M width were stained with Toluidine Blue for glycosaminoglycan content and subsequent histopathological scoring or with Picrosirius

Red (0.1% Sirius red in picric acid) for staining of collagen fibril content and organization[23]. Polarized light microscopy was used to evaluate size and organization of collagen fibrils, and light intensity and tissue angle were kept identical between samples as per [23].

Toluidine Blue stained sections were evaluated by 2 blinded observers, and cartilage degeneration and subchondral bone damage in four quadrants of the knee were graded using the Osteoarthritis Research Society International (OARSI) histopathologic scale[24].

3.3.4 Statistical Analysis

All statistics were performed in GraphPad Prism v.6.2. Blood glucose, liver weight and incapacitance-testing were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Weight change over time was analyzed using a one-way ANOVA with repeated measures. Open Field Testing for spontaneous locomotor behaviour was analyzed using a two-way repeated measures ANOVA with Bonferroni post-hoc test. OARSI histopathological scoring was analyzed using a Kruskal-Wallis test followed by Dunn's post-hoc.

3.4 Results

3.4.1 Rats treated with PPAR δ inhibitors do not demonstrate systemic abnormalities

PPAR δ is a regulator of metabolism in many tissues, and to evaluate whether systemic delivery could cause physiological and anthropometric changes we measured weight change, blood glucose and liver weight. There was no difference in body weight over the

time-course of four weeks post-surgery between rats that received DMSO, GSK3787, GSK0660 or the SHAM operation (Fig 3-1). Similarly, there were no differences in blood glucose or liver weight between groups at 4 weeks post-surgery.

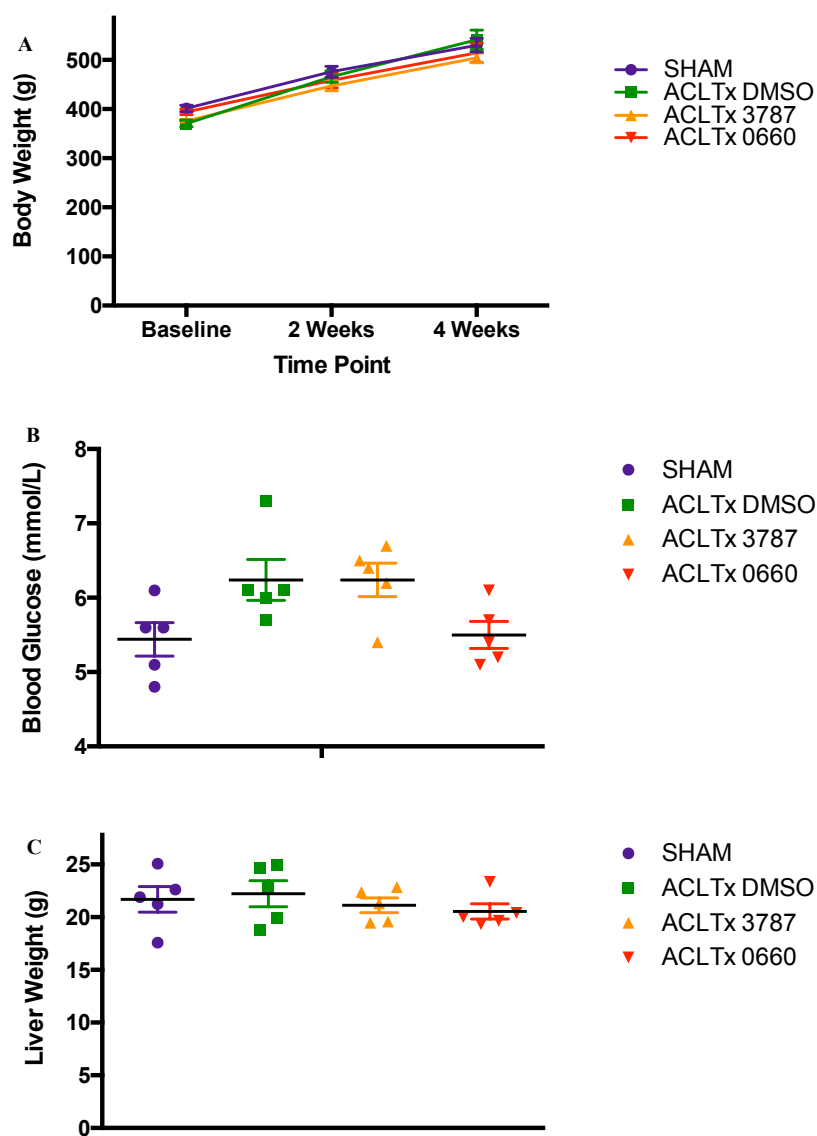


Figure 3-1 Anthropometric and Physiological Characteristics after PPAR δ inhibition and surgical induction of OA

Over the course of 4 weeks post-ACLT/PMMx or SHAM surgery, rats do not demonstrate differences in body weight (g) regardless of vehicle control (DMSO), GSK3787, or GSK0660 treatment (A). Similarly, there were no significant differences in blood glucose (mmol/L) or liver weight (g) four weeks post-surgery between any of the treatment groups (B, C). N=5 per group, data shown are mean \pm SEM.

3.4.2 Rats receiving PPAR δ inhibitors are protected from OA-induced behavioral changes

To evaluate changes in behavior that were indicative of pain, rats were examined through Open Field Testing to measure changes in spontaneous locomotor activity. Throughout the course of 4 weeks, as rats developed more severe structural pathology, rats who underwent ACLT/PMMx and were administered DMSO significantly modified their behavior by resting more (Fig 3.2), and performing less vertical activity (Fig 3-2). This increase in pain behavior was not experienced by rats who received either PPAR δ inhibitor, or rats who underwent the SHAM operation, suggesting that they were not experiencing the same level of pain.

3.4.3 Rats receiving PPAR δ inhibitors do not alter load distribution in hind-limbs

In order to investigate whether there were changes in load distribution between the surgically operated limb and contralateral limb, Incapacitance testing was employed. As OA progresses, behaviour is modified to place less weight on the joint that is affected; accordingly 4 weeks post-surgery rats in the ACLT/PMMx group treated with DMSO compensated, increasing the weight on the contralateral limb and thus decreasing the ipsilateral:contralateral ratio (Fig 3-3).

3.4.4 Histopathological Scoring does not indicate significant differences in disease progression with PPAR δ inhibition

At 4 weeks post-surgery, rats were euthanized and their joints were harvested for histopathologic analyses to determine the effect of PPAR δ inhibition on the progression

of OA. Eight serial frontal sections per rat were assessed semi-quantitatively by 2 blinded observers using the OARSI recommendations[24]. Cartilage degeneration was scored by

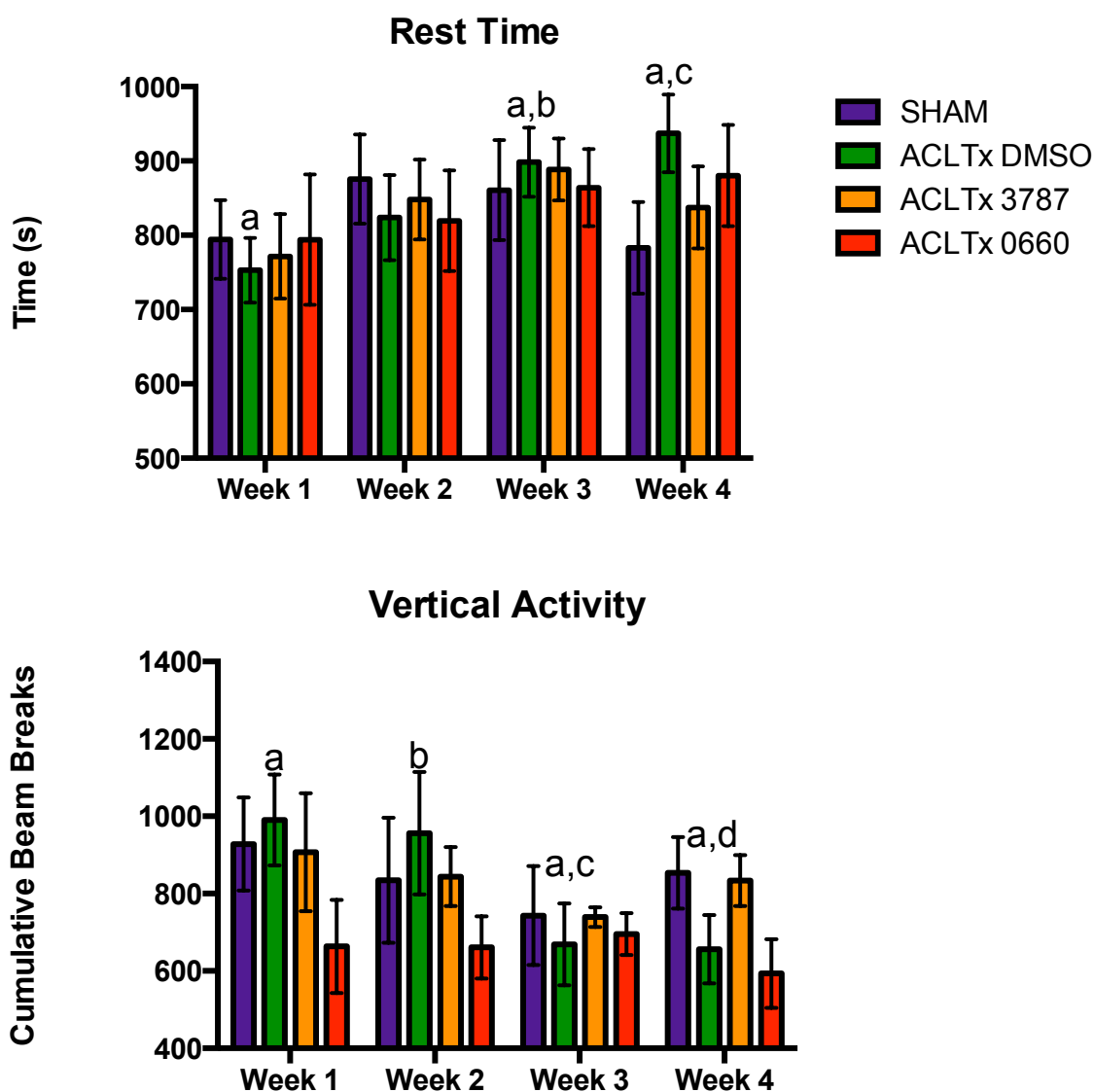


Figure 3-2 PPAR δ inhibition prevents rats from OA pain like behavioural modification post-surgery

Open Field Testing over the course of four weeks demonstrates changes in spontaneous locomotor activity. Rats receiving DMSO daily for four weeks post-ACLT/PMMx (green bar) demonstrate pain invoked behavior modification such as increased rest time (**A**) and decreased vertical activity (**B**). These changes were not seen in SHAM operated rats (purple bars) or rats undergoing post-ACLT/PMMx surgery with either PPAR δ inhibitor (orange, red bars). N=5 per group, data shown are mean \pm SEM, $p \leq 0.05$.

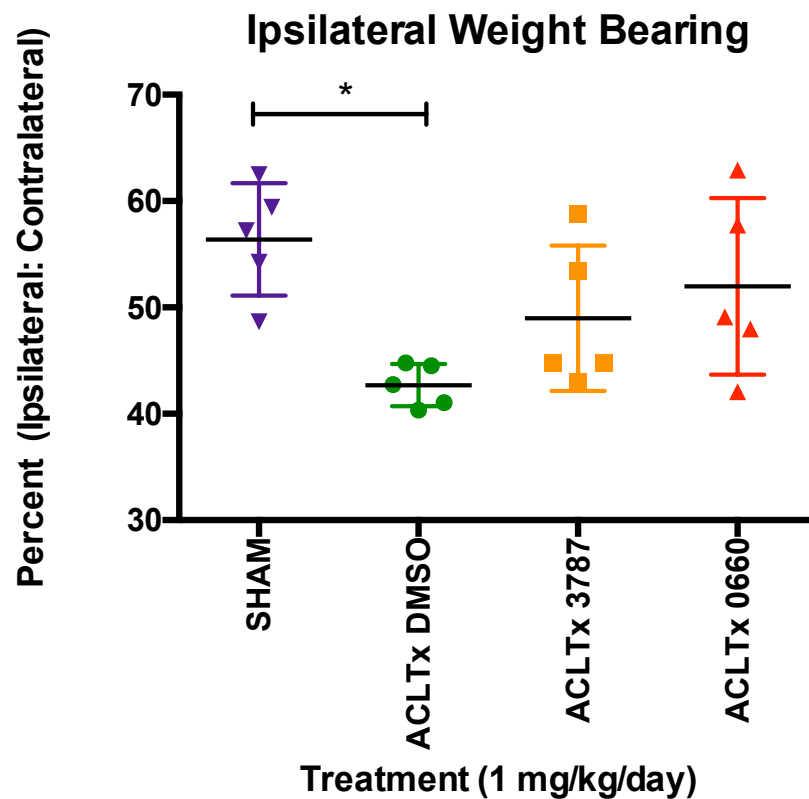


Figure 3-3 PTOA causes load redistribution in hind-limbs

4 weeks post ACLT/PMMx surgery, rats underwent Incapacitance testing to determine load placed on each of the hind limbs. Rats who underwent surgical induction of OA, and were administered vehicle control DMSO significantly favoured their un-operated limb, demonstrating decreased ipsilateral: contralateral weight bearing. N=5 per group, data shown are mean \pm SEM, $p \leq 0.05$.

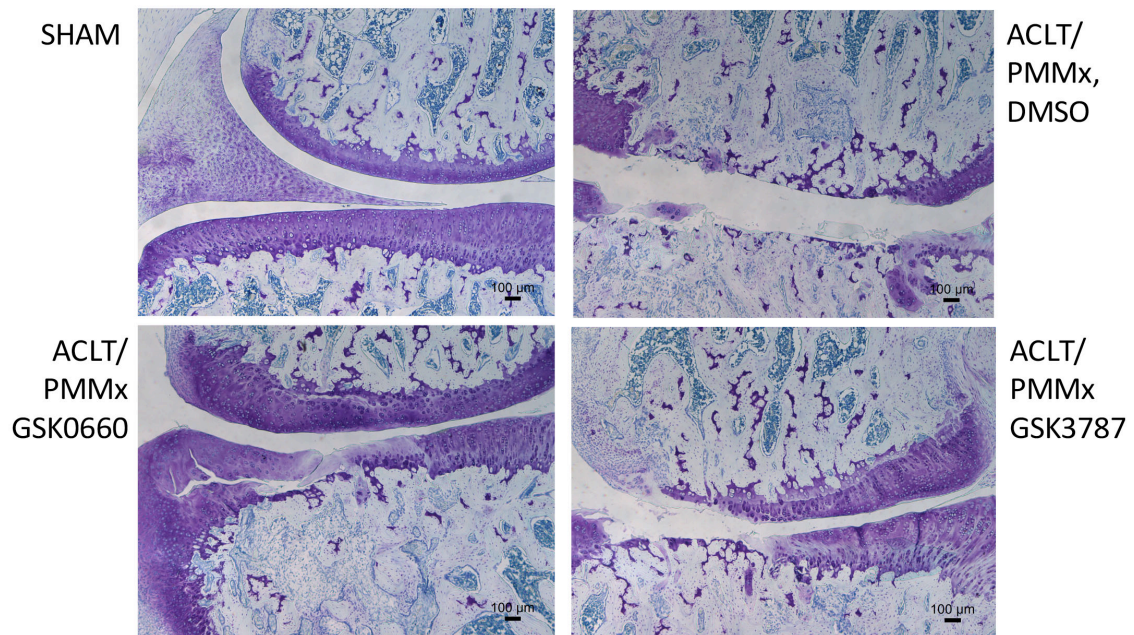


Figure 3-4 Rats receiving PPAR δ inhibitors still experience cartilage degeneration and subchondral bone changes

Toluidine Blue staining on serial frontal sections indicates severe cartilage erosion in the medial femoral condyle and medial tibial plateau of rats treated with DMSO after surgical induction of OA via ACLT/PMMx. Articular cartilage collapse into subchondral bone and chondrogenesis is apparent. GSK0660 treated rats have observed cartilage shearing and focal cartilage defects as well as fractures of the tidemark and marrow changes. Similarly, GSK3787 treated rats experience cartilage shearing, proteoglycan loss, subchondral bone thickening and chondrogenesis in underlying bone. SHAM operated rats present with healthy cartilage, subchondral bone and menisci.

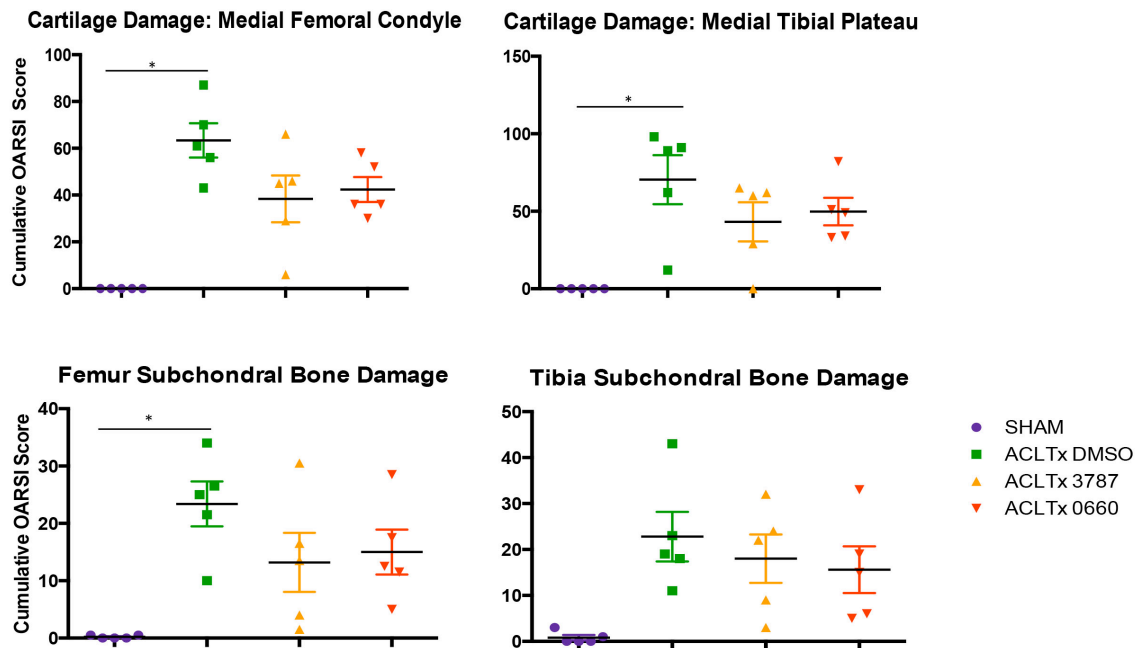


Figure 3-5 Semi-quantitative assessment of knee joint histopathology after surgical induction of PTOA

Structural progression of OA was semi-quantitatively assessed via OARSI scoring for cartilage degeneration, and subchondral bone damage. DMSO (vehicle control) treated rats had significantly more cartilage, and subchondral bone damage in either quadrant of the medial side versus SHAM operated animals. N=5 per group, data shown are mean±SEM, $p \leq 0.05$.

subdividing the quadrant into thirds (outer, mid, inner) and scoring each third from 0-5, with 0 being no degeneration and 5 indicating severe degeneration with more than 75% of cartilage lost. Each of the thirds is summed, and the aggregate score is out of 15. Subchondral bone and calcified cartilage was also evaluated with a score of 0-5; 0 indicating no pathology and 5 indicating severe pathology with articular cartilage collapse into subchondral bone and the formation of bone cysts. Rats that underwent the SHAM operation had healthy cartilage and subchondral bone. Rats that underwent ACLT/PMMx followed by treatment with vehicle control DMSO, GSK3787 or GSK0660 experienced varying degrees of focal fibrillation, glycosaminoglycan loss, and subchondral bone remodeling (Fig. 3-4) This damage was most severe in the ACLT/PMMx DMSO group which was significantly different compared to the SHAM operated group in terms of cartilage degeneration and subchondral bone damage in the medial femoral condyle, and medial tibial plateau (Fig 3-5). This damage was not significant between either PPAR δ inhibitor treated group and the surgically induced OA DMSO treated rats, or the SHAM group. These data suggest that the effects of PPAR δ inhibition might be somewhat protective but not statistically significant within the experimental parameters.

3.4.5 Picosirius red staining indicates bone remodeling in vehicle control rats but not in PPAR δ inhibitor treated rats

Picosirius red staining was performed on frontal sections from rats that underwent surgical induction of OA and SHAM rats. Stains were imaged under polarized light, where healthy articular cartilage showed mixed tones of orange with green and yellow

birefringence. Interestingly, the articular cartilage from the rats after surgery had largely orange birefringence, indicating a change in fibril organization. The subchondral bone had clear trabeculae. In contrast, the ALCT/PMMx vehicle controls presented with strong yellow and green fibers oriented in the same plane, indicating remodeling activities (Fig. 3-6).

3.5 Discussion

OA is a common, debilitating problem affecting individual quality of life and places an enormous economic burden on health and social services[25]. PTOA comprises 12% of OA cases and follows a distinct pathogenesis with immediate and acute phases of the disease largely contributing to irreversible joint damage[4, 6]. Arguably, early interventions have tremendous potential to limit or delay damage.

Our study examines whether inhibition of the nuclear receptor PPAR δ can delay the onset or progression of OA after surgical induction of PTOA through ACLT/PMMx. We discovered that systemic treatment of rats with PPAR δ inhibitors demonstrate a suppressed behavioral response, and maintenance of normal function over the course of four weeks.

In a previous paper published by our group, we demonstrate functional deficits in vertical activity and rest time after ACLT/PMMx[22]. Our study replicated this finding; vehicle control rats after surgery had decreased vertical activity and increased rest time (decreased movement time).

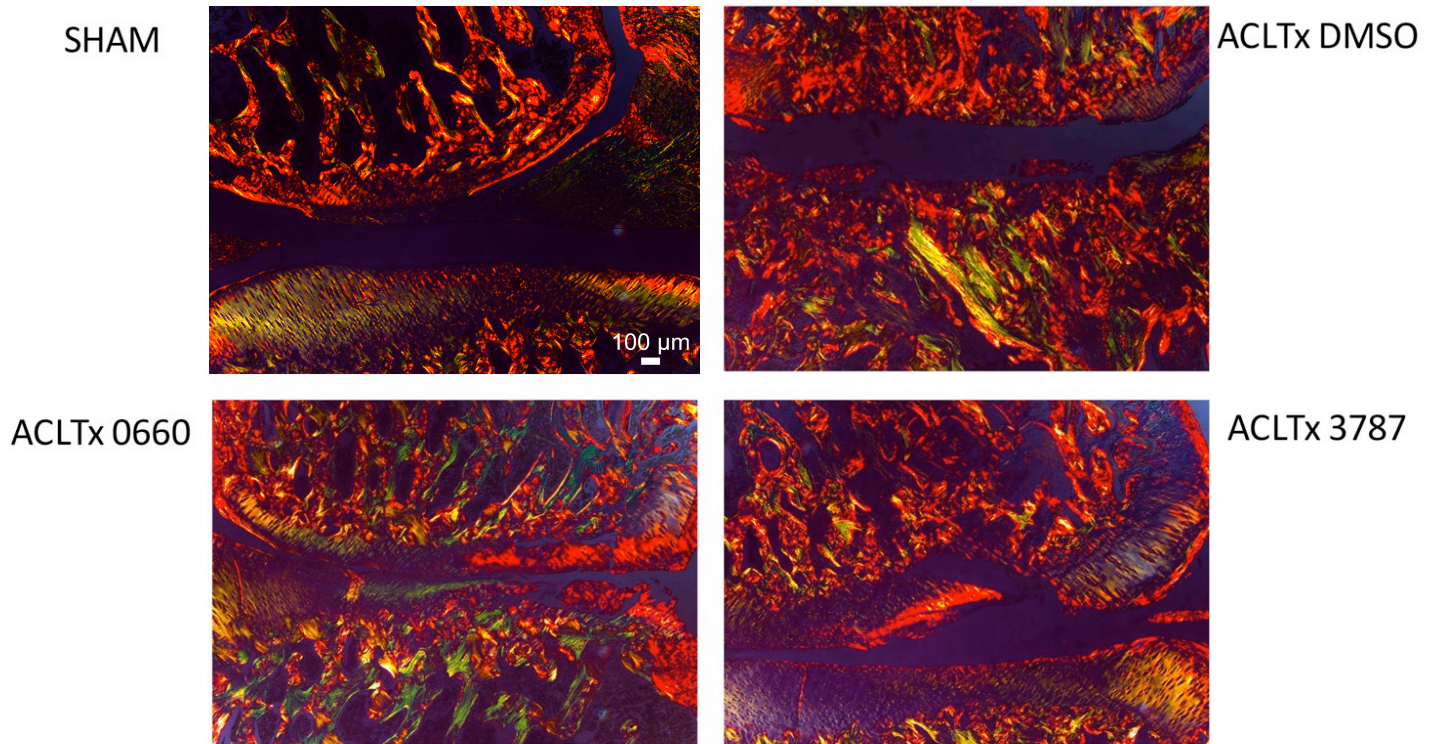


Figure 3-6 Examination of Collagen fibre structure and organization

Picosirius red staining under polarized light reveals changes in subchondral bone organization and structure. Strong yellow and green birefringent band in ACLT/PMMx DMSO group indicates change in collagen fibril organization and bone structure. (N=5 per group).

We also showed inhibitor treated rats and SHAM rats do not, further cementing improved functional outcomes with PPAR δ inhibition. Together these data suggest that PPAR δ inhibition protects from pain caused by surgical induction of PTOA.

Joint pain in OA has a complex pathophysiology; chronic pain can result in mechanical allodynia where even innocuous stimuli can elicit a pain response[26]. It can also result in hyperalgesia, where stimuli distant to the joint can cause pain[27]. We first considered whether the pain could be correlated with structural protection from the progression of OA. Indeed, the severity of cartilage degeneration and subchondral bone damage appeared less advanced in the PPAR δ inhibitor treated animals, but it did also not confer significant protection according to semi-quantitative histopathological assessment. Our group has previously demonstrated that PPAR δ agonism results in the induction of matrix degrading enzymes, significant upregulation of fatty-acid oxidation and inhibitors of anti-oxidants in chondrocytes [Ratneswaran et al, submitted]. These data indicate that inhibition of PPAR δ could protect cartilage and possibly bone by reducing aberrant beta-oxidation and oxidative stress. It is therefore likely that some of the pain relief that we observe is correlated with attenuated structural pathology.

However, it is important to note that structural progression is not always associated with joint pain in OA. A number of individuals with established radiographic disease are asymptomatic for pain[28]. It is also probable that PPAR δ acts directly on the nervous system to influence pain behaviour. PPAR δ is the most highly expressed PPAR protein in the central nervous system, and is expressed throughout the brain, in oligodendrocytes

and neurons[29]. In particular, it is present in the thalamus, indicating that it may play a role modulating response to pain[30]. Further, COX-2 inhibitors have been shown to suppress PPAR δ expression and have been used clinically (Rofecoxib) as NSAIDs in osteoarthritis to ameliorate inflammation associated pain, indicating a possible mechanism for the changes we see[30, 31].

PPAR δ is broadly expressed in the body, and has roles in glucose and lipid metabolism, cell proliferation, apoptosis, and immune regulation[32]. Although systemic delivery of PPAR δ inhibitors did not result in deleterious physiological changes, it was only over a time course of four weeks. If PPAR δ inhibitors are a feasible therapeutic target for OA, they need to be locally delivered to the joint, and longer time courses must be evaluated. Additionally, we will need to evaluate the quantity of drug that reaches the cartilage. We will also need to increase the N of our experiments to examine whether the observed trend towards structural protection by PPAR δ inhibitors reaches statistical significance.

It would also be informative to evaluate the role of PPAR δ inhibition in early OA, and in other subtypes of OA such as metabolic OA and age-associated OA to investigate whether the protection conferred would be similar. Our study has elucidated the feasibility of PPAR δ inhibition in post-traumatic OA. We have shown that PPAR δ inhibition results in decreased pain-associated behaviour, and this may be modulated through delayed structural progression. The molecular mechanisms underlying OA remain poorly understood, and in order to deliver an effective treatment for PTOA we must continue to explore these mechanisms, concurrent with methods of local drug delivery.

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Chapter 4

4 Nuclear receptors regulate lipid metabolism in chondrocytes

These data have been submitted to Arthritis and Rheumatology and are awaiting review.

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4.1 Abstract

Objective: Failure of joint homeostasis can result in osteoarthritis (OA). Metabolic OA is characterized by dysregulation of lipid and cholesterol metabolism which can detrimentally affect cartilage. Currently, there are no available treatments to alter disease progression in OA, but targeting early changes in cellular behaviour has great potential. Recent data show that nuclear receptors contribute to the pathogenesis of OA and could be viable therapeutic targets, but their molecular mode of action in cartilage is incompletely understood. The purpose of the present study was to examine global changes in gene expression after treatment with agonists of four nuclear receptors implicated in OA (LXR, PPAR δ , PPAR γ and their heterodimeric partner RXR).

Methods: Immature murine articular chondrocytes were treated with pharmacological agonists for LXR, PPAR δ , PPAR γ or RXR and underwent microarray analysis, qPCR and cell lipid assays to evaluate changes in gene expression, expression, and lipid profile. Immunohistochemical analysis was conducted to compare presence of one identified differentially expressed target (Txnip) in WT mice and cartilage-specific PPAR δ knockout mice subjected to surgical destabilization of the medial meniscus (DMM).

Results: Nuclear Receptor agonists increased expression of several genes regulating lipid metabolism, but each agonist induced a differential profile of responses. LXR activation downregulated gene expression of proteases involved in OA, whereas RXR agonism decreased gene expression of ECM components, and increased gene expression of *Mmp13*. Functional assays indicate increases in cell triglyceride accumulation after PPAR γ , LXR and RXR agonism but a decrease after PPAR δ agonism. PPAR δ and RXR both downregulate the antioxidant GSTA4, and PPAR δ upregulates Txnip. WT, but not PPAR δ -deficient mice display increased staining for Txnip after surgical induction of OA.

Conclusions: These data demonstrate that nuclear receptor activation in chondrocytes primarily affects lipid metabolism. In the case of PPAR δ this change might lead to increased oxidative stress, possibly contributing to OA-associated changes described earlier.

Keywords: Cartilage, chondrocyte, joint, lipid metabolism, osteoarthritis, oxidative stress

4.2 Introduction

Joint homeostasis is an integral process determining the functional load bearing capabilities of the joint that are essential to ensuring mobility and preventing morbidity. Dysregulation of this process can result in osteoarthritis (OA), a collective of heterogeneous pathologies culminating in joint failure. OA presents with similar pathological end points but mechanisms of initiation and progression vary among subtypes of this disease, which is one of the leading causes of disability worldwide[1, 2]. Its varied presentation influences whether it is symptomatic or not, and even whether it

can be diagnosed radiographically. Multiple tissues such as the articular cartilage, subchondral bone, synovium, meniscus, and fat pads are involved in this condition, and initiation of this disease can stem from mechanical, metabolic, or age-associated factors although none of these are mutually exclusive.

The main function of cartilage is to act as a shock absorber, mediating load bearing through the influx and efflux of water attracted to the proteoglycan aggregates forming the main protein component of the extracellular matrix, and tensile strength through collagen fibril organization[3]. Although cartilage cells contribute to a small percentage of the volume of the entire joint, they are sensitive to changes that occur to changes in gene expression in models of OA, thus underscoring their importance in joint homeostasis.

Metabolic OA has been classified as a distinct subtype of OA associated with disorders such as dyslipidemia, hypertension and obesity. Imbalances in systemic lipid and cholesterol metabolism, nutrient exchange, accumulation of advanced glycation end products, and increases in adipokines contribute to this condition. Changes in lipid metabolism, in particular, may directly affect joint homeostasis through ectopic lipid deposition in chondrocytes[4-7]. In fact, both chondrocyte specific cholesterol accumulation and high fat diet have caused increased disease severity in murine models[8-10]. Collectively these data suggest direct regulation of cartilage homeostasis by lipid metabolism.

Nuclear Receptors are a class of proteins that are activated by small molecule ligands and can up or downregulate the expression of target genes through the recruitment of co-

factors or co-repressors. They have been reported as attractive potential targets for pharmacological therapy because of their ability to bind synthetic or natural ligands that regulate transcriptional activity[11]. As such, synthetic agonists for nuclear receptors have been developed targeting metabolic conditions such as dyslipidemia, atherosclerosis, and diabetes [12, 13]. PPARs (peroxisome proliferated activated receptors) are typically involved in the control of lipid metabolism and activated by the binding of endogenous fatty acids, whereas LXR (liver X receptor) is principally involved in cholesterol metabolism. Recently, we have shown that cartilage-specific ablation of the gene encoding the nuclear receptor PPAR δ has a protective effect on cartilage after surgical induction of OA, demonstrating that PPAR δ promotes post-traumatic OA. Conversely PPAR γ and LXR are protective and necessary for normal joint function and skeletal development [14-17]. Interestingly, all three of these receptors act in heterodimers with the common partner RXR, positioning RXR at the centre of a complex network of nuclear receptors. All of these proteins are expressed in cartilage[16, 18, 19].

In this study we have used microarray analysis paired with functional validation to identify gene targets of LXR, PPAR γ , PPAR δ and RXR in articular chondrocytes, in order to elucidate their potential role in OA pathogenesis. There is strong evidence implicating the involvement of nuclear receptors in the progression or prevention of OA, and here we provide insight as to how they may be involved in altering the gene expression profile and phenotype of mature, healthy chondrocyte cultures. We are also the first, to our knowledge, to quantify changes in neutral lipid and free cholesterol mass in chondrocytes *in vitro*. This information is essential in uncovering the early changes

that occur in chondrocytes before irreversible phenotypic changes within the joint, and is vital since we currently have no effective biomarkers or treatment to alter the course of OA progression. Our data demonstrate that changes in gene regulation after nuclear receptor agonist treatment primarily affect lipid metabolism, suggesting a close link between lipid metabolism within chondrocytes and the progression of OA.

4.3 Methods

4.3.1 Primary Cell Culture and Isolation

Immature murine articular chondrocytes (IMACs) were isolated from the femoral head, femoral condyle, and tibial condyles of 5-6 day old CD1 mice (Charles River Laboratories). The tissue was then subjected to one hour (3 mg/ml) followed by 24 hour (0.5 mg/ml) incubations in Collagenase D diluted in Dulbecco's Modified Eagles Medium supplemented with 2 mM l-glutamine, 50 U/ml penicillin and 0.05 mg/ml streptomycin) at 37°C under 5% CO₂. The tissue fragments were then agitated and cells were isolated and cultured as per [20]. On the seventh day after isolation, cells were treated with either PPAR δ agonist (GW501516), PPAR γ agonist (Rosiglitazone), LXR agonist (GW3965), RXR agonist (SR11237), or control (DMSO) all at concentrations of 1 μ M for 72 hours.

4.3.2 RNA extraction, purification and qPCR

Total RNA was isolated from cells using TRIzol® (Invitrogen). Cells were first lysed in TRIzol® reagent, phases were separated using chloroform (20%), and supernatant was removed. RNA was precipitated using 100% isopropanol (0.5%) and RNA was washed using 70% ethanol followed by air-dry and resuspension in RNase free water, as per

manufacturer's instructions. RNA was quantified using a Nanodrop 2000 spectrophotometer. RNA integrity was confirmed with Aligent 2100 BioAnalyzer Data Review Software (Wilmington, DE) at the London Regional Genomics Centre and RNA samples with RNA integrity number (RIN) values greater than 8 were used for microarray analysis.

Real-time PCR (qPCR) was performed as per [17]. In brief, qPCR was performed using a One-Step RT qPCR Master Mix kit and TaqMan Gene Expression Assays (Applied Biosystems), with 40 cycles on an ABI Prism 7900HT sequence detector (PerkinElmer), or on a Bio-Rad CFX384 RT-PCR system with 15 μ l reaction volumes of iQ SYBR Green Supermix (Biorad) with diluted cDNA equivalent to 200 ng of input RNA per reaction, as well as 25 μ M forward and reverse primers[21]. Probes for *Acan*(Mm00545794_m1), *Actb* (Mm02619580_g1), *Adamts4*(Mm00556068_m1), *Adamts5* (Mm00478620_m1), *Angptl4*(Mm00480431_m1), *Col2a1* (Mm01309565_m1), *Fabp3*(Mm02342495_m1), *Fabp4*(Mm00445878_m1), *LPL*(Mm00434764_m1), *Mmp2* (Mm00439498_m1), *Mmp3* (Mm00440295_m1), *Mmp13*(Mm00439491_m1), *Pdk4*(Mm01166879_m1), *Sox9* (Mm00448840_m1) were purchased from Life Technologies. Gene expression was normalized relative to ActB. Relative gene expression was calculated using the $\Delta\Delta C_t$ method [22] as described [23]. Statistical analysis was conducted using GraphPad Prism 6.0. Values were transformed, and a one way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparisons tests.

4.3.3 Microarray and Data Analysis

Total RNA (200 ng per sample) was subject to 2 rounds of amplification followed by labeling and hybridization to Affymetrix GeneChip® Mouse Gene 2.0 ST Array containing 35,240 probes at the London Regional Genomics Centre (London, Ontario, Canada) as described [24]. Three independent cell and RNA isolations were used for each treatment. Probe data was analyzed, and gene level, ANOVA p-values and fold changes were determined using Partek Genomics Suite v6.6. Genes with at least 1.5-fold change, with $p < 0.05$ were considered significant and used for subsequent analyses. The complete array data set has been made publicly available through Gene Expression Omnibus (GEO). The Venn diagrams were created using the online plotting tool Venny 2.0.1[25]. Gene Ontology biological processes were classified through GO consortium available at geneontology.org. Biological processes identified with more than 3 genes involved were included in the table.

4.3.4 Cell Lipid Mass

IMACs were isolated, cultured, and treated with nuclear receptor agonists as described above. At the 72 hour time point, cells were washed with 0.2% BSA in phosphate-buffered saline (PBS), followed by 3 washes in PBS. Lipids were extracted using 3:2 hexane:isopropanol solvent and pooled. Hexane:isopropanol solvents were evaporated to dryness under N_2 and resuspended in 1.4ml of chloroform-triton (0.5% triton v/v). Solvent was re-evaporated, and lipids were re-solubilized in 350 μ l water. Two 50 μ l aliquots were used per sample to determine total cholesterol (TC), free cholesterol (FC), and triglyceride (TG) mass, spectrophotometrically as per [26]. Cholesteryl esters (CE) were calculated by subtracting FC from TC. Proteins were extracted using 0.2 NaOH

overnight incubation to digest chondrocytes and quantified using a standard BCA protein assay (Pierce, Thermo Fisher Scientific). All cell lipid measures reported are standardized to mg of cell protein. Values were normalized relative to vehicle control DMSO, and statistical analyses were performed using GraphPad Prism 6.0. Values were transformed, and a one-way analysis of variance (ANOVA) was performed followed by Tukey's multiple comparisons tests.

4.3.5 Animals and Surgery

All animal experiments were approved by the Animal Use Subcommittee at The University of Western Ontario and were conducted in accordance with the guidelines from the Canadian Council on Animal Care. Mice were group housed (6 mice per cage) in colony cages, on a standard 12h light/dark cycle with free access to standard mouse chow, water, and running wheels. Surgical destabilization of the medial meniscus (DMM) or SHAM surgery was performed on 12 week old C57/bl6 male mice (N=9 per group), as described in [14]. Mice were euthanized at 10 weeks post-surgery for preparation of paraffin sections, and subsequent histological analysis. Another cohort of 20 week old male cartilage specific *Ppard* knockout mice and wild-type littermate controls underwent DMM surgery (N=5 per group) and were harvested for histological analyses 8 weeks later as in [14]. Paraffin sections from these studies were employed to evaluate the presence of Thioredoxin Interacting Protein (*Txnip*).

4.3.6 Immunohistochemistry

Immunohistochemistry was performed on frontal sections of paraffin embedded knee joints as described[27]. Txnip rabbit polyclonal antibody was purchased from proteintech (18243-1-AP). Slides without primary antibody were used as controls, antigen retrieval

was performed in 0.1% Triton in H₂O, primary antibody was used at a concentration of 1:300.

4.4 Results

4.4.1 Global changes in chondrocyte gene expression in response to nuclear receptor agonist treatment

We have previously reported that treatment of articular chondrocytes with the PPAR δ agonist GW501516 results in increased gene expression of matrix-degrading enzymes, and robust fatty-acid oxidation. We have also determined that treatment of embryonic tibiae with the LXR agonist GW3965 suppresses chondrocyte hypertrophy[14, 17]. Identifying which genes are responsible for these phenotypes, and how they interact with each other, is key to understanding signaling pathways responsible for joint homeostasis and the prevention of osteoarthritis. We first examined global changes in chondrocyte gene expression in response to 1 μ M treatment with LXR agonist GW3965, RXR agonist SR11237, PPAR δ agonist GSK501516, or PPAR γ agonist Rosiglitazone. RNA was isolated from IMACs cultured with agonists for 72 hours, then hybridized to Affymetrix microarrays representing the mouse genome.

We compiled a list of genes changed by more than 1.5 fold (refer to supplementary data for full list). LXR agonism significantly altered 128 genes (97 upregulated, 31 downregulated), RXR agonism differentially regulated a total of 108 genes (67 upregulated, 41 downregulated), PPAR δ agonism induced changes in 58 genes (48 upregulated, 10 downregulated), while PPAR γ agonism changed 32 genes (29 upregulated, 3 downregulated). The most robust and significantly upregulated and downregulated genes after nuclear receptor agonist treatment are shown in Figure 4-1.

LXR induced ATP binding cassette transporter subfamily A member 1 (Abca1), Stearoyl CoA desaturase 1 (Scd1), Insulin induced gene 1 (Insig1), ATP binding cassette transporter

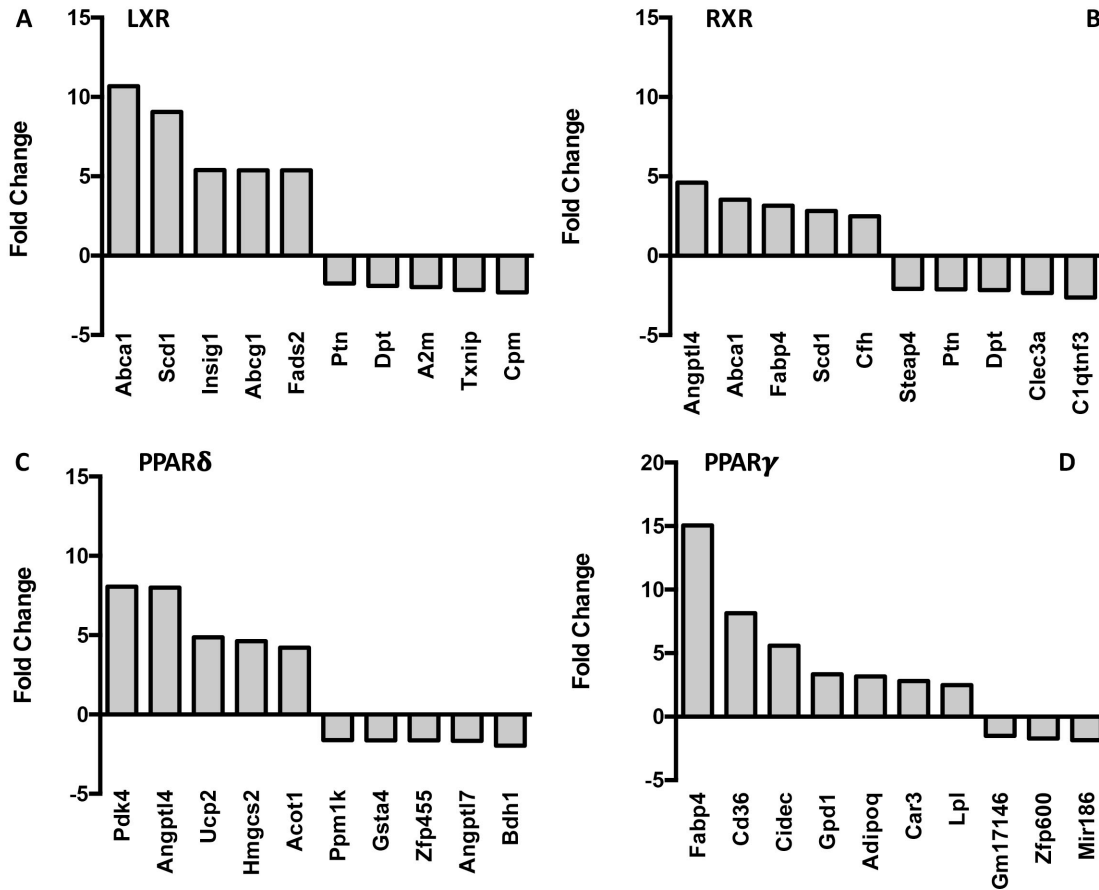


Figure 4-1 Microarray analyses of nuclear receptor agonist effects on chondrocyte gene expression

Microarray analysis of RNA isolated from immature murine articular chondrocytes treated for 72 hours with 1 μ M LXR agonist GW3965 (**A**), RXR agonist SR11237 (**B**), PPAR δ agonist GSK501516 (**C**), or PPAR γ agonist Rosiglitazone (**D**). The most highly upregulated and downregulated genes are shown with fold change relative to vehicle control DMSO (1 μ M).

subfamily G member 1 (Abcg1), Fatty acid desaturase 2 (Fads2), and downregulated Pleiotrophin (Ptn), Dermatopontin (Dpt), Alpha-2-macroglobulin (A2m), Thioredoxin interacting protein (Txnip), and Cpm (carboxypeptidase) (Fig. 1A). Activation of RXR, the heterodimeric partner for LXR, PPAR δ and PPAR γ , significantly upregulated Angiopoietin like 4 (Angptl4), Abca1, Fatty-acid binding protein 4 (Fabp4), Scd1 and Complement factor H (Cph) (Fig. 4-1B). Conversely, RXR significantly reduced expression of Six transmembrane epithelial antigen of prostate family member four (Steap4) (also known as Tumor necrosis alpha inducing protein 9), Ptn, Dpt, C1q and tumor necrosis factor related protein 3 (C1qtnf3) (Fig. 4-1B). PPAR δ significantly induced Pyruvate dehydrogenase kinase isoenzyme 4 (Pdk4), Angptl4, Uncoupling protein 2 (Ucp2), 3-hydroxy 3-methylglutaryl CoA synthase 2, Acyl CoA thioesterase 1 (Acot1), and significantly downregulated Protein phosphatase Mg²⁺/Mn²⁺ dependent 1K (Ppm1K), Glutathione S-transferase A 4 (Gsta4), Zinc finger protein 455 (Zfp455), Angiopoietin like 7 (Angptl7), and 3 Hydroxybutyrate dehydrogenase type 1 (Bdh1) (Fig. 4-1C). Lastly, PPAR γ agonism upregulated Fabp4, CD36 molecule (CD36), Cell death inducing DFFA like effector C (Cidec), Glycerol 3 Phosphate Dehydrogenase 1 (Gpd1) and Adiponectin C1Q and collagen domain containing (Adipoq), Carbonic anhydrase 3 (Car3) and Lipoprotein lipase (Lpl) and downregulated predicted gene 17146 (Gm17146), Zinc finger protein 600 (Zfp600), microRNA 186 (Mir186) (Fig. 4-1D).

4.4.2 Nuclear Receptors share common gene targets in chondrocytes

Comparison of gene expression profiles induced by various nuclear receptor agonists revealed several common hits. We therefore decided to evaluate shared functional roles by identifying similar biological processes through Gene Ontology. Table 4-1 indicates the biological processes regulated by agonist treatment for each nuclear receptor, common processes are highlighted with the same colour. Both LXR and RXR agonism altered cholesterol biosynthetic processes, while LXR and PPAR γ regulated triglyceride metabolism, and RXR and PPAR γ increased metabolic processes in chondrocytes. In order to compare relationships between nuclear receptor agonist treatments, we created a Venn diagram to illustrate the number of genes induced by multiple receptors (Figure 4-2). The two genes upregulated by all four nuclear receptor agonists were *Pdk4* and *Angptl4*. *Pdk4* functions as an inhibitor of the pyruvate dehydrogenase complex. Thus it plays a key regulatory role in shifting energy utilization from glycolytic to fatty-acid metabolism in the cell[28]. *Angptl4* is a well known direct target of PPARs that is upregulated by hypoxia, and has been characterized as an adipocytokine[29]. It has also been identified as a potential pro-angiogenic mediator of arthritis, is involved in ECM remodeling, and is upregulated in the cartilage of RA and OA patients[30-32]. Since PPAR δ has opposite effects on OA progression than PPAR γ and LXR, we were also interested in genes showing opposite responses to the respective agonists. However, the only gene that was differentially regulated greater than 1.5 fold between any of the treatments was *Txnip*, which encodes the Thioredoxin interacting protein. *Txnip* inhibits Thioredoxin and contributes to ER stress, inflammasome activation, and the accumulation of reactive oxygen species (ROS)[33]. This gene was upregulated by

PPAR δ agonist GW501516 and downregulated by LXR agonist GW3965 treatment. Based on the common genes identified, we next validated changes in the expression of these genes by qPCR.

Table 4-1

GO Biological Process (GW3965 Tx)	# of Genes Involved	Fold Enrichment
cholesterol biosynthetic process (GO:0006695)	12	82.46
regulation of cholesterol biosynthetic process (GO:0045540)	4	49.82
acetyl-CoA metabolic process (GO:0006084)	5	39.86
isoprenoid biosynthetic process (GO:0008299)	5	33.21
response to fatty acid (GO:0070542)	6	29.16
regulation of plasma lipoprotein particle levels (GO:0097006)	6	28.47
triglyceride metabolic process (GO:0006641)	7	24.47
fatty acid biosynthetic process (GO:0006633)	13	23.99
regulation of fatty acid metabolic process (GO:0019217)	7	18.6
positive regulation of lipid biosynthetic process (GO:0046889)	7	18.12
cellular response to starvation (GO:0009267)	9	15.73
lipid homeostasis (GO:0055088)	7	12.24
lipid localization (GO:0010876)	9	7.57
oxidation-reduction process (GO:0055114)	21	4.85

GO Biological Process (SR11237 Tx)	# of Genes Involved	Fold Enrichment
cholesterol biosynthetic process (GO:0006695)	5	42.29
positive regulation of lipid metabolic process (GO:0045834)	7	12.53
anion transport (GO:0006820)	11	6.35
regulation of phosphorus metabolic process (GO:0051174)	20	3.07
negative regulation of biological process (GO:0048519)	38	2.12

GO Biological Process (GW501516 Tx)	# of Genes Involved	Fold Enrichment
carnitine metabolic process (GO:0009437)	4	> 100
long-chain fatty acid metabolic process (GO:0001676)	5	23.53
alcohol metabolic process (GO:0006066)	7	12.16
regulation of lipid metabolic process (GO:0019216)	8	11.95
small molecule biosynthetic process (GO:0044283)	9	10.82
lipid biosynthetic process (GO:0008610)	9	9.63

GO Biological Process (Rozglitazone Tx)	# of Genes Involved	Fold Enrichment
regulation of sequestering of triglyceride (GO:0010889)	3	> 100
lipid particle organization (GO:0034389)	3	> 100
regulation of macrophage derived foam cell differentiation (GO:0010743)	3	> 100
positive regulation of lipid storage (GO:0010884)	3	> 100
regulation of lipid storage (GO:0010883)	4	92.81
triglyceride metabolic process (GO:0006641)	5	75.3
fat cell differentiation (GO:0045444)	4	33.67
positive regulation of lipid metabolic process (GO:0045834)	5	31.33
chemical homeostasis (GO:0048878)	8	8.31

Biological processes regulated by nuclear receptor agonists as indicated by Gene Ontology (GO) Bioinformatics Analysis. Commonly regulated processes between treatments are indicated by the same colour.

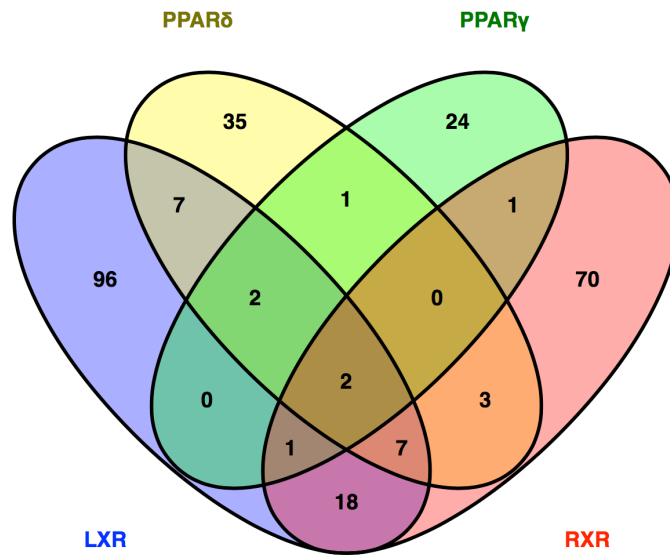


Figure 4-2 Comparison of nuclear receptor agonist effects on chondrocyte gene expression

Comparison of all genes regulated by the four different nuclear receptor agonist treatments on chondrocytes demonstrate that 2 genes are commonly regulated by all four nuclear receptors. 9 genes are commonly regulated by LXR, PPAR δ , and RXR, while 4 genes are commonly regulated by LXR, PPAR δ and PPAR γ . 3 genes are regulated by LXR, PPAR γ , and RXR, and 2 genes are commonly regulated by PPAR δ , PPAR γ , and RXR.

4.4.3 LXR, RXR and PPAR agonism promote changes in genes involved in ECM homeostasis and chondrocyte metabolism

Genes induced in the microarray were primarily metabolic, or involved in extracellular matrix component production and turnover. We chose to validate a subset of these genes which were shared between nuclear receptor agonist treatments. Aggrecan and Fibrillin 2 are extracellular matrix proteins encoded by the *Acan* and *Fbn2* genes. In concert with our microarray results, gene expression of *Acan* was significantly lower than vehicle control (DMSO) with RXR agonist treatment. Similarly, both LXR and RXR agonism significantly lowered gene expression of *Fbn2* (Figure 4-3). Gene expression of Collagen 2 (*Col2a1*) remained unchanged in response to any of the treatments. We also validated proteases that were changed by some of the nuclear receptor agonists, and accordingly found that gene expression of *Adamts4*, *Mmp2* and *Mmp13* were significantly reduced by LXR agonism. Interestingly, RXR agonism decreased gene expression of *Adamts4* while increasing that of *Mmp13* (the primary collagenase of OA), implying a preferential pathway for ECM remodeling and degradation.

LXR, RXR, and PPARs are involved in the regulation of metabolism in a number of tissues. In a previous study we showed that chondrocytes express functional PPAR δ and are capable of responding to GW50516 stimulation with increased fatty acid oxidation[14]. All four nuclear receptor agonists induced strong metabolic effects.

Angptl4 and *Pdk4*, the two common genes induced by all four nuclear receptors in the microarray, demonstrated a similar robust upregulation in qPCR validation (Figure 4-4).

Abca1, *Cidea*, *Lpl* and *Cpt1a* were significantly increased by PPAR δ , LXR and RXR agonist treatment. Gene expression of cytoskeletal fatty-acid transporter *Fabp3* was significantly increased by PPAR δ activation, while *Gsta4*, a gene encoding an enzyme

for cellular defense against reactive electrophiles, was significantly reduced by both PPAR δ and RXR agonism[34].

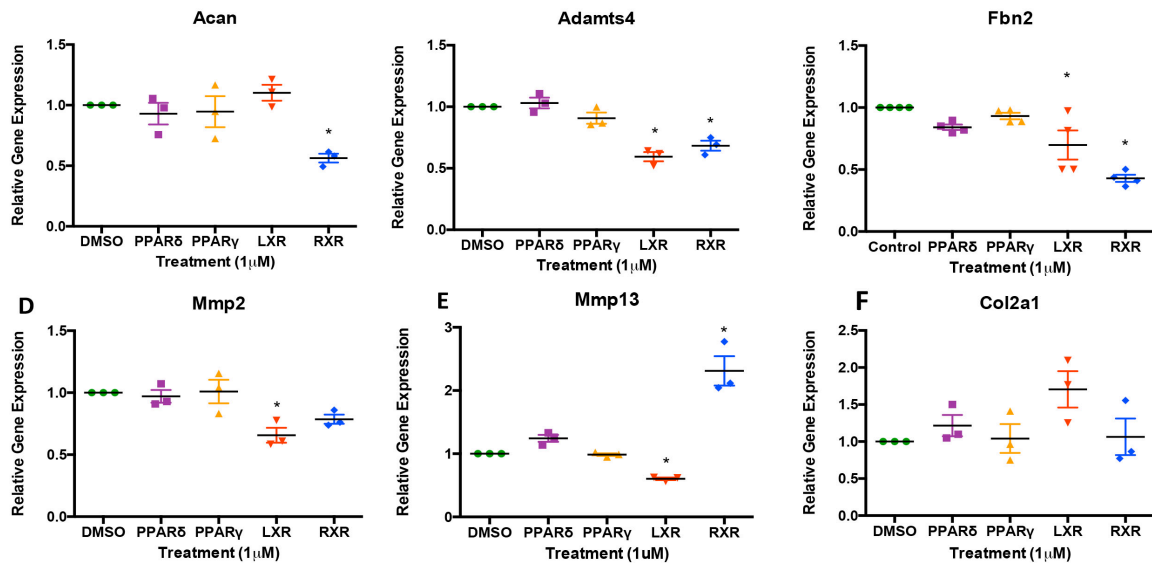


Figure 4-3 Effects of nuclear receptor agonist treatments on extracellular matrix gene expression in chondrocytes

IMACs were incubated for 72 hours with 1 μM DMSO (vehicle control), PPARδ agonist GW501516, PPARγ agonist Rosiglitazone, LXR agonist GW3965, or RXR agonist SR11237. **(A)** Relative gene expression of *Acan* gene is significantly reduced by treatment with the RXR agonist. **(B, C)** Relative gene expression of *Adamts4* and *Fbn2* are significantly reduced by LXR and RXR agonist treatment. **(D, E)** Relative gene expression of matrix metalloproteinases *Mmp2* and *Mmp13* is decreased by LXR agonist treatment, while gene expression of *Mmp13* is significantly elevated by RXR agonist treatment. **(F)** *Col2a1* gene expression remains unchanged by all treatments. Values represented are the mean ± SEM of ≥ 3 independent cell isolations. * = p < 0.05.

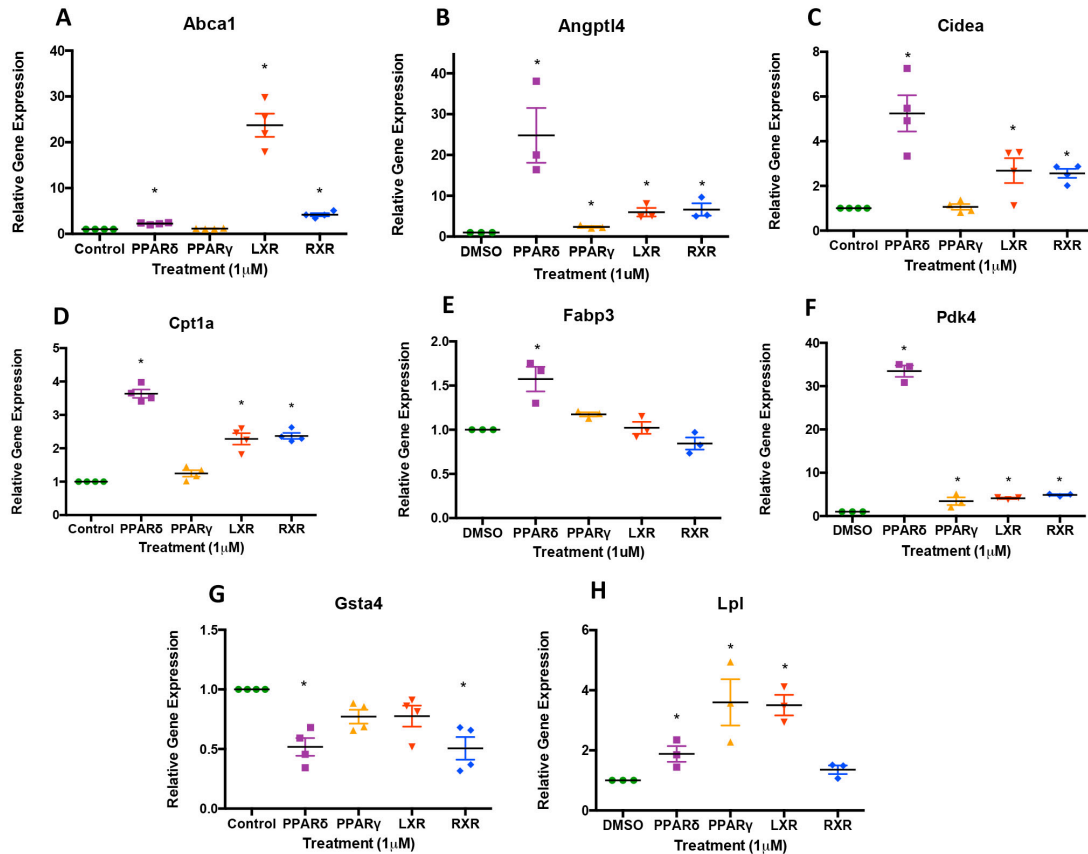


Figure 4-4 Effects of nuclear receptor agonist treatment on metabolic gene expression in chondrocytes

IMACs were incubated for 72 hours with 1 μM DMSO (vehicle control), PPARδ agonist GW501516, PPARγ agonist Rosiglitazone, LXR agonist GW3965, or RXR agonist SR11237. **(A,C,D,H)** Relative gene expression of *Abca1*, *Cidea*, *Cpt1a* and *Lpl* is significantly increased by PPARδ, LXR and RXR treatments. **(B,F)** Relative gene expression of *Angptl4* and *Pdk4* is elevated by all four treatments. **(E)** *Fabp3* gene expression is significantly upregulated by PPARδ agonism. **(G)** RXR and PPARδ treatment significantly decrease relative gene expression of *Gsta4*. Values represented are the mean ± SEM of ≥ 3 independent cell isolations. *p<0.05.

4.4.4 Increased expression of Thioredoxin binding protein in osteoarthritic cartilage

Txnip plays an important regulatory role in mediating oxidative stress and inflammation in a number of tissues[33]. *Txnip* was the only gene differentially regulated between nuclear receptor agonists. LXR agonist treatment downregulated gene expression, while PPAR δ highly induced *Txnip*. These patterns observed in microarray analyses were validated by qPCR, where PPAR δ agonism significantly increased gene expression of *Txnip* while LXR agonist-treated cells demonstrated trends toward decreased gene expression, and RXR and PPAR γ agonism showed no change (Figure 4-5a). Immunohistochemistry for TXNIP was performed on frontal sections of mice 8 and 10 weeks post DMM surgery (Figure 4-5b,c). Wild-type mice 10 weeks post surgery had increased numbers of positively stained chondrocytes compared to mice that underwent SHAM surgery. To validate the effects of PPAR δ on *Txnip* expression, we compared protein expression in cartilage-specific *Ppard* KO mice and wild-type littermates 8 weeks after DMM surgery. Wild-type mice demonstrated increased staining for *Txnip*, particularly in areas of osteophyte growth at joint margins, whereas both sham-operated control mice and KO mice after either surgery showed little to no staining. The increase of *Txnip* expression in the process of OA implies an imbalance in regulatory processes governing oxidative stress and inflammation, potentially linking changes in metabolism to osteoarthritic changes.

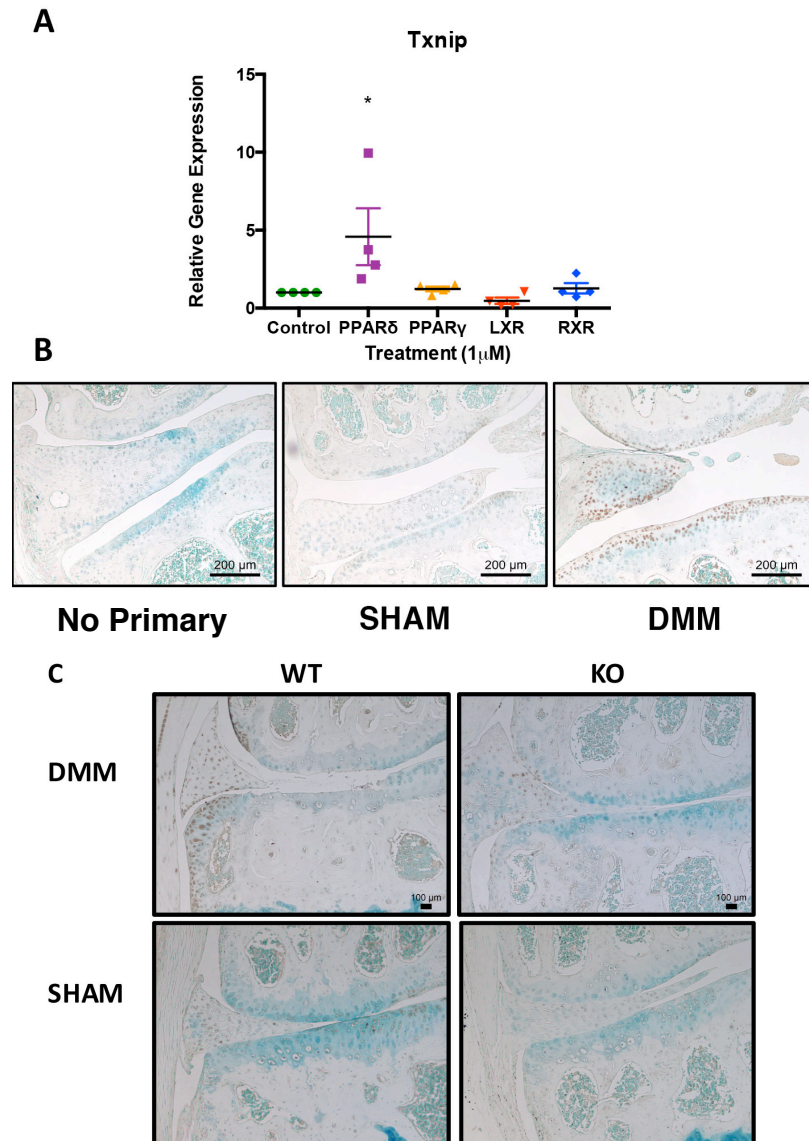


Figure 4-5 Effects of nuclear receptor agonist treatment on *Txnip* gene expression

(A) IMACs were incubated for 72 hours with 1 μ M DMSO (vehicle control), PPAR δ agonist GW501516, PPAR γ agonist Rosiglitazone, LXR agonist GW3965, or RXR agonist SR11237. PPAR δ treatment significantly increased gene expression of *Txnip*. Values represented are the mean \pm SEM of ≥ 3 independent cell isolations. * $p < 0.05$. (B) Immunohistochemistry (IHC) for *Txnip* demonstrates increased cellular staining in the cartilage of WT DMM mice 10 weeks post-surgery relative to SHAM mice. (C) IHC for *Txnip* in cartilage-specific *Ppard* KO mice vs WT littermate controls 8 weeks post DMM surgery. *Ppard* KO mice display less staining than WT mice with greater OA severity.

4.4.5 Changes in gene expression correspond with functional changes in chondrocyte lipid profile

In light of the number of genes involved in lipid metabolism that were identified in our gene expression analyses, we assessed neutral lipid and cholesterol accumulation in chondrocytes. Using the same nuclear receptor agonist treatment protocols, we harvested IMACs for cellular lipid mass assays. These assays allowed us to directly quantify triglycerides and cholesterol *in vitro*. There were significant changes in cell triglycerides, but not total cholesterol, free cholesterol or cholesteryl esters (Figure 4-6). These data suggest that changes in lipid metabolism upon agonist treatment are likely related to lipogenesis and fatty acid oxidation, rather than cholesterol transport or accumulation. In particular, triglycerides were significantly decreased with PPAR δ agonist treatment, and were significantly increased with LXR, PPAR γ and RXR agonism. These changes are consistent with the known effects of activation of these nuclear receptors on triglyceride metabolism in other cell types and suggest that PPAR δ may have an opposing functional role in lipid metabolism in chondrocytes relative to the other nuclear receptors examined here[35].

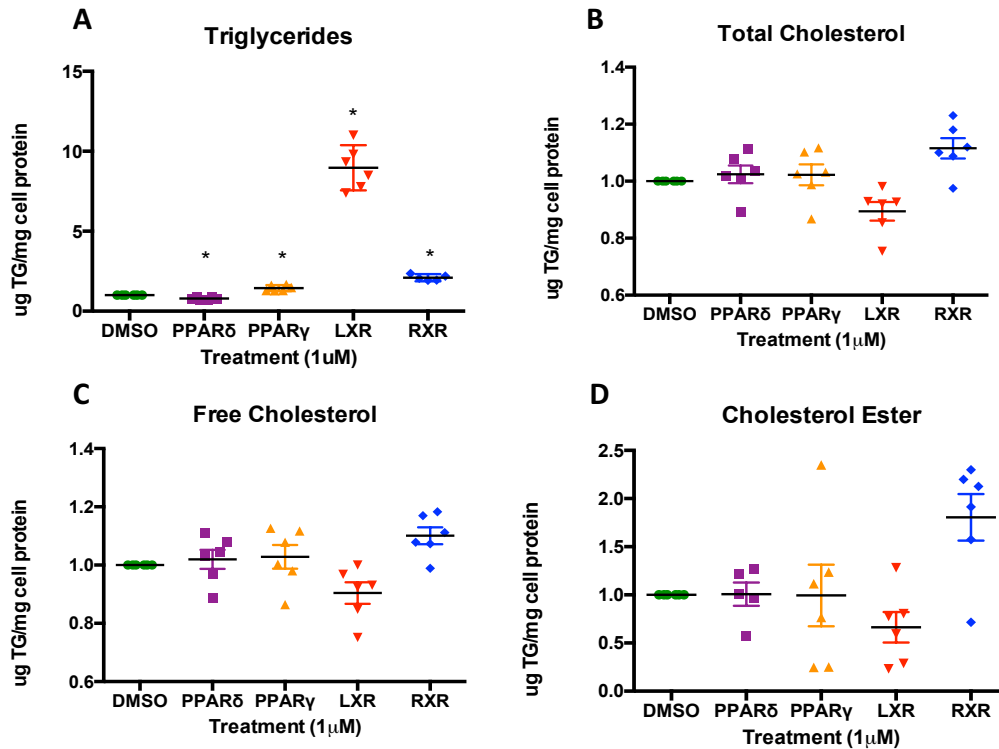


Figure 4-6 Quantification of cellular lipid mass

IMACs were incubated for 72 hours with 1 μ M DMSO (vehicle control), PPAR δ agonist GW501516, PPAR γ agonist Rosiglitazone, LXR agonist GW3965, or RXR agonist SR11237. Lipids were extracted, isolated and mass was measured spectrophotometrically. Proteins were isolated and quantified using BCA. Measurements are reported relative to mg of cell protein. **(A)** Cell triglycerides (μ g) are significantly elevated by PPAR γ , LXR, and RXR treatment, and are significantly decreased by PPAR δ agonism. **(B,C,D)** Total cholesterol, free cholesterol, and cholesterol ester remain unchanged after nuclear receptor agonist treatment. Values represented are the mean \pm SEM of ≥ 5 independent cell isolations. * $p < 0.05$.

4.5 Discussion

This is amongst the first studies to examine changes in global gene expression in chondrocytes after nuclear receptor agonist treatment paired with concurrent functional analysis. It provides compelling evidence that nuclear receptors are involved with early changes in cell metabolism that can influence deleterious changes in cell phenotype leading to the progression of OA. Nuclear receptors have been increasingly linked to the progression of OA. We have previously established the degenerative changes promoted by PPAR δ agonism in cartilage, as well as the beneficial and necessary role of PPAR γ in cartilage [14, 15]. We and others have characterized the protective role of LXR in osteoarthritis [17, 36, 37]. However, in order to establish how or whether these ligand-activated receptors are feasible therapeutic targets we must examine what molecular changes cause the phenotypic changes characteristic of OA.

We used IMACs treated with LXR, RXR, PPAR γ or PPAR δ agonists for 72 hours. Immature murine articular chondrocytes provide a large number of cells for analyses on fully differentiated primary chondrocytes while minimizing dedifferentiation [38]. Microarray analyses of IMACs revealed changes in metabolic and ECM genes in response to these agonists, changes which were largely confirmed by qPCR. Agonism of RXR decreased gene expression of the major ECM component aggrecan, and increased the expression of ECM protease *Mmp13*, while LXR agonism decreased the gene expression of proteases *Adamts4*, *Mmp2*, and *Mmp13*. Of particular interest were the increases in expression of genes involved in lipid metabolism, since they showed greater induction than those regulating ECM turnover. Amongst these genes, two were induced by all four agonists, *Pdk4* and *Angptl4*, suggesting that they might play central roles in

cartilage metabolism. Interestingly, in an earlier study we had also demonstrated increased expression of *Pdk4* in response to dexamethasone, a ligand for the glucocorticoid receptor which is another member of the nuclear receptor family[39].

Functional evaluation of lipid metabolism using cellular lipid mass assays demonstrated a significant decrease in triglycerides after PPAR δ agonist treatment. Conversely, triglycerides were significantly increased with PPAR γ , LXR, and RXR agonists. This is not surprising as PPAR γ can often act antagonistically to PPAR δ with regard to lipogenesis [40], while LXR mediates fatty acid biosynthesis through activation of genes such as *Srebf1*, *Fasn*, and *Scd1* which corroborate our data (see supplementary)[41, 42]. Quantification of cell lipids *in vitro* is advantageous because it permits us to directly measure the amount of lipid being stored in chondrocytes. This enables us to assess differences in some aspects of lipid metabolism between treatments. In fact, it is plausible that the dysregulation in lipid metabolism that we observed could initiate metabolic changes in the cell that eventually lead to apoptosis, inflammation, or changes in cell behaviour such as synthesis of catabolic factors. It has been shown that there is increased lipid deposition in osteoarthritic cartilage, but also that increased reactive oxygen species (ROS) can cause lipid peroxidation, which in turn could cause oxidative stress resulting in degenerative changes to the matrix through oxidation of collagen II[6, 43].

In addition to dysregulation of cell lipids, we also see significantly decreased *Gsta4* expression after PPAR δ or RXR agonist treatment. The encoded enzyme Glutathione S-transferase A 4 protects against HNE (4 Hydroxynonal)-induced damage in chondrocytes. HNE is an extremely reactive aldehyde produced from ROS and lipid peroxidation, and is

increased in synovial fluid from OA patients[42]. HNE can also post-transcriptionally modify Collagen 2 and MMP13 to induce degradative changes in cartilage. On the other hand, decreased levels of GSTA4 are present in human OA cartilage, making cartilage more susceptible to damage [34].

One gene of particular interest, *Txnip* (thioredoxin interacting protein) was highly induced by PPAR δ agonism, but appeared repressed by LXR agonism, in agreement with the opposing effects of these nuclear receptors on OA progression. Thioredoxin is an important antioxidant, but binding of Txnip to thioredoxin inhibits its ability to scavenge for ROS[44]. In our study, we demonstrate increased gene expression of *Txnip* after PPAR δ agonism in chondrocytes. We also show that cartilage-specific *Ppard* knockout mice that are protected against cartilage degeneration in OA have decreased Txnip staining after DMM surgery. In contrast, control mice have increased staining for Txnip 8 and 10 weeks post-surgery when compared to SHAM-operated mice. A similar effect has previously been demonstrated in other tissues where silencing Txnip abrogated palmitate induced inflammasome activation and proapoptotic activity in retinal endothelial cells[45]. Additionally, Txnip has been shown to link oxidative stress and inflammation, and it can directly activate NF-kB and downstream inflammatory cytokines[45]. In chondrocytes, recent work has shown that *Redd1* can form a complex with Txnip to regulate autophagy[46]. Taken altogether, these data help to form a cohesive picture of how changes in cell metabolism could influence the development of early osteoarthritis. Nuclear receptors appear to play a key role in these processes by regulating the expression of central players such as *Txnip* and *Gsta4*.

Current treatment strategies for OA are largely ineffective or inconclusive. It is possible that we are missing a critical temporal period during which chondrocyte homeostasis is disrupted, later leading to matrix degeneration. Recent evidence demonstrates that nuclear receptors are key regulators of OA pathogenesis, and the data presented here suggests that their primary targets are metabolic regulation. Metabolic deregulation, in turn, can trigger events leading to oxidative stress and inflammation, protease activation, and ultimately cartilage degeneration. Targeting these critical processes could be a promising avenue for treatments that alter disease progression.

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Chapter 5

5 Discussion

5.1 Overview

The overall objective of my thesis was to characterize the role of the nuclear receptor PPAR δ in osteoarthritis (OA). Our laboratory first identified the PPARs as potential targets in OA through microarray studies comparing cartilage from rats with surgically induced OA to SHAM operated rats[1]. Subsequently, our group investigated PPAR γ and demonstrated that it was necessary for cartilage homeostasis; cartilage-specific depletion of PPAR γ either during skeletal development or in adult tissue is severely debilitating[2, 3]. Our laboratory next decided to investigate the role of PPAR δ in-vitro, since these two nuclear receptors can have either opposing or complementary context-dependent actions[4-6]. We found that PPAR δ agonism by synthetic agonist GW501516 results in significant increases in gene expression of matrix degrading proteases (*Mmp 2*, *Mmp3*, *Adamts2*, *Adamts5*) in mouse primary chondrocytes. Similarly, GW501516 treatment of murine joint explants resulted in glycosaminoglycan (GAG) loss and an OA-like phenotype. This data indicated a strong catabolic role for PPAR δ in cartilage and provided the rationale for investigation of this target as a mediator of OA.

The first study in my thesis investigates the role of PPAR δ in OA. It built upon the in-vitro work, and quantitated GAG loss in cartilage explants through a dimethylmethylene blue binding (DMMB) assay. This assay revealed that a significant number of GAGs are released from the joint after treatment with GW501516 and cemented the theory that PPAR δ has catabolic effects in the joint. We believed that if this nuclear receptor's

activity was destructive in the joint, then inhibiting it could attenuate some of these detrimental effects. Therefore, I generated a cartilage specific *Ppard* knock-out mouse to study the effects of PPAR δ inactivation in skeletal development and osteoarthritis. Mice with conditional ('floxed') *Ppard* alleles (*Ppard*^{f/f}) were bred to mice expressing Cre recombinase under the control of the Collagen 2 promoter. The mice were born in normal Mendelian ratios with no obvious defects. I examined skeletal development through analysis of gross skeletal morphology, and measurement of anatomical markers of the growth plate and long bones at time points of p0, p10, p21, and 5 months of age. These mice displayed no deformities in gross morphology, and had similar cellular organization and zonal lengths of growth plate cartilage as well as overall long bone length. As there were no congenital defects affecting anatomical or mechanical factors that predisposed these mice to OA development, we next surgically induced OA in mice at 20 weeks of age through a destabilization of medial meniscus surgery (DMM). This surgery is a widely used and accepted method for inducing secondary OA that develops gradually in the medial tibial plateau of mice[7-9]. 8 weeks post-surgery we compared the WT to KO mice through OARSI histopathology scoring and immunohistochemistry for cartilage breakdown products. I discovered that while WT mice had moderate-severe cartilage breakdown in the medial tibial plateau and medial femoral condyles of the knee, as expected, the *Ppard* KO mice showed much less damage. Cartilage was significantly protected from the progression of OA. This was recapitulated in my staining for cartilage neoepitopes, which demonstrated increased staining for these products in the lesions of WT mice after DMM surgery, while the KO mouse cartilage had little to no staining[10]. I concluded that genetic PPAR δ inhibition in cartilage was protective in post-traumatic

OA. I suspected this protective effect was in part due to a reduction of fatty acid oxidation (and resulting oxidative stress) seen in our early studies, since cartilage primarily metabolizes energy through glycolytic pathways.

Next, I investigated pharmacological inhibition of PPAR δ in post-traumatic OA in rats. The genetic model provided a strong foundation for our studies, but in order for this evidence to be translated into a useful therapeutic strategy, we needed to find a treatment. I administered PPAR δ inhibitors (GSK0660, GSK3787), or vehicle control DMSO after surgical induction of OA through anterior cruciate ligament transection (ALCT) and partial medial meniscectomy (PMMx). These drugs were administered daily (6 days a week) for four weeks. Concurrently, behavioural assessments were also conducted weekly on rats to evaluate behaviour modifications associated with pain. Changes in pain-related behaviour were observed in rats who had surgically induced OA, but no PPAR δ drug treatment. These rats rested more and performed less vertical activity. They also redistributed their limb loads by compensating with the contralateral limb. All these changes were blocked by the PPAR δ inhibitors. I also investigated structural pathology through toluidine blue staining paired with OARSI scoring. I discovered that rats without drug treatment had severe cartilage loss and substantial bone remodeling. In contrast, rats who were administered PPAR δ inhibitors experienced focal fibrillation, cartilage shearing and bone remodeling. When semi-quantitatively assessing these pathologies, cartilage degeneration and subchondral bone damage after surgical induction of OA was significantly different than SHAM operated rats in the DMSO group, while PPAR δ inhibitor treated rats fell somewhere in the middle. It may be necessary to find other measures and time points to quantitate damage, or to increase the N, to elucidate whether

there are substantial differences between treatment groups. I concluded that PPAR δ inhibition results in protection from pain-related behaviours after surgical induction of PTOA in rats, and this may be in part related to milder structural progression of OA.

Lastly, I identified novel gene targets for PPAR δ in chondrocytes. We conducted microarray studies paired with real-time PCR validation on immature murine articular chondrocytes treated with PPAR δ agonist GW501516, PPAR γ agonist Rosiglitazone, LXR agonist GW3965 and RXR agonist SR11237. These studies revealed that genes induced after treatment were largely metabolic in nature, primarily affecting lipid metabolism. Genes involved in oxidative stress and extracellular matrix turnover were also identified. Next, functional cellular lipid assays were performed to quantify cellular triglycerides, total cholesterol, free cholesterol and cholesterol esters. I observed a significant decrease in triglycerides after treatment with GW501516. This result, paired with our earlier in-vitro investigations, implied that chondrocytes were undergoing significant fatty-acid oxidation in response to PPAR δ stimulation. It is also probable that this shift in metabolic pathway could cause an increase in oxidative stress; and so we investigated one of the genes significantly induced in our arrays, *Txnip*. This gene encodes thioredoxin interacting protein, which binds and inhibits thioredoxin - a potent antioxidant. We investigated the presence of this protein in OA by performing immunohistochemistry on knee sections from mice who underwent surgical induction of OA through DMM. 10 weeks post-surgery TXNIP was present and intensely stained damaged cartilage, whereas there was little to no staining in the SHAM operated mice. We also wondered whether this could be a potential mechanism underlying PPAR δ 's effects in OA, so we stained *Ppard* KO mice and WT littermate controls 8 weeks post-

DMM surgery with the same antibody. We found increased staining in WT mice that had more severe damage, with prominent staining at joint margins and in osteophytes. In contrast, the *Ppard* KO mice that were protected from OA progression had little to no staining. From this I concluded that early OA changes caused by PPAR δ may be metabolic, and could be due to increased oxidative stress paired with lipid peroxidation.

Overall, these data demonstrate a significant role for PPAR δ in the promotion of OA and establish PPAR δ inhibitors as a potential therapeutic agent for structural pathology as well as pain-related behaviour.

5.2 Contributions and Significance of Findings

5.2.1 Contributions to the Field of Osteoarthritis

In this thesis, I characterize for the first time the role of PPAR δ in OA. This was accomplished through cartilage specific deletion of *Ppard*. In Chapter 2, I present work where I discovered that PPAR δ did not contribute significantly to joint or cartilage development, in contrast to *Pparg* which is essential for skeletal development[2]. Additionally, PPAR δ had never previously been examined in the context of PTOA. Here, I demonstrate that inactivation of PPAR δ in cartilage is beneficial, and chondro-protective after surgical induction of OA by DMM. Again this is in contrast to studies examining cartilage-specific deletion of *Pparg* which induces OA; indicating that these nuclear receptors have differential roles in both skeletal development and OA progression[3]. Through these studies, I establish PPAR δ as a potential therapeutic target in OA.

In Chapter 3, I draw upon my findings from the previous study indicating that genetic PPAR δ inactivation attenuates disease progression in PTOA and translate this into a preclinical model using pharmacological inhibitors of PPAR δ , to examine whether drug treatment will recapitulate this protective effect. Currently there are no drugs available that stop or delay disease progression in OA. In this study, I employed rat model paired with ACLT/ PMMx surgery to induce more rapidly progressing OA. This model is advantageous because it permits the evaluation of pharmacological agents in a shorter duration of time, and is commonly used and accepted[1, 11-13]. I discovered that rats treated with PPAR δ inhibitors do not experience the same behavioural modifications indicative of pain after surgical induction of OA, as untreated rats. It also appears that the inhibitors could confer some protective effects in both the cartilage and subchondral bone, but this will need to be more thoroughly quantitatively assessed. In addition to directly assess the role of PPAR δ , this study is among the first in the world to correlate changes in disease progression at a structural level with functional behavioural modification in a rat model. We build upon our own previous data validating the methods of behavioural testing (spontaneous locomotor activity and incapacitance testing) with histopathological progression of disease[14]. We also show that there may be a disconnect between significant effects of our PPAR δ inhibitors on pain behaviour but not on structural progression, similar to how a portion of patients who have radiographic osteoarthritis are asymptomatic for pain[15]. However, this needs to be studied in more depth before we can reach firm conclusions. Further we are able to show similar effects (at least qualitatively) in two different animal animal models, strengthening the foundation for PPAR δ 's potential as a feasible therapeutic target.

Chapter 4 identifies novel gene targets in chondrocytes after treatment with the PPAR δ agonist GW501516. These genes are highly involved in lipid metabolism, and our studies demonstrate that these changes in gene induction are functionally executed by chondrocytes. It has previously been established that chondrocytes can store lipids, and lipid or cholesterol abnormalities can influence the progression of OA[16-18].

Commonly, OA studies use Oil-Red O staining to visualize lipid deposition[19-21]. Here we quantitate the amount of lipids being stored and secreted by chondrocytes, in-vitro.

This is beneficial because it allows us to form a greater understanding of the changes occurring at a molecular level. We measure significantly less triglycerides in chondrocytes after PPAR δ treatment. In our earlier studies, we had observed significant upregulation of beta-oxidation after treatment with the same PPAR δ agonist.

Cumulatively this indicates that chondrocytes respond to PPAR δ by increasing their energy utilization from triglycerides. This is of interest since usually energy metabolism in chondrocytes is thought to be largely glycolytic[22, 23].

In this study we also observed a significant decrease in gene expression for *Gsta4*, the gene that encodes Gluthathione s-transferase A 4, in response to PPAR δ activation.

Gluthathione s-transferase A 4 protects against HNE (4 hydroxynonal) induced damage in chondrocytes. HNE is produced from ROS and lipid peroxidation and increased in OA.

It can post-transcriptionally modify the ECM and activate proteases to degrade the matrix. It is possible that the decreased levels of *Gsta4* in combination with increased fatty acid oxidation in response to PPAR δ activation causes severe oxidative stress and ultimately chondrocyte dysfunction. This stress can be further enhanced by increased levels of *Txnip*, since this gene is induced by the PPAR δ agonist. Increased presence of

TXNIP in OA cartilage in our mice supports this model and is indicative of increased oxidative stress and lipid peroxidation stemming from the dysregulation of lipid metabolism. Recently, another group also identified TXNIP as a regulator of autophagy in chondrocytes[24]. It is likely that an imbalance in regulatory processes mediating oxidative stress and inflammation promotes the OA phenotype that we observe (Figure 5-1).

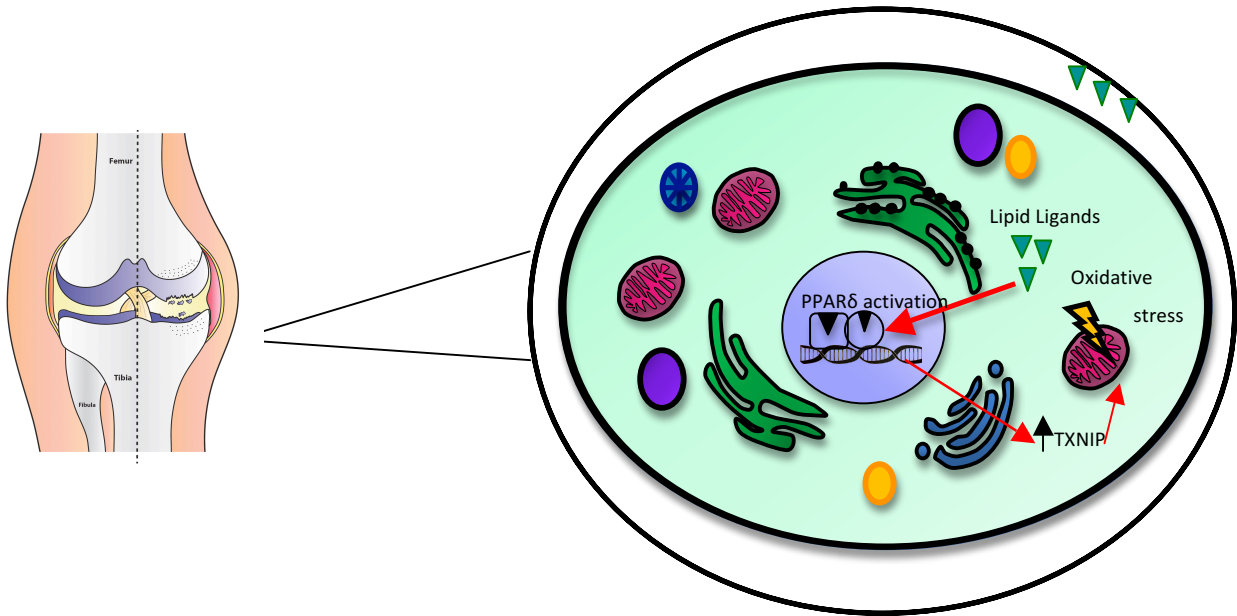


Figure 5-1 Schematic of PPAR δ function in OA

PPAR δ activation in chondrocytes by lipid ligands increases gene expression of *Txnip*, and results in increased production of thioredoxin interacting protein (TXNIP) which promotes oxidative stress by binding to anti-oxidant thioredoxin. Unregulated oxidative stress can cause chondrocyte dysfunction and this process may be in part responsible for what we observe during OA. Knee provided by Dr. M. Pest

5.3 Limitations of Research

5.3.1 Limitations in In-vitro Models

It is important to note and carefully consider the limitations in the studies described in this thesis. Firstly, although the immature murine articular chondrocytes (IMACs) are advantageous in that they allow us to investigate interventions on fully differentiated chondrocytes, with minimal dedifferentiation into other cell types, they are still limited in their ability to reproduce an in-vivo environment[25, 26]. The joint is a functional unit, and the cartilage, synovium and subchondral bone all communicate with each other, and are necessary to each other's functions. This in-vitro system lacks that feature, as well as normal mechanical stimulation. Even when examining the cartilage alone, chondrocytes plated in monolayer are not representative of cartilage tissue with its ECM, and different oxygen tensions throughout the tissue. Cells that normally interact with the matrix, instead interact with each other in culture, which subsequently can result in altered signaling[27, 28]. However, some of the mechanistic studies done here are almost impossible to do in-vivo, requiring the use of in-vitro surrogates with an awareness of their limitations.

In our array studies, we investigated one time-point; 72 hours. Genes may be differentially induced at earlier and later time-points. Further we only used one concentration of agonist for each of the nuclear receptors. While the concentration of 1 μ M was effective at inducing known target genes, different concentrations could have had a more potent effect on downstream target genes. Additionally, microarrays are not specific for direct gene targets. Chromatin Immunoprecipitation with subsequent DNA

sequencing (ChIP seq) would be ideally used to elucidate direct targets of PPAR δ when appropriate antibodies become available – unfortunately, the lack of ChIP grade antibodies is a common problem in the nuclear receptor field.

5.3.2 Limitations of In-Vivo Models

In-vivo models enable us to evaluate interventions and gain valuable information about behaviour and pathophysiology but they also have substantial limitations. In Chapter 2, I generated a tissue specific knockout of *Ppard*. The Cre-driver used in this model was Col-2 Cre which has since been characterized to be not completely cartilage specific with recombination occurring in a portion of osteoblasts and other joint cells as well[29, 30]. This might be a difficult problem to overcome in light of the strong evidence for chondrocyte-osteoblast trans-differentiation; however, the use of an inducible Cre driver (e.g. Aggrecan CreER) and postnatal tamoxifen injection could minimize these concerns[29].

In my study investigating the progression of PTOA with systemic treatment of PPAR δ inhibitors one of the major limitations was the method of drug delivery, which was daily subcutaneous injections. Firstly, we do not know how much of the drug actually reaches the joint tissues and how long it stays there. They must penetrate through the synovial membrane or subchondral bone in order to reach the cartilage. A solution to this would be to give the drug intraarticular, however this is complicated in a rodent because the injection itself could risk damaging the joint. Additionally, systemic delivery could have long term detrimental consequences because PPAR δ is broadly expressed in a number of tissues where it serves to regulate metabolism[6, 31].

Both of the in-vivo studies only used one-time point, whereas early and late stage OA- particularly PTOA are quite different[32]. While PPAR δ may have one effect at one time point, it may not act the same way at different stages of OA, with different levels of inflammation and mediators present. Therefore, it would be beneficial to characterize its effects at different time-points from early to late stage OA. Another common factor between these studies, is that they both employ rodent models. While these rodent models are economically efficient, and enable evaluation of disease progression and interventions over a shorter period of time, they do not fully recapitulate the biomechanics of human OA, as they are quadrupeds and have a tail. Rodents have growth plates that do not fuse as they become skeletally mature and their size limits tissue discrimination and availability. Larger animal studies, though also quadrupeds with tails, may be more beneficial for imaging studies due to their size, and also have more tissue for biochemical analyses, but they are far more costly, difficult to house and manage, and do not have the option for genetic modification[33]. Lastly, both in-vivo models only used male animals. It has been well documented in human studies that the incidence of OA, progression, and severity are greater in post-menopausal females, implying that sex-hormones could be responsible for chondroprotective effects in females[34]. It has also been demonstrated that male mice develop the most severe OA following surgical destabilization of the medial meniscus, followed by orchietomized males, ovariectomized females and then female mice[35]. It would therefore be more encompassing to account for the effects of sexual dimorphism on OA progression by investigating both sexes.

5.4 Future Directions

The foundation established by this thesis can be further expanded and built upon in several ways, some of which can directly address the limitations discussed above.

Molecular studies could use RNA-sequencing to form a more complete picture of what is happening in chondrocytes after they are treated with the PPAR δ agonist. This would encompass non-coding RNAs as well as alternative splicing. Executing these studies in multiple tissue types including synovium and osteoblasts would add to its comprehensiveness. Moreover, additional shorter and longer time points would add important information. As mentioned above, if ChIP grade antibodies to PPAR δ become available, this technique would ideally complement RNA-sequencing to distinguish direct and indirect targets.

As described earlier, the Col-2 Cre driver used in the transgenic mouse studies was not specific; to evaluate the role of PPAR δ in cartilage we could instead employ an aggrecan Cre driver which is more specific and temporally controlled[34]. However, as OA affects multiple tissue types, it would be equally important to evaluate the role of this candidate in other joint tissues such as bone by using an osteoblast-specific Cre driver such as one driven by the osteocalcin promoter[35].

In addition to examining multiple tissue types through transgenic mouse studies, it would be beneficial to investigate changes in bone mineral density, and subchondral plate thickness through MicroCT imaging after administration of PPAR δ in a model of PTOA. Similarly, looking at changes in the synovium, such as inflammatory markers and synovial membrane thickness, would be helpful to obtain a more cohesive picture of

disease progression and to evaluate whether PPAR δ inhibition was helpful or detrimental in one tissue versus another. The dorsal root ganglion demonstrates changes in innervation and macrophage infiltration during the progression of OA, and future studies could include these changes in their assessment[36], especially in light of the dis-connect between behavioral and structural outcomes seen in chapter 3. Next steps would also involve assessing outcomes at different stages of OA, early vs late, as well as the use local delivery combined with a vehicle that promotes long-term release, so inhibitors do not need to be administered every day. In addition, an increase in the number of animals tested in our rat model (chapter 3) will be required to examine whether the trend towards structural benefits of the PPAR δ inhibitors reflects true beneficial effects.

The in-vivo studies described here focus on post-traumatic osteoarthritis which is only representative of 12% of all OA cases[37]. Further studies should investigate primary age-associated osteoarthritis and metabolic OA. Some of these studies are in progress in our laboratory already. PPAR δ is an important regulator of metabolism in many tissues, and has promoted changes in metabolism in our own studies. Therefore it would be worthwhile to evaluate the role of PPAR δ in high-fat diet induced OA, and in combination with OA promoting fatty acids. Lastly, it would also be important to evaluate differences in PPAR δ expression and activity in human tissue samples from different sub-types of OA. This would add another dimension and potentially enhance our rationale for the continued investigation of this target.

In conclusion, the data shown here suggest that PPAR δ is an important promoter of post-traumatic OA, possible through induction of lipid metabolism and associated oxidative

stress. Future studies will need to expand on these results to determine the relative importance of this player in OA, and to further evaluate its potential as therapeutic target.

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Appendix A: Animal Use Protocols

Sent: June 30, 2015 2:17 PM

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AUP Number: 2015-018

PI Name: Beier, Frank

AUP Title: Tgfa/egfr Signaling In Osteoarthritis

Approval Date: 06/30/2015

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



AUP Number: 2015-031

PI Name: Beier, Frank

AUP Title: Regulation Of Endochondral Bone Growth By Hormones

Approval Date: 07/10/2015

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2. Animals for other projects may not be ordered under this AUP number.
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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
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University Council on Animal Care

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Curriculum Vitae

Anusha Ratneswaran

Education

- 2011-Present The University of Western Ontario
 London, Ontario, Canada
 Ph.D. Candidate
- 2006-2010 McMaster University
 Hamilton, Ontario, Canada
 BSc. Honours

Publications

Ratneswaran, A., Sun, MMG., Dupuis, H., Sawyez, C., Borradaile, N., and Beier, F. Nuclear receptors regulate lipid metabolism in chondrocytes. Submitted to Arthritis and Rheumatology. May 2016.

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Editorials

Ratneswaran A., and Beier F. A top-notch dilemma: the complex role of NOTCH signaling in osteoarthritis. *Science Signaling*. 386 (8): fs14. 21 July 2015

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2015 Gordon Mogenson Award, Physiology

2015 Physiology and Pharmacology Research Day, 2nd Place Poster, Clinical Pharmacology

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2015 Lucille and Norton Woolf, Best Publication Award

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- 2012-15 Canadian Institute of Health Research Strategic Training Program Musculoskeletal Health Research and Leadership, Joint Motion Program, Graduate Fellowship
- 2014 Osteoarthritis Research Society International Collaborative Scholarship
- 2013 Arthritis Alliance of Canada, Best Poster, Graduate Student
- 2013 Canadian Connective Tissue Conference, Best Poster, Clinical and Fundamental Aspects of Connective Tissue Disease
- 2013 London Health Research Day, Best Poster, Musculoskeletal Health Research

Professional Development

- 2012-2013 Richard Ivey School of Business, Health Sector Leadership, Joint Motion Program

Teaching

- 2013-2016 Physiology 4980- 4th Year Honours Thesis Course, UWO
- 2011-2013 Physiology 3130Y- Physiology Laboratory, UWO
- 2008-2010 Human Anatomy and Physiology, McMaster University

Supervision

- 2015-2016 3 Undergraduate students (1- 4th year Honors, 1-2nd year scholars elective, 1-2nd year scholars elective)
- 2014-2015 1 Summer Student
- 2013-2014 1-4th Year Honors Student
- 2012-2013 1-4th Year Honors Student
- 2011-2012 1-3rd Year Undergraduate Student

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- 2015 Gordon Cartilage Biology and Pathology, Galveston, TX, USA
- 2014 Matrix Biology Society of Australia and New Zealand, Queenscliff, VIC, Australia
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- 2015 Bone and Joint Institute Trainee Conference, London, ON, Canada
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- 2015 Gordon Research Seminars, Galveston, TX, USA
- 2014 Matrix Biology Society of Aus. and NZ., Queenscliff, VIC, AUS
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