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Le rôle spécifique de PPARγ *in vivo* et sa voie de signalisation dans la pathophysiologie de maladie l'arthrose

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The specific *in vivo* role of PPARγ and its downstream signaling pathway in the pathophysiology of Osteoarthritis

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RESUME

L'arthrose est une maladie articulaire dégénérative, avec une pathogenèse inconnue. Des études récentes suggèrent que l'activation du facteur de transcription du récepteur activateur de la prolifération des peroxysomes (PPAR) gamma est une cible thérapeutique pour ce maladie. Les agonistes du PPARγ inhibent l'inflammation et réduisent la synthèse des produits de dégradation du cartilage *in vitro* et *in vivo*. Cependant, des études utilisant des agonistes du PPARγ n'élucident pas les effets exacts médiés par ce gène complexe. En effet, certains de ces agonistes ont la capacité de régulariser d'autres voies de signalisation indépendantes de PPARγ, ainsi entraînant des effets secondaires graves. Afin d'obtenir une efficacité thérapeutique avec potentiellement moins de problèmes de sécurité, il est donc essentiel d'élucider, *in vivo*, le rôle exact de PPARγ dans la physiopathologie OA. Mon projet de thèse permettra de déterminer, pour la première fois, le rôle spécifique de PPARγ *in vivo* dans la physiopathologie OA. Les souris utilisées pour l'étude avaient une délétion conditionnelle du gène PPARγ dans le cartilage. Ces dernières ont été générées en employant le système LoxP/Cre.

Pour tester cette hypothèse, j'ai généré deux types de souris avec une délétion au PPARγ, (a) une suppression du gène PPARγ spécifiquement dans le cartilage germinale pour l'étude de l'arthrose liée au développement et à l'âge et (b) la suppression inductible du gène PPARγ spécifiquement dans le cartilage chez la souris adulte pour les études OA. L'étude précédente dans notre laboratoire, utilisant ces souris ayant une délétion au gène PPARγ germinales, montre que ces souris présentent des anomalies du développement du cartilage. J'ai également exploré si ces souris qui présentent des défauts précoces du développement ont toutes les modifications phénotypiques dans le cartilage au cours du vieillissement. Mes résultats ont montré que les souris adultes, ayant une délétion au gène PPARγ, ont présenter un phénotype de l'arthrose spontanée associée à une dégradation du cartilage, l'hypocellularité, la fibrose synoviale. Cette étude a montré que PPARγ est un régulateur essentiel pour le cartilage, et c'est le manque (l'absence) de ce dernier qui conduit à un phénotype de l'arthrose spontanée accélérée (American Journal of Pathologie).

A partir de ce but de l'étude, on n'a pas pu vérifier si ces souris présentaient l'OA spontanée en raison des défauts de développement ou à la suite de la délétion du gène PPARγ.

Pour contourner les défauts de développement, j'ai généré des souris ayant une délétion du gène PPARγ spécifiquement dans le cartilage inductible avec le système Col2rTACre. Ces souris ont été soumises à modèle de la chirurgie OA (DMM: déstabilisation du ménisque médial) et les résultats révèlent que les souris PPARγ KO ont une dégradation accélérée du cartilage, une hypocellularité, une fibrose synoviale et une augmentation de l'expression des marqueurs cataboliques et des marqueurs inflammatoire.

La perte de PPAR dans le cartilage articulaire est un évènement critique qui initie la dégradation de cartilage dans OA. Les études récentes suggèrent que le procès d'autophagie, une forme de survie cellulaire programmée, est altéré pendant l'OA et peut contribuer vers une protection diminuée des cellules, résultant la dégradation du cartilage. J'ai donc exploré le rôle de PPARγ dans la protection des cellules en déterminant l'effet de manque de PPARγ dans le cartilage par l'expression de mTOR (régulateur négatif principal d'autophagie) et les gènes d'autophagie durant OA. Mes résultats ont montré que les souris KO PPARγ présentent également une augmentation sur l'expression de mTOR et une diminution sur l'expression des marqueurs autophagiques en comparaison avec les chondrocytes articulaires isolés des souris contrôles OA. J'ai suggéré l'hypothèse que PPARγ contrôle la régulation de la signalisation de mTOR/autophagie, et finalement la mort des chondrocytes et l'expression des facteurs cataboliques et les facteurs inflammatoire. Pour tester cette hypothèse, j'ai fait la transfection des chondrocytes arthrosiques PPARγ-KO avec le vecteur d'expression de PPARγ pour déterminer si la restauration de l'expression de PPARγ peut sauver le phénotype des cellules PPARγ-KO OA. J'ai observé que la restauration de l'expression de PPARγ dans les cellules PPARγ-KO en présence du vecteur d'expression PPARγ, a pu considérablement régulariser négativement l'expression de mTOR et mettre en règle positivement l'expression des gènes autophagiques ainsi que le sauvetage significative de l'expression du collagène de type II et l'aggrecan et de baisser de manière significative l'expression de marqueurs cataboliques critiques et des marqueurs inflammatoires. Pour prouver que l'augmentation de la signalisation de mTOR et la diminution de l'autophagie est responsable du phénotype OA accélérée observée dans les souris PPARγ KO *in vivo*, j'ai généré les souris doubles KO PPARγ- mTOR inductible spécifique du cartilage en utilisant le système Col2 - rtTA -Cre et soumis ces souris à DMM modèle de l'arthrose. Mes résultants démontrent que les souris avec PPARγ- mTOR doubles KO ont été significativement protégés contre les OA DMM induites associées à une

protection significative contre la destruction du cartilage, la perte de protéoglycanes et la perte de chondro-cellularité par rapport aux souris témoins. Considérant que mTOR est un répresseur majeur de l'autophagie, j'ai trouvé que l'expression de deux marqueurs de l'autophagie critiques (ULK1 et LC3B) a été significativement plus élevée dans les chondrocytes extraits les souris doubles KO PPARγ-mTOR par rapport aux souris témoins. En plus, les études de sauvetage *in vitro* en utilisant le vecteur d'expression PPAR et les études *in vivo* utilisant les souris doubles KO PPARγ- mTOR montrent que PPARγ est impliqué dans la régulation de la protéine signalant de mTOR/autophagie dans le cartilage articulaire.

Ces résultats contournent PPARγ et sa signalisation en aval de mTOR/autophagie en tant que cibles thérapeutiques potentielles pour le traitement de l'arthrose.

Mots clés: Arthrose, les souris knock-out, cartilage, PPARγ, catabolisme, inflammation, Vieillissement, chondro-protection, m-TOR, autophagie

SUMMARY

Osteoarthritis (OA) is an age related degenerative joint disease with unknown pathogenesis. Recent studies suggest that the activation of the transcription factor Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is a therapeutic target for OA. Agonists of PPARγ inhibit inflammation and reduce the synthesis of cartilage degradation products both *in vitro* and *in vivo***.** However, studies using agonists of PPARγ do not elucidate the exact effects mediated by this complex gene. Indeed, some of these agonists have the ability to regulate, *in vivo*, various other signaling pathways independent of PPARγ, resulting in serious side effects**.** It is therefore vital, in order to achieve therapeutic efficacy with potentially less safety concerns, to elucidate the exact *in vivo* role of PPARγ in OA pathophysiology. Thus, the aim of my PhD project was to determine the specific *in vivo* role of PPARγ in OA pathophysiology using cartilage-specific PPARγ knockout (KO) mice and subjecting these mice to surgical model of OA.

I generated two separate PPARγ KO mice harboring a (a) constitutive cartilage-specific germ-line deletion of PPARγ gene for developmental and age-related OA study and (b) inducible cartilage-specific deletion of PPARγ in adult mouse specifically for OA studies using LoxP Cre system. Previous study in my laboratory using germ-line PPARγ KO mice shows that these mice exhibit cartilage developmental defects. I further explored if these mice which exhibit early developmental defects have any phenotypic changes in the articular cartilage during ageing. My results showed that adult PPARγ KO mice exhibited a spontaneous OA phenotype associated with enhanced cartilage degradation, hypocellularity, synovial fibrosis, and increased expression of catabolic and inflammatory factors. This study showed that PPARγ is a critical regulator of cartilage health, the lack of which leads to an accelerated spontaneous OA phenotype (Vasheghani et al, 2013; *American Journal of Pathology*). From this aim of the study, I could not ascertain if cartilage-specific germline PPARγ KO mice exhibited spontaneous OA because of developmental defects or as a result of PPARγ deficiency. To bypass the developmental defects, I then generated inducible cartilage-specific PPARγ KO mice using Col2rTACre system and subjected these mice to destabilization of medial meniscus (DMM) model of OA surgery. My results revealed that

PPARγ KO mice showed accelerated cartilage degradation, hypo-cellularity, synovial fibrosis and increased expression of catabolic and inflammatory factors during OA.

Loss of chondrocyte cellularity within the articular cartilage is one of the critical events that initiate the degradation of the cartilage during OA. Recent studies suggest that the process of autophagy, a form of programmed cell survival, is impaired during OA and may contribute towards decreased chondro-protection resulting in cartilage degradation. Thus, I further explored the role of PPARγ in chondro-protection by determining the effect of PPARγ deficiency in the cartilage on the expression of mTOR (master negative regulator of autophagy) and autophagy genes during OA. My results revealed that PPARγ-deficient chondrocytes exhibit significantly enhanced expression of mTOR and decreased expression of genes that initiate autophagy process compared to chondrocytes extracted from control OA mice. I then hypothesized that PPARγ controls mTOR/autophagy signaling and ultimately the fate of chondrocytes and the expression of catabolic and inflammatory factors in the articular cartilage. To test this, I transfected PPARγ KO OA chondrocytes with PPARγ expression vector to determine if restoration of PPARγ expression can rescue the phenotype of PPARγ KO OA cells. I observed that restoration of PPARγ expression in PPARγ KO cells significantly down-regulated the expression of mTOR and up-regulate the expression of autophagy genes along with significant rescue in the expression of collagen type II and aggrecan and significant down-regulation in the expression of critical catabolic and inflammatory markers. To validate our *in vitro* finding that enhanced mTOR signalling and resultant decrease in autophagy is responsible for accelerated OA phenotype observed in PPARγ KO mice, I generated inducible cartilage-specific PPARγ-mTOR double KO mice and subjected these mice to DMM model of OA. My results clearly demonstrate that PPARγ-mTOR double KO mice exhibit significant protection against DMM-induced OA associated with significant protection from cartilage destruction, proteoglycan loss and loss of chondro-cellularity compared with control mice. Since mTOR is a major repressor of autophagy, I found that the expression of two critical autophagy markers (ULK1 and LC3B) was significantly elevated in PPARγ-mTOR double KO mice compared to control mice. My in vitro rescue studies using PPARγ expression vector and *in vivo* studies using PPARγ- mTOR double KO mice clearly show that PPARγ is involved in the regulation of mTOR/autophagy signalling in the articular cartilage. Therefore, deficiency of PPARγ upregulates mTOR

signalling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA. This study for the first time provides direct evidence on the role of PPARγ in chondroprotection by modulation of mTOR/autophagy signalling in the articular cartilage.

These findings outline PPARγ and its downstream signalling by mTOR/autophagy as potential therapeutic targets for the treatment of OA.

Keywords: Osteoarthritis, Knockout mice, cartilage, PPARγ, catabolism, inflammation, aging, chondro-protection, mTOR, Autophagy

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LIST OF ABBREVIATIONS

ACL: anterior crucial ligament
ADAMTS: a disintegrin and metalloproteinase with thrombospondin motif
AP-1: activator protein-1
Bcl-2: B-cell lymphoma 2
Bcl XL: B-cell lymphoma-extra large
BMI: body mass index
BMP: bone morphogenetic proteins
BSA: bovine serum albumin
C1-2C: Col2-3/4Cshort
cAMP: cyclic adenomonophosphate
C/EBP: CCAAT/enhancer-binding protein
COX-2: cyclooxygenase-2
Celecoxib: selective COX-2 inhibitors
CRE : Cyclic AMP response element
CR-CHUM: University of Montreal Hospital Research Centre
CT-Scan: computed tomography- scan
DJD: Degenerative Joint Disease
DMM: destabilization of medial meniscus
DBD: DNA-binding domain
DMOADs: disease modifying osteoarthritis drugs
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic scid
Egr-1: early growth response protein 1
ER: endoplasmic reticulum
FAK: focal adhesion kinase
FBS: fetal bovine serum
FGF: fibroblast growth factor
FRAP-1: FK506 binding protein 12-rapamycin associated protein 1
GHP-1: growth hormone protein-1
HIF2-α: hypoxia inducible factor 2-alpha
IGF-1: insulin growth factor-1
IgG: immunoglobulin G
IHC: immunohistochemistry
IL: interleukin
IL-1ß: interlukine-1 beta
IL-1R: interlukine -1-receptor
IIa: splice form of type II collagen
iNOS: inducible nitric oxide synthase
JNK: c-Jun N-terminal kinase
KO: knockout
LBD: ligand binding domain
LC-3: microtubule-associated protein 1 light chain 3
MAPK: mitogen activated protein kinase
MCL: medial colateral ligament
MMPs: matrix metalloproteases
MMTL: medial meniscotibial ligament
mPGES-1: microsomal prostaglandin E synthase-1
MRI: magnetic resonance imaging
mRNA: messenger ribonucleic acid
MSCs: mesenchymal stem cells
mTOR: mammalian terget of rapamycin
N-cadherin: class-1 transmembrane protein in cell adhesion
N-cam: natural-cell adhesion molecule
N-CoR: nuclear receptor co-repressors
NF-κB: nuclear factor- kappa B
NO: nitric oxide
NSAID: non-steroidal anti-inflammatory drug
OA: osteoarthritis
OARSI: osteoarthritis research society international
PBS: phosphate buffered saline
PE: phosphatidylethanolamine
PGE2: prostaglandin E2
PI3K/AKT: phosphatidylinositide 3-kinases/ protein kinase B
ROS: reactive oxygen species
PPARs: proxisome proliferator-activated receptors
PPREs: proxisome proliferator hormone response elements
RXR: retinoid X receptor
SDF-1ß: stromal derived factor-1 beta
SEM: standard error of the mean
sPLA2: soluble phospholipase A2
TACE: tumor necrosis factor converting enzyme
TBS: tris buffered saline
TBS-T: tris buffered saline containing Tween-20

TD: thanatophoric dysplasia
TGF: transforming growth factor
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
TGF-βR: TGF-beta receptor
TZDs: Thiazolidindiones
ULK-1: Unc-51–like kinase 1
WT: wild type
9c-RA: 9-cis-retinoic acid
15d-PGJ2:15-deoxy-Δ12,14-prostaglandinJ2
15-HETE: hydroxyl-eicosatetraenoic acid

DEDICATION

To my parents, my brothers whose boundless love and continuous encouragement was unremitting source of inspiration for this work. No word can express how grateful I am for your love and support. And to my love, who has supported me throughout entire process, both by keeping me harmonious and helping me putting pieces together. I will be grateful forever for your love. This thesis is dedicated to them.

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CHAPTER I:

INTRODUCTION

I- Osteoarthritis

Osteoarthritis (OA) belongs to the "Arthritis family of diseases" in which'Arth' means joint and 'itis' means inflammation. OA is also referred as "osteoarthrosis" or degenerative joint disease (DJD). OA is among the most prevalent chronic human health disorders and the most common form of arthritis. It is a leading cause of disability in Canada and with increasing life expectancy; OA is a major socioeconomic and clinical concern. OA is a slowly progressing disease that can affect all the joints of the body, but the knees are most commonly affected. Over 27 million people are affected by OA in North America and majority of them are over 65 years old, with radiological signs of OA [1-3]. Roughly 2% of women and 1.4% of men develop radiographic OA per annum [4]. OA is one of the major socioeconomic and clinical concerns with huge annual health and economical expenses.This joint degenerative disease with significant costs including direct costs such as resources used in the research, prevention, detection, and treatment of disease and indirect costs like work absenteeism and arthritis-related hospitalization poses a economic and humanistic burden on society [5].

OA is categorized into two groups: primary (idiopathic) and secondary. Primary OA is the most common form of OA that is associated with aging and heredity. Secondary OA is associated with trauma, metabolic diseases and developmental disorders [6]. Aetiology and pathogenesis underlying OA is poorly understood, thus making it difficult to identify potential disease-modifying targets. OA is clinically described by joint pain and reduced mobility, associated radiographically with joint space narrowing, subchondral bone remodelling and osteophyte formation [7, 8]. While OA is described by articular cartilage deterioration and damage, it is a disease that affects the whole joint. Other affected parts of the joint consist of

the peri-articular and subchondral bones, synovial membrane, and adjacent supporting connective tissue elements [9, 10].

I-1-Risk Factors

OA is a multifaceted disease with a variety of underlying biochemical and physical causes. There are numerous risk factors implicated in OA including aging, obesity, metabolic factors, hereditary and genetic susceptibility, joint injuries and fractures, trauma, altered mechanical load, inflammation and weight [7]. Some of these factors are systemic including age, sex, ethnicity, genetic, and nutritional factors. Other factors like obesity and joint injury are classified as local biomechanical factors (Table-i). Typically, some factors such as genetics, metabolism, and obesity are believed to promote the initiation of OA while some factors like aging and hereditary lead to the progression of this disease [1, 9, 11, 12].

I-1-1-Systemic risk factors:

The most critical systemic susceptibilities are aging and female gender. Disease incidence and prevalence increase radically with age [4]. The Framingham study has shown that 27% of 63 to 70 years old aged had radiographic evidence of knee OA, increasing to 44% in the over 80 age group [4]. On a biomechanical level, this probably arises from changes like decreased muscle tone, and altered gait. On a cellular level, it may correlate with reduced ability of cartilage to carry our anabolic activity, or decreased responses to growth factors and enhanced apoptosis in chondrocytes, the only cell type of the cartilage [12].

I-1-2- Biomechanical risk factors:

Obesity, body mass index (BMI) above 30, is deemed as one of the significant risk factors for knee OA because of the effect of over load on joint [9]. According to Felson and

his colleagues, there is a significant connection between obesity and radiographic knee OA [13]. Cicuttini *et al*. have shown that per kg increase in body weight in women is associated with significant increase (9-13%) in the risk of developing hand and knee OA [14].

Other systemic and biomechanical risk factors such as metabolic factors like hypertension, hyperglycemia, lipid abnormality, and insulin resistance have also been implicated in the pathophysiology of OA [15].

Systemic factors	Local biomechanical factors
Age	**Obesity**
Gender	**Trauma**
Ethnicity	**Joint injury**
Hormonal status	**Bone density**
Diet	**Exercise**
Genetic	

Table-i: ***OA Risk Factors***

1-2- Symptoms

The first sign of OA that appear in joints is pain, specifically after physical work or exercise. Progressively, other most common symptoms including progressive pain, tenderness, and swelling appear in the joint areas. Pain associated with OA leads to reduction of motivation and functional limitation in affected person and is usually followed by stiffness, inflammation and crepitus in the joint area. Articular cartilage degradation in the joint area is the main feature of OA. In early stage of disease, roughening of cartilage and sclerosis of sub-chondral bone are followed by expanding of cartilage fissures and bone remodeling in the

intermediate stage of deterioration. However, in the late stage of the disease, complete loss of articular cartilage, osteophyte and subchondral cyst formation are accompanied by synovitis. OA related pain reduces the quality of life and limits physical functions like housework as well as leisure activities [16, 17].

I-3- Diagnosis

Swelling and deformities in OA are detectable by physical examination. However, to define the diagnosis, conventional radiographs are necessary to evaluate characteristic modifications found in OA joints. By using the radiographic diagnostic tools such as X-ray, computed tomography- scan (CT-Scan), and magnetic resonance imaging (MRI) several features of OA can be detectable including osteophytes formation on the joint margins, narrowing of joint spaces, pseudocystic area in subchondral bone, and bone malformation [18]. Since 1980s, MRI has become a potent and effective methodology for diagnosis of OA due to its ability to take images in three dimensions with a high degree of soft-tissue contrast.

1-4- Treatment

Previous clinical studies to treat established OA by inhibiting cartilage ECM degradative and catabolic factors such as MMPs as well as inflammatory cytokines such as IL-1β, TNF-α *etc.* have met with disappointing results associated with low efficacy and serious side effects [19], as a result there is no disease-modifying OA drugs (DMOADs) currently available. MMPs are one of the major catabolic/destructive factors implicated in OA pathophysiology. To control the level of MMP synthesis/activity, various synthetic approaches have been the focus of very intensive research. Synthetic MMP Inhibitors (MMPIs) were first developed as a

result of studies aimed at interpreting the physiological damage caused by MMPs. They generally block the proteolytic effects of activated MMP enzymes and prevent pro-enzyme activation [20]. MMPIs first entered clinical trials in humans for the treatment of cancer. Batimastat, a peptidomimetic and a broad-spectrum inhibitor that blocks MMP-1, -2, -3, and -9, was a first generation potent MMPI while Marimastat was a second generation synthetic inhibitor [21, 22]. Metastat (COL-3), another synthetic MMPI, is a tetracycline derivative that reached phase II clinical trials [23]. However, none proved to be therapeutically effective for reasons such as poor tolerability and musculoskeletal pain. To improve bioavailability and pharmacokinetics, non-peptidomimetics were synthesized. Among them is Prinomastat, which is described mainly as a gelatinase inhibitor that also demonstrated musculoskeletal side effects [21]. Another, Bay 12-9566, which, unlike other MMPIs, has a carboxyl zinc-binding group as opposed to a hydroxamate-binding group, also underwent clinical trials for cancer and OA; trials were halted due to disease progression and mortality [24, 25]. Similarly, a recombinant form of IL-1Ra, named anakinra, that inhibits IL-1 activity when tested in a multicenter, randomized, placebo-controlled study to evaluate the efficacy of anakinra in patients with symptomatic OA of the knee demonstrated that despite a slight pain relief at four days following a single intra-articular injection of anakinra, it was not effective at relieving the symptoms of knee OA at 12 weeks [26]. Moreover, the drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors prescribed for OA treatment only reduce inflammatory symptoms but don't cure the underlying pathology of the disease [27-31].

Surgery is the last solution for the patients with significant joint destruction. This includes joint replacement, uni-compartment knee replacement, joint lavage (to rinse out any

blood, fluid or loose debris from inside the joint space), arthroscopic debridement (taking out areas of loose, synovitis and joint lining from the joint) and osteotomy (bone cutting to remove a wedge of the tibia). As development of cartilage destruction and damage is a hallmark in OA progression, looking for potential drugs to retard or cease this process is essential.

II- Synovial Joint

A synovial joint is the most flexible type of joint located in the wrists, elbows, hips and knees. Synovial joints contain articulating bones, articular cartilage, synovial membrane and synovial cavity or joint capsule filled with synovial fluid. Synovial fluid acts as a protective barrier in the joint which provides nutrients to the articular cartilage [32]. Articular cartilage is a lubricious and load-bearing tissue at the end of bones in synovial joints provides low-friction gliding surface with solid compressive strength and resistance to assist the movement of bones without friction [33].

II-1- Cartilage

Cartilage is a solid connective tissue with flexible matrix made up of chondrocytes, collagen, proteoglycans and water. Cartilage is avascular and is located in the joints between bones and derived from mesoderm during embryogenesis. Shock absorbance is the utmost vital role of cartilage throughout the body. It absorbs shock in the joint area with unique mechanical behavior. Cartilage tissue consists of specialized cell types called chondrocytes. Chondrocytes along with a large amount of ECM form articular cartilage tissue. ECM consists of 70% of water and 30% of collagen, proteoglycans and elastic fibers that maintain the

cartilage homeostasis [34]. Homeostasis of cartilage depends on a tightly regulated balance between anabolism and catabolism of the matrix.

Cartilage is typically divided in four zones. The superficial zone contains the highest amount of collagen among other zones and occupies 10 to 20% of the articular cartilage volume and provides a smooth gliding surface. Middle or transitional zone occupies about 40 to 60% of articular cartilage and consists of thicker, less organized collagen fibers, and round chondrocytes. The deep zone with capacity up to 30% of the articular cartilage contains large collagen fibers, columnar chondrocytes. This zone has the highest proteoglycan content and the lowest water concentration among all other zones. The Calcified zone consists of smaller cells in a chondroid matrix which is directly attached to the subchondral bone [35, 36].

Although there are numerous types of collagen like collagen type VI, IX, X and XI; collagen type II is the major form of collagen present in the cartilage ECM. Cartilage ECM consists of two phases: solid phase and fluid phase. Solid phase with low permeability contains collagen (mostly type II with up to 20% of wet weight of cartilage) and proteoglycan consisting of aggrecan protein core with chondroitin sulphate and keratan sulphate glycosaminoglycan chains [37]. Fluid phase is composed of water and ions. Interestingly, in fluid phase, water with abundance of up to 80% of the cartilage wet weight, creates intra-fibrillar space in solid matrix and associates with osmotic pressure in collaboration with negative charge of proteoglycans [38].

II-2-Chondrocytes

Chondrocytes are the only specific cell types present in the cartilage. Chondrocytes produce cartilage matrix and maintain ECM under appropriate condition of low turnover. Chondrocytes secrete catabolic and anabolic cytokines for maintaining equilibrium between matrix synthesis and degradation factors which allows the preservation of the structure of the cartilage ECM [37]. This balance can be broken by a combination of mechanical and biochemical factors during early OA. Imbalance in expression, activity and signaling of catabolic and anabolic factors causes the degradation process progression, resulting in a loss of articular cartilage and severe OA [39]. One of the components of the cartilage ECM, aggrecan, is relatively replaceable. Therefore, its destruction is not highly critical until a certain point. However, collagen is not released abundantly from the tissue; its loss is essentially irreversible [1, 40].

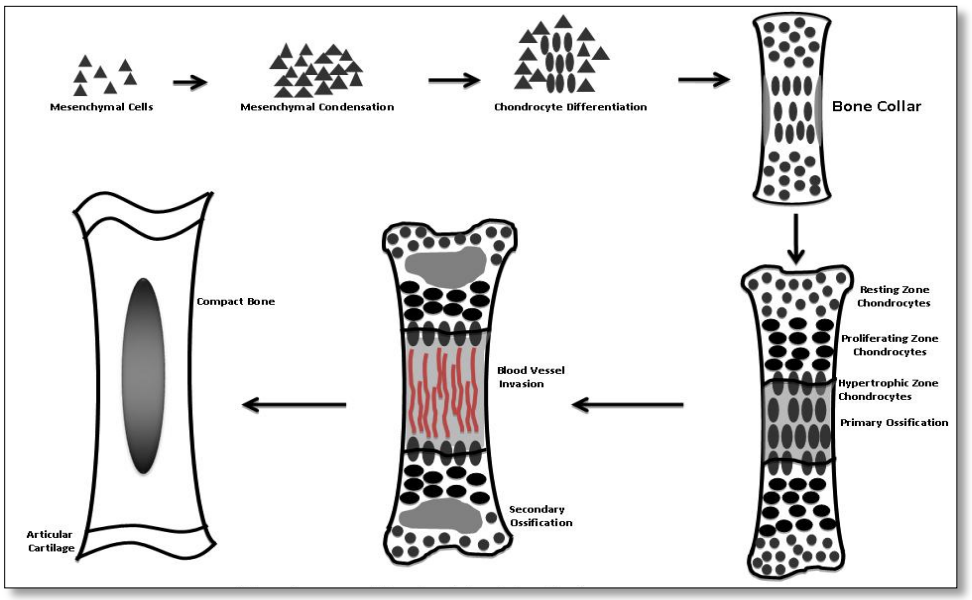

Figure-1: ***Process of chondrogenesis.*** The first step in creating intermediate cartilage template is through the condensation of mesenchymal cells. Differentiated chondrocytes express specific markers allowing the two cell types to be easily distinguishable. Undifferentiated cells remain at the border of condensations and form perichondrium, a layer of connective tissue surrounding the cartilage.

During chondrogenesis, cells become spherical and begin expressing transcription factors such as Sox 9, 5, and 6, all of which regulate genes encoding for collagen type II and aggrecan [39, 40]. The first step in creating this intermediate cartilage template is through the condensation of mesenchymal cells (Figure-1). In these condensations, mesenchymal cells differentiate to chondrocytes [41]. Differentiated chondrocytes express specific molecular markers that undifferentiated mesenchymal cells do not, thus allowing the two cell types to be easily distinguishable. Undifferentiated cells remain at the border of condensations and form a perichondrium, a layer of connective tissue surrounding the cartilage [42]. The density of the initial condensations is directly proportional to the level of chondrogenic differentiation [43].

Chondrocytes are organized into three zones within the epiphyses of the cartilage: resting, proliferating, and hypertrophic. The chondrocytes belonging to each zone are distinguishable by behavior, cell shape, function, and the molecular markers they express. Upon differentiation from mesenchymal cells to chondrocytes, chondrocytes will either remain as chondrocytes or differentiate to hypertrophic chondrocytes. It is the chondrocytes within the cartilage anlagen that proliferate while central chondrocytes differentiate to hypertrophy. Chondrocytes that do not differentiate form the cartilage on the articular surface of joints [44].

II-3- Synovial membrane

The synovium is a well vascularized and innervated membrane that lines the joint capsule and surrounds all non-cartilaginous components within articular joints. The membrane consists of two thin layers of specialized cells termed synoviocytes [45].There are two types of synoviocytes within the synovium, type A and type B. Type A synoviocytes are macrophage-

like cells and type B synoviocytes are synovial fibroblasts. Type A cells are responsible for eliminating excess material and pathogens from the joint, and producing and secreting some enzymes and cytokines/chemokines that contribute to inflammation and cartilage degeneration. Type B cells are presumably responsible for producing hyaluronan and for performing as a barrier that keeps synovial fluid in the joint capsule. Overall, both type A and B cells function as integral players in the maintenance of a healthy environment, which is essential for the proper function of all tissues.

The synovial membrane secretes synovial fluid and has two fundamental physiological roles. Firstly, it determines what can and cannot enter the joint space. Secondly, synovial cells are responsible for producing substances such as hyaluronan and lubricin. These are the main components of synovial fluid as they contribute to its mechanical properties. Synovial fluid is responsible for joint lubrication and transportation of nutrients and oxygen to cartilage.

II-4-Subchondral bone

The rounded end of a long bone is referred as epiphysis which is filled with red bone marrow. At the joint, the epiphysis is roofed with the articular cartilage. Subchondral bone consists of the subchondral bone plate and the underlying trabecular and subarticular bone The subchondral bone overlying the articular cartilage is located adjacent to the deepest area of the articular cartilage consisting of calcified cartilage and a thin layer of cortical bone [46, 47].

Subchondral bone imparts supportive function to the joint area and plays a critical role in maintaining the integrity of the articular cartilage [46]. It reduces the weight exerted on the knee joints and plays a key role in shock absorbance within the joint structure. Besides other function, subchondral bone is essential supplier for nutrients to the cartilage [46, 48].

Subchondral bone products can transfer between the bone-cartilage interfaces [49]. It is now believed that subchondral bone is a key joint structure implicated in the pathophysiology of OA [50]. However, it is still unclear if subchondral bone changes occur before or after cartilage degradation during the development of the OA. Some recent *in vivo* studies using animal models reveal that subchondral bone changes may occur before, during or after cartilage destruction [51-53]. Changes in the joint area during OA cause damage to the articular cartilage and thickening of the subchondral bone. Degradation of articular cartilage, leads to exposure of subchondral bone to external surface[54]. During the OA development, subchondral bone goes through structural and molecular changes including amplified bone turnover (20-fold increase compared to normal bone [55]), microfractures, the appearance of new vessels and sclerosis [46].

III- Pathophysiology of OA

Despite extensive research, the exact pathogenesis of OA is largely unknown. In addition, the exact sequence of pathological events resulting in OA is not clearly defined. However, it is now well established that OA manifestations are not only confined to the articular cartilage but affect the whole joint including subchondral bone, joint space, synovial fluid and the synovial membrane. During OA, gradual cartilage changes such as fibrillation, erosion and softening contribute to joint deformity, joint space narrowing, subchondral bone remodeling, pain, inflammation and decreased mobility. Further, hypertrophic bone changes and osteophyte formation accelerate pain and discomfort in the patient [34].

OA is an outcome of complex interaction of mechanical and inflammatory processes resulting in imbalance between anabolic and catabolic processes. OA was previously defined

as a non-inflammatory arthritis. However, this notion has changed and it is now obvious that inflammatory and immune responses play a critical role in the pathophysiology of OA [56-59]. Synovial inflammation namely, synovitis, results from the synthesis and release of various cytokines and pro-inflammatory mediators. In OA, synovial inflammation affects areas adjacent to damaged cartilage and bone, and in addition to being a contributor to inflammation and cartilage degradation, synovitis also contributes to pain. Arthroscopic studies have demonstrated that localized proliferative and inflammatory changes of the synovium occur in up to 50% of OA patients, and the activated synovium can produce both proteases and cytokines, which contribute to disease progression. Synovitis is evident at the clinical stage of disease and is a predictor of disease progression [60, 61]. Histological studies reveal that in early OA, the first affected zone of cartilage is superficial zone. This is followed by loss of subsequent cartilage layers, chondrocyte necrosis, cartilage clefts and chondrocytes cloning [62]. Proteolytic degradation of proteoglycans in OA cartilage decreases the length of the proteoglycans chain resulting in increased permeability of solid matrix and reduction of the hydraulic pressure leading to decreased compressive stiffness and early chondromalacia [63, 64]. *In vivo* and *in vitro* studies show that chondrocytes along with other joint cells produce and respond to various cytokines, inflammatory mediators and proteases to create a vicious cycle of destructive events in the joint leading to the destruction of joint architecture (Figure-2) [9].

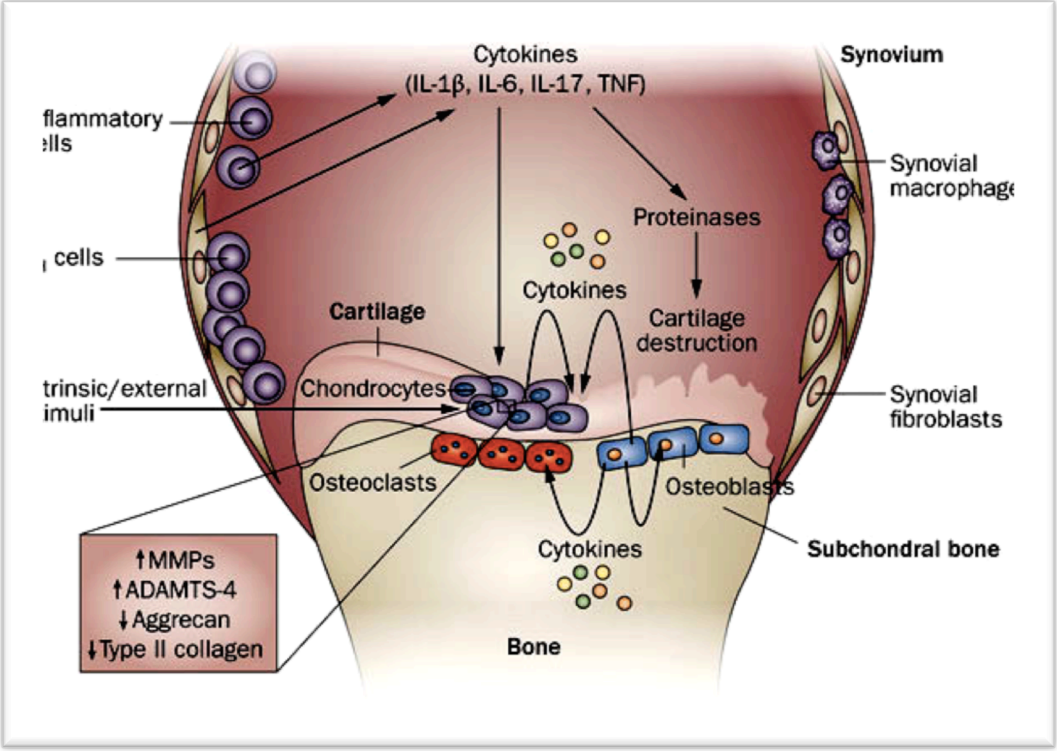

Figure-2: Schematic figure of pathogenesis of OA. ***Kapoor et al.; 2010. Nature Rev. Rheumatology.*** The joint structures affected in OA include articular cartilage, sub-chondral bone and the synovium. Cells from these structures produce several catabolic and pro-inflammatory factors such as IL-1β and TNF-α which further increase the the expression and production of key catabolic factors such as MMPs and ADAMTSs resulting in articular cartilage degradation, subchondral bone remodeling and synovial inflammation and fibrosis [57].

IV- Cartilage metabolism in OA

In normal cartilage, a balance between catabolic and anabolic factors is maintained to preserve the structural integrity of the cartilage ECM. However, during initiation and progression of OA, chondrocytes can be stimulated by different types of catabolic, degradative and inflammatory factors. OA is the result of the action of numerous different factors, which lead to cartilage degradation (Table-ii). The catabolic factors involved in the pathophysiology of OA include the proteases, the pro-inflammatory cytokines and growth factors. The matrix metalloproteases (MMPs) are key family of proteases that degrade ECM key components

aggrecan and collagen [65, 66] and damage the cartilage matrix macromolecules as a result are among the major destructive factors implicated in OA pathology [67].

Anabolic factors		**Catabolic factors**			
Cartilage Matrix Proteins	***Cytokines***	***Degradation Factors***		***Inflammatory Enzymes***	***Cytokines***
Collagens Aggrecan	TGF-ß FGF-2,18 BMPs IGF-1	Matrix-metalloproteinases MMP-13 MMP-1 MMP-3 MMP-2 MMP-8 MMP-9 MMP-11 MMP -14 MMP -16 MMP-28	Aggrecanases ADAMTS-4 ADAMTS-5	iNOS COX-2	IL-1β TNF-α IL-6 IL-11 IL-17 IL-18 LIF

Table-ii: ***Key anabolic and catabolic factors implicated in the pathogenesis of OA.*** ABBREVIATIONS; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs, BMPs: bone morphogenetic proteins, COX-2: cyclooxygenase-2, FGF: fibroblast growth factor, IGF: insulin-like growth factor, IL: interleukin, iNOS: inducible nitric oxide synthase, LIF: leukemia inhibitory factor, MMPs: matrix metalloproteinases, TGF-ß: transforming growth factor-beta, TNF-α: tumor necrosis factor- alpha.

IV-1- Anabolic Factors

IV-1-1- Extra Cellular Matrix Proteins

- **Collagen**

Matrix of normal cartilage is predominantly composed of collagen type II, responsible for imparting tensile strength to the tissue [12]. Collagen structure of articular cartilage is established during development and cannot be replaced or recreated. This framework structure differs between the different zones (superficial or transitional, deep, and calcified) in the articular cartilage [68]. The amount of the collagen is directly dependent on the density of the

chondrocytes in the articular cartilage. ECM of articular cartilage contains collagen type II fibril as the main structural backbone, collagen type IX on the fibril surfaces and type XI collagen within the fibrils and/or on the surface of the fibrils besides leucine-rich proteoglycans including fibromodulin and decorin [12]. In the calcified zone of the cartilage, collagen type X is present [69]. In OA, increased expression of catabolic markers specifically collagenase-3 (MMP13) results in enhanced collagen damage in chondrocytes [70] . Hollander *et al.* have shown that the collagen type II damage during OA begins around chondrocytes at the articular surface, and spreads into the cartilage with advanced deterioration [71]. After the initial cleavage of type II collagen by collagenases it is denatured and lost. Chondrocytes subsequently become hypertrophic and start secreting type X collagen. Collagen type II chains contain hydroxylysine, glucosyl and galactosyl residues which facilitate the collagen interaction with proteoglycans specially aggrecan [72]. Pro-inflammatory cytokines IL1 and TNF-α are involved in cartilage damage in OA via inducing the expression of proteinases specially MMPs in chondrocytes [73, 74]. It has been suggested that IL-1 and TNF-α are directly involved in the cleavage of collagen as IL1 receptor antagonist protein (IL1Ra) and a soluble TNF-α p55 (type I), a chemically modified receptor protein, arrest excessive cleavage of collagen by collagenase [75].

- **Aggrecan**

Proteoglycans are key proteins of the ECM that are inserted in the fibrous collagen network of the cartilage ECM [76]. Aggrecan with a mass of about 230 kilo Daltons forms up to 90% of the proteoglycan content in the articular cartilage [77]. Aggrecan consists of cartilage specific proteoglycan core or chondroitin sulfate proteoglycan 1 protein with

attached glycosaminoglycan (GAG) chains. The core protein consists of three globular domains (G1, G2, and G3). There is a large extended region between G2 and G3 for attachment of glycosaminoglycan chain. Branches of condroitin sulfate and keratan sulfate chains are attached to this core protein [78]. Aggrecan interacts with hyaluronic acid (HA) and link protein to form large aggregates in ECM. The complex of aggrecan-HA has a negative charge and is highly hydrated [76]. Aggrecan's concentration is directly proportional to the depth of articular cartilage and the amount of aggrecan is higher in the deep zone of the cartilage [12].

Aggrecan plays a vital role in chondrocyte-chondrocyte and chondrocyte-matrix interaction. G1 domain of aggrecan core protein interacts with hyaluronan acid and link protein. This interaction forms stable ternary complexes in the cartilage ECM [79]. In addition, aggrecan enhances glycosaminoglycan modification and secretion via G3 domain of the core protein [79]. Different types of proteases such aggrecanases can cleave aggrecan at different sites [12]. ADAMTS-4 and -5 (A Disintegrin and Metalloproteinase with Thrombospondin motifs) are two important aggrecanases involved in the degradation of the ECM, particularly aggrecan [80]. It has been shown that proteolysis of aggrecan is facilitated by ADAMTSs in the articular cartilage during OA [81, 82]. It has also been reported that ADAMTS-5 is involved in the degradation of aggrecan in mouse models [82]. Other metalloproteinases like MMPs are not significantly involved in aggrecan degradation [83].

IV-1-2- Cytokines

- **Transforming Growth Factor-β (TGF-β)**

There are 35 members in the TGF-β family, including TGF-βs, activins, and bone morphogenetic proteins [84]. These members play an important role in various physiological processes and regulate cell proliferation, differentiation, apoptosis and migration [85]. TGF-β exists in three isotypes, β1, β2, and β3. Each isotype possesses different promoter sequences and, as a result, their expression is differentially regulated at the transcriptional level. Chondrocytes secrete TGF-β in an inactive form, which comprises of a TGF-β dimer, its propeptide latency associated peptide and latent TGF-β binding proteins. TGF-β can act through Smad signaling pathways or Smad independent signaling pathways [86, 87]. In Smad dependent pathway, TGF-β binds to type II receptor (TβRII), which phosphorylates and activates TGF-β type I receptor (TβRI), resulting in the phosphorylation of cytoplasmic Receptor Activated SMAD proteins (R-Smads) such as Smad 2 and 3. The activated R-Smads (phosphorylated Smad 2/3) bind to Co-Smad also known as Smad 4 to form a hetero-complex which eventually trans-locates in to the nucleus and regulates the gene expression in association with other co-activators (CBP and p300), and co-repressors [88]. However, Smads alone do not activate transcription in TGF-β/Smad signaling, which is tightly controlled by MAPK signaling cascades [89]. In PI3K/AKT pathway, TGF-β activates AKT signal transduction through p38-MAPK and Focal Adhesion Kinase (FAK). Activated FAK recruits p85 (regulatory subunit of PhosphoInositide-3 Kinase (PI3K) leads to phosphorylation and activation of AKT via PI3K. Activated AKT signaling pathway has different roles in cell differentiation, inflammatory responses and cell survival [87].

TGF-β plays a regulatory role in chondrogenesis. It controls fibronectin expression, which demonstrates its involvement in mesenchymal cell condensation [90-92]. As well, TGF-β is essential in osteoblast differentiation. For example, osteoblasts overexpressing TGF-β2 exhibit low bone mass and an increase in osteocytes [93]. Furthermore, TGF-β is responsible for stimulating chondrocytes in order to induce aggrecan and collagen type II production [94].

The TGF-β signaling pathway is essential in postnatal joint growth and development and for the maintenance of the articular cartilage. For example, TGF-β-deficient mice display a cartilage phenotype similar to the cartilage pathology in OA [85]. Both Smad 3 and TGF-β are expressed in chondrocytes and in the perichondrium [95]. During adolescence, TGF-β inhibits chondrocyte differentiation [96, 97]. Animal mouse models have confirmed this by demonstrating that a disruption in Smad 3 causes a degenerative joint disease as a result of TGF-β's inability to inhibit chondrocyte differentiation [98]. TGF-β also works against inflammatory cytokines such as interleukin (IL)-1, which suppress ECM synthesis. TGF-β is able to prevent this by stimulating ECM synthesis and down-regulating cytokine receptor and MMP expression [85, 99] .

- **Bone Morphogenic Proteins (BMPs)**

Bone morphogenic proteins (BMPs) are homodimeric molecules with molecular weight of 25-30 Kd. During embryonic and skeletal development, BMPs play a key role in bone and cartilage formation. BMPs are involved in cartilage repair by stimulating the synthesis of the cartilage matrix proteins in adult articular chondrocytes. Multiple indications of BMP-2 and BMP-7 (osteogenic protein-1/OP-1) in the bone fracture repair have been suggested [9]. BMP-

7 regulated metabolism of chondrocytes [100] and stimulates synthesis, organization and maintenance of the ECM [101, 102]. A recent study showed that weekly intra-articular injection of BMP-7 inhibits progression of OA in rabbit model of OA [100]. BMPs also promote endochondral ossification, regulate chondrocyte hypertrophy and osteophyte formation [9] BMP-6, another key member of BMP family, is expressed endogenously in normal and OA human articular cartilage and plays a key role in maintaining cartilage homeostasis. It has been shown that BMP-6 controls cartilage retention and repair through stimulation of proteoglycan synthesis [103].

- **Fibroblast Growth Factor (FGF)**

Fibroblast growth factors (FGFs) are a family of polypeptide growth factors with molecular weight ranging between 17 to 34 kDa and majority of the members of this family share a common core region of 28 highly conserved and six identical amino-acid residues [104, 105]. FGF signal transduction consists of a complex of growth factors with a proteoglycan and a trans-membrane tyrosine kinase receptor (FGF-receptor) [106]. FGF receptors include four different high affinity types of receptors including FGFR (1, 2, 3 and 4) that have an affinity for FGF ligands [107]. FGFs are known to play a vital role in cell differentiation, proliferation, angiogenesis and tissue repair.

FGFs play a vital role in various stages of bone formation including the early stages of endochondral ossification as they have shown to be involved in inducing Sox 9 expression in primary chondrocytes and in undifferentiated mesenchymal cells [108, 109]. *In vitro* study on human cartilage explants suggests that FGF-2 has a chondroprotective role in the articular cartilage due to fact that it inhibits interleukin-1-driven aggrecanase activity [110, 111]. FGF8

is a key member of FGF family and it has been shown that FGF8 may contribute to the destruction of the cartilage during OA [112]. Studies suggest that degradation of the ECM in the articular cartilage is enhanced by FGF8 produced by injured synovium [112]. FGF8 can increase the production of protease and PGE2 from inflamed synoviocytes [112]. FGF-18, another member of FGF growth factor family, plays a key role in skeletal growth and development [113]. Studies using Fgf18 knockout mice have revealed bone and cartilage abnormalities such as dys-regulated joint development and delay in osteogenic differentiation [113]. Further, intra-articular injections of FGF-18 in a rat meniscal tear model of OA has been shown to have a protective role associated with decreased cartilage degradation in the tibial plateau of animal model of OA [114].

IV-2- Catabolic Factors

IV-2-1- Degradation Factors

- **Matrix metallo-proteinases (MMPs)**

The specific proteases that play a major role in cartilage ECM degradation are matrix metalloproteases (MMPs). These enzymes are metalloenzymes from the metzincin superfamily with a signal peptide, a pro-peptide, a catalytic domain, a hinge region, and a hemopexin C-terminal domain, all MMPs share a common domain structure [115]. Based on substrate specificity, primary structure, and cellular location, MMPs are divided into functional groups including collagenases, stromelysins, gelatinases, and membrane-type MMP (MT-MMP). Other protein families implicated in cartilage ECM degradation include astacins, ADAM (a protein with a disintegrin and metalloprotease domain), and ADAMTS (ADAM with a thrombospondin-like motif). Since the discovery of the first MMP member in 1962

[116], 23 MMPs have been described in humans, all of which are zinc- and calcium-dependent endopeptidases.

In terms of gene expression, MMP synthesis is regulated in a tissue-specific manner. MMP-1, -3, -9, and -13 are all induced by interleukin (IL)-1β and tumor necrosis factor-α (TNF-α), which are stimulants that regulate gene expression through signal transduction pathways such as mitogen-activated protein kinase (MAPK) pathways. Their promoters contain an activator protein 1 (AP-1) binding site, a key regulator of MMP gene expression [117].

The collagenases, MMP-1 and -13 are major mediators in cartilage degradation and are the main targets for OA therapeutic agents. Other MMPs, namely, MMP-2, -3, -8, -9, -11, -14, -16, and -28 are also highly expressed in OA cartilage. Three classical collagenases (MMP-1, -8 and -13) have been identified in human cartilage and MMP-1 was the first to be discovered [118]. MMP-1, an interstitial collagenase, and MMP-8, a neutrophil collagenase, are found in the superficial zone of cartilage while MMP-13 is found in deeper layers. It is important to note that the MMP-1 present in mice is not a direct orthologue of that present in humans [119]. In addition, pro-MMP-1 activated by APMA (4-aminophenylmercuric acetate), an organomercurial chemical, exhibits up to 25% of maximal activity while pro-MMP-1 activated by MMP-3, a stromeolyin (discussed later), results in full activation [120]. All collagenases are capable of cleaving the collagen triple helix. However, each human collagenase shows distinct substrate specificity toward different collagen types [119]. MMP-13 has the greatest specificity for type II collagen, the key structural protein in cartilage [121]. MMP-1 preferentially cleaves type III collagen [122] while MMP-8 is most effective against type I collagen [123], the principal interstitial collagen of tendon and bone. [124-129].

Mouse models overexpressing MMP-13 in articular cartilage display OA-like characteristics [130]. In addition, MMP-13 knockout mice demonstrate a significant inhibition in cartilage damage in mouse OA model. Furthermore, MMP-13 deficiency has been shown to inhibit cartilage erosion in the presence of aggrecan depletion [131]. This supports the potential of MMP-13 inhibitors as potential therapeutic targets for OA.

- **Aggrecanase**

Aggrecan is one of the most critical constituent of the articular cartilage ECM and its degradation is an important manifestation of OA. ADAMTS family belonging to zinc metalloproteinases exhibit aggrecanase activity [132]. ADAMTSs contain signal sequence, prodomain, catalytic domain, spacer domain, trombospondin motifs and sub motifs. The ADAMTS consists of two disintegrin loops and three C-terminal TS motifs. The ADAMTS are thoroughly correlated to the ADAMs family; however, they do not bind to the cell surface and contain a thrombospondin motif. The most prominent members of this family of proteases are ADAMTS-4 and ADAMTS-5. They are responsible for the degradation of the aggrecan structure in the ECM and cleave the aggrecan molecules at glutamic residues, specifically at the residues GLu373–Ala374 [66]. Both of them are the shortest members of ADAMTS family having only Thrombospondin motifs (TSP).

ADAMTS-4 and -5 have become of considerable interest as potential therapeutic targets in OA. Both are expressed in normal human cartilage, and data from mouse studies suggest that ADAMTS-5 is involved during OA pathophysiology. Studies using chondrocytes and cartilage explants reveal that ADAMTS-4 can be induced by IL-1, TNF-α or TGF-β [133].

Mouse models lacking ADAMTS-5 and subjected to surgically-induced OA showed a protection against the erosion of cartilage, occurrence of arthritis and subchondral bone changes [134, 135]. Mice lacking ADAMTS-4 when subjected to surgically-induced OA showed no effect on the progression or severity of OA [136]. However, in human chondrocytes and cultured human cartilage explants, both ADAMTS-4 and -5 were shown to be involved in aggrecan degradation [137].

IV-2-2- Cytokines

Cytokines are a group of small proteins that act as a messenger for cell to cell information. Among all cytokines, IL-1β, TNF-α and IL-6 can be regarded as the most critical cytokines involved in the pathophysiology of OA. Other cytokines that have also been implicated in OA pathophysiology include IL-15, IL-17, IL-18, IL-21, leukemia inhibitory factor (LIF) and chemokines [138].

- **Interleukins**

Interleukins can be regarded as one of the most widely studied cytokines in various physiological and pathological processes. They regulate inflammatory responses, play a key role in cell growth and differentiation and are also involved in the regulation of catabolic and anabolic processes. IL-1 is the major pro-inflammatory cytokine implicated in OA pathophysiology [57]. In OA, IL-1 plays a prominent role in cartilage degradation and stimulation of nociceptive pain pathways [139]. It is produced by chondrocytes, mononuclear cells, synovial tissues and osteoblasts [57, 140]. There are two types of IL-1β receptors (type I IL-1R and type II IL-1R). However, the affinity of type I IL-1R is higher for IL-1β and is responsible for signal transduction. Also, it has been revealed that the level of type I IL-1R is

elevated in OA chondrocytes [141]. IL-1 receptor antagonist (IL-1Ra) is produced by chondrocytes and synovial fibroblasts and exhibits anti-inflammatory activity through inhibition of IL-1 [142].

In OA chondrocytes, the level of IL-1 is enough to induce the expression of catabolic proteases including MMPs and aggrecanases [9, 143]. In OA cartilage, IL-1 is located in regions of matrix depletion. It is co-localized with other catabolic mediators such as MMPs, TNF-α and neo-epitopes of type II collagen in the superficial zone of OA cartilage [144]. In addition to direct effects of IL-1β in OA, it induces a multiplicity of other cytokines such as IL-6, IL-8, and leukemia inducing factor (LIF) [139] to initiate a cascade of catabolic events resulting in inflammation and joint destruction. Studies using human OA specimens as well as *in vivo* studies using mice model OA suggest that IL-1 is a key initiator of pro-inflammatory and catabolic process during OA and blocking this cytokine may yield therapeutic benefit. However, clinical studies using anti-IL1 therapy have been unsuccessful and most cases have shown to have serious side effects.

IL-6 is another catabolic cytokines involved in OA pathophysiology. The exact role of IL-6 in OA is unclear. However, studies suggest that this cytokine is involved in cartilage degradation, subchondral bone remodeling as well as hyperalgesia and hypersensitivity in joint tissues [145]. Although, the level of IL-6 is low in normal human chondrocytes, it is induced by IL-1β and TNF-α in joint chondrocytes and synovial cells during OA [138]. The levels of IL-6 are substantially elevated in serum and synovial fluid of OA patients [146]. IL-6 has a critical role in subchondral bone. It stimulates osteoclastogenesis and bone resorption mechanism and increases the interactions between osteoblasts and osteoclasts [147].

IL-17 is another familiar pro-inflammatory cytokine with known involvement in cartilage degradation. Published data has shown that IL-17 in combination with IL-1β induces collagenase-3 in human OA chondrocytes via activator protein (AP)-1 [128]. In addition, it has been shown that IL-17 can increase the expression of NO in human OA cartilage [148]. Explant study using human OA knee menisci has revealed that NO and PGE2 production is elevated by IL-17 [149].

- **Tumor Necrosis Factor (TNF)- α**

Apart from IL-1β, TNF-α is another major cytokine implicated in OA pathogenesis. TNF-α is first synthesized in an inactive state. Subsequently, it is activated by the TNF-α converting enzyme (TACE). It has been shown that the level of TACE is increased in OA [150]. During its activation, TNF-α molecule binds to another TNF-α to form a complex in order to subsequently bind with one of two TNF-α receptors (TNFR), TNFR55 or TNF57 [151]. TNFR55 is the central receptor of TNF-α in articular cartilage during OA and its expression is increased in OA chondrocytes and synovial fibroblasts [54, 152]. TNF-α can be produced by chondrocytes, synovial tissues, mononuclear cells and osteoblasts and induces the production of several inflammatory and catabolic mediators in the joint environment [57]. It also regulated several signal transduction pathways including NF-κB activation, Mitogen Activated Protein Kinase (MAPK) pathway, and death signaling induction.

TNF-α is involved in maintaining the homoeostasis of articular cartilage ECM in combination with other cytokines and metabolic mediators such as IL1, IGF-1 and TGF-β [153]. It has been shown that the expression level of iNOS, COX-2 and mPGES-1 is increased in chondrocytes treated with IL-1β and TNF-α. Interestingly, in these treated chondrocytes, the

level of NO and PGE2 are elevated which further increase the activation of other catabolic factors such as MMPs and suppress the synthesis of ECM components and stimulated apoptosis in chondrocytes [57]. Kobayashi *et al.* have shown that inhibition of IL-1 and TNF-α by their antagonists block the amplification of the cleavage of type II collagen (CII) and glycosaminoglycan (GAG) in human OA cartilage [75]. Published data suggest that the activities of TNF-α and IL-1 in articular cartilage may control the deterioration of articular cartilage ECM by stimulating the expression of proteases especially MMPs expression in chondrocytes [73-75].

IV-2-3- Inflammatory Enzymes

- **Cyclooxygenase (COX)**

Cyclooxygenase, also known as PGH_2 synthase (PGH_2S), catalyzes the formation of PGs and TXs (collectively known as prostanoids) from arachidonic acid (AA). The AA pathway starts with the activation of PLA_2 which catalyzes the release of free AA from membrane phospholipids. This is followed by the conversion of free AA to PGG_2 by the cyclooxygenase activity of the COX enzyme and then to PGH_2 by its peroxidase. With the action of individual PG synthase enzymes, PGH_2 is converted into TXA_2, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 [154]. COX inhibition is the site of action of aspirin and a wide range of NSAIDs. Inhibition of COX can account for both the therapeutic and side effects of NSAIDs. Current studies reveal that COX exists in three isoforms namely COX-1, COX-2 and COX-3. COX-1 is constitutively expressed in majority of cells and tissues and regulates the production of PGs and thromboxanes (TXs) involved in the regulation of vascular, gastrointestinal (GI) and renal

homeostasis [155]. In contrast, COX-2 is induced in response to variety of pro-inflammatory stimuli and regulates the production of PGs involved in inflammation, pain and fever, especially PGE_2 and prostacyclin (PGI_2) [156-159]. Existence of COX-3 as a novel COX isozyme, predominantly expressed in cerebral cortex and heart has also been reported [160] but its existence in humans has been questioned by a subsequent study [161].

COX-2 is not expressed in un-stimulated normal human articular chondrocytes, but is induced by inflammatory mediators including IL-1β, IL-17, TNF-α, leukemia inhibitory factor (LIF) and bacterial lipopolysaccharide (LPS) [162-164]. COX-2 is expressed in high levels in cartilage from OA patients [162, 165, 166]. The increased expression of COX-2 is associated with the release of PGE_2. Selective COX-2 inhibitors significantly inhibit the pathophysiological symptoms including paw edema, spontaneous pain, and hyperalgesia of adjuvant induced arthritis in rat (AIA) [167]. However, a COX-1 selective inhibitor does not inhibit the inflammation and PGE_2 production in the paw of AIA model [168]. In addition, a selective COX-2 inhibitor, but not a selective COX-1 inhibitor, reduces the severity of symptoms in a type II collagen-induced arthritis model (CIA) of mice [169]. Furthermore, COX-2 deficient mice, but not COX-1 deficient mice, display a significant reduction in both clinical and histological signs of CIA [170]. Since the production of proinflammatory PGs, especially PGE_2, at sites of inflammation coincides with the up-regulation of COX-2 expression in activated articular cells; therefore COX-2 has long been a key target for the treatment of OA and other forms of arthritis.

- **Inducible Nitric Oxide Synthase (iNOS)**

Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline and nitric oxide. NO is formed from the terminal guanidine nitrogen atom of L-arginine, which accepts five electrons in an oxidation process requiring molecular oxygen, resulting in the production of NO and L-citrulline [171]. In this conversion, L-hydroxy-arginine is formed as an intermediate product. NOS are homodimeric, flavoprotein enzymes containing haem, that are linked to nicotinamide-adenine-dinucleotide phosphate (NADPH) driven electron transport by flavine adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The NOS family is comprised of three isoforms located on separate chromosomes. These include inducible NOS (iNOS or Type II NOS), neuronal NOS (nNOS or Type I NOS) and endothelial NOS (eNOS or Type III NOS. nNOS and eNOS are the Ca^{2+} dependent constitutive enzymes while iNOS, that is the Ca^{2+} independent inducible one, induced by a variety of cytokines and stimuli. Inducible NOS (iNOS) or type II NOS is not normally expressed but is highly inducible in response to inflammatory cytokines, bacterial endotoxins, microbes or microbial products and results in significant sustained production of high levels of NO [172]. Levels of NO and iNOS are elevated in the synovial fluid and articular cartilage during OA [173-175]. The presence of NO in the articular cartilage has been shown to promote cell apoptosis [176]. *In vitro* studies illustrate that the activation of pro-inflammatory factors through iNOS pathway can stimulate NO production [177]. In joints, chondrocytes are the main source of NO and iNOS can be induced by various stimuli. Interestingly, undifferentiated mesenchymal stem cells do not produce iNOS, but after differentiation to chondrocytes, they express iNOS and produce NO upon IL-1 stimulation [178].

V- Peroxisome Proliferator-Activated Receptors (PPARs):

Nuclear hormone receptors belong to a group of transcription factors that bind to DNA in a ligand dependent manner and regulate gene expression. Peroxisome Proliferator-Activated Receptors (PPARs with three subtypes α, β/δ, γ) are ligand activated transcription factors that belong to class II nuclear hormone receptor superfamily including classical steroids, vitamin D, retinoic acid, and thyroid hormone receptors [179]. PPARs were discovered for the first time in rodents in 1990 [180]. The name PPAR derives from PPARα (first PPAR subtype) that increases the size and numbers of peroxisome in rodents liver [181]. Peroxisome is an organelle in all eukaryotic cell types that regulates metabolic functions including H2O2-based respiration, fatty acid ß-oxidation and cholesterol metabolism [182]. In rodent liver, PPAR α enhances the size and number of peroxisomes with increased fatty acids metabolism in this organelle [181]. Originally, PPARs were identified as receptors that induce peroxisome proliferation in Xenopus frog cells [183].

Chromosomal positions of PPARs has been identified in human and mouse. In humans, PPARα has been assigned to telomeric part of chromosome 22q [184], PPARγ gene was mapped on chromosome 3p21 [185], while PPARβ is located at position 6p21.1-p21.2 on chromosome 6 [186]. In mouse, PPARα and PPARβ were mapped on chromosomes 15 and 17 and PPARγ was found on chromosome 6 [187]. The mouse and human PPAR genes have similar structural characteristics as steroid or thyroid hormone receptors. Their organization of the translated region contributes to six coding exons. They have common functional domains including a N-terminal region that contains A/B domain, two exons for DNA-binding domain

(DBD), flexible hinge exon, ligand binding domain (LBD), and C-terminal region, thus sharing structural similarities [188] (Figure-3).

V-1- PPARs subtypes

PPARs play a key role in glucose homeostasis, lipid and lipoprotein metabolism. Each PPAR isoform is encoded by different genes and characterized by a specific tissue expression pattern. All three subtypes of PPARs play pivotal roles in a variety of physiological processes [189]. PPARα plays a vital role in fatty acid metabolism and exists in liver, muscle and heart [190]. PPARβ/δ plays an important role in lipid homeostasis, epidermal maturation, skin wound healing and brain development [191, 192]. PPARγ is the third member of this family. PPARγ plays a role in the regulation of lipids metabolism and affects on skeletal muscle, heart, adipose tissue and liver [193]. It must be mentioned that PPARγ is the most extensively studied member of this family that regulates expression of various genes [189]. It is expressed in both brown and white adipocytes. PPARγ has a vital role in adipocyte differentiation, peripheral glucose utilization and insulin sensitization [194].

Figure-3: ***Schematic structure of PPARs gene sequence.*** PPARs structure has two zinc finger motifs in the DBD domain and A/B domain in the N-terminal.

V-2- Peroxisome Proliferator Activated Receptor gamma (PPARγ)

PPARγ is the third member of PPAR subfamily that is expressed by different range of cells such as adipocytes, skeletal muscle cells, chondrocytes, osteoclasts, osteoblasts and several immune cell types. It has been identified in many species like salmon, frogs, pigs,

monkey and human. PPARγ protein is remarkably conserved in contrast to the isotypes α and β/δ. It has been suggested that this conservation probably is associated with an important regulatory role of PPARγ in lipid and glucose homeostasis [195].

Synthetic ligands of PPARγ such as rosiglitazone and pioglitazone belong to the family of thiazolidinediones and are clinically used for the treatment of insulin resistance in Type 2 diabetes mellitus[196-199]. Recent studies reveal that PPARγ has a role in inflammatory responses, cell differentiation and apoptosis, beside its vital role in adipogenesis and lipid storage [200]. In addition, PPARγ has been implicated in inflammation, wound healing, angiogenesis, atherosclerosis, osteoporosis and cancer [197, 201, 202].It also plays a role in bone homeostasis by contributing to osteoclastogenic and osteoblastogenic pathways [203].

Studies using somatic cell hybridization and linkage analysis have shown that PPARγ is located on chromosome 3p25 in human [185]. PPARγ gene extends over more than 100 kb of genomic DNA in human and mouse and gives rise to two different isoforms (γ1, γ2), depending on different splicing and promoter alteration [204]. PPARγ2 isoform differs from PPARγ1 in just 30 additional amino acids at the N-terminal region [204, 205]. PPARγ2 is mainly expressed in adipose tissues [205], whereas PPARγ1 is expressed ubiquitously in all tissue types [206].

V-2-1- PPARγ functions

PPARγ acts in heterodimer form with Retinoid X Receptor (RXR) and regulates gene transcription in ligand dependent or ligand independent manners.

Ligand independent function

Studies show that A/B domain of PPARγ gene is responsible for the ligand independent activity of PPARγ [207]. A/B domain is located in the additional 30 amino acids in N-terminal (a major phosphorylation site) of PPARγ2. The A/B domain can interact with p300 co-activator to activate gene transcription in a ligand independent manner specifically in PPARγ2 [208]. However, the exact physiology of this action is still unknown.

a) Ligand dependant function

PPARγ as a ligand-activated transcription factor has DNA Binding Domain (DBD). The DBD is a highly conserved domain in all nuclear receptors. It is formed by two zinc finger like motifs folded in a globular structure. This motif can distinguish a DNA target composed of 6 nucleotides that is named "functional hormone response elements" [209]. These functional hormone response elements are named peroxisome proliferator hormone response elements (PPREs) upon PPARs function. PPREs are a specific region on the DNA of target genes and contain DR1, a consensus direct repeat of two sequence AGGTCA spaced by one nucleotide [210]. These DR1s help differentiate between PPREs and other direct repeat response elements such as vitamin D receptor (VDR). Functional PPREs are represented by DR1 elements. PPRE sequence is located in upstream regulatory side of metabolic pathway genes [211].

PPAR binds to the Retinoid X Receptor (RXR). RXR is a receptor of 9-cis-retinoic acid (9c-RA). The heterodimer of PPAR: RXR binds to PPRE in the upstream region of target genes and regulates gene expression in the presence of RNA polymerase type II, co-activators and co-repressors [189, 212] (Figure-4).While PPARγ:RXR heterodimer binds to PPRE sequence, the ligand joins the heterodimer in the PPARγ Ligand Binding Domain (LBD) to

promote co-activators (Src-1 and Tif-2) connectivity with this complex. Next, in presence of RNA pol II, the complex regulates the transcription of target genes resulting in adipose differentiation, glucose homeostasis lipid trafficking in monocytes and anti-inflammatory effects. In the absence of ligand, co-repressors like silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressors (N-CoR) [213] will be activated. These co-repressors can bind to heterodimer and inhibit gene expression.

PPARγ activation in brown adipocytes depends on the activation of Src-1 co-activator. High level of free fatty acids in plasma activates PPARγ followed by Src-1 activation. On the other hand, Src-1 requires the PGC-1α (a co-activator that mediates thermogenesis) activation. Due to the great number of Src-1s recruited by the PPARγ even with a high level of free fatty acids in plasma, thermo-genesis decreases in brown adipocytes. In white adipocytes, Tif-2 is the dominant co-activator in uptake of plasma free fatty acids in presence of PPARγ. Some ligands recruit special co-activators to induce different functional responses based on interaction. For example 15-deoxy prostaglandin J2 (the natural ligand of PPARγ), promotes PPARγ interaction with SRC-1, TIF2, TRAP220, and p300, while synthetic ligands recruit other co-activators.

In addition, PPARγ can act independent of PPARE in gene regulation by suppressing growth hormone protein-1 (GHP-1) or interfering with the function of AP-1 (activator protein-1), the activator of STAT-1 and NF-κB [214].

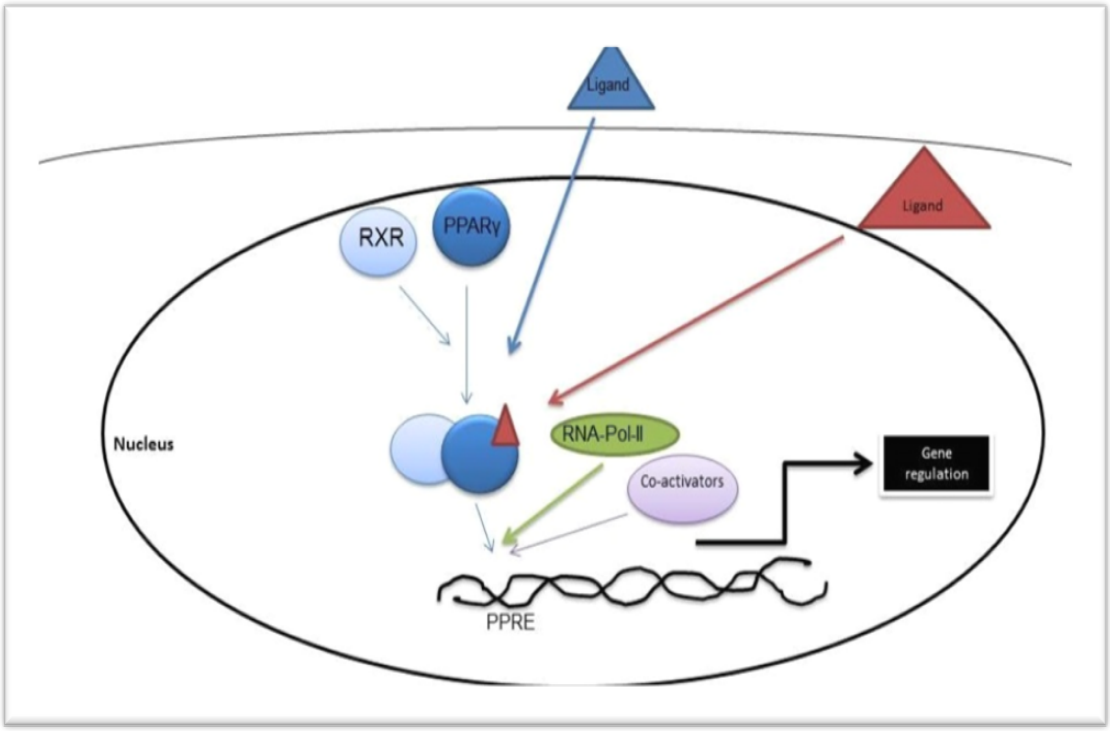

Figure-4: ***PPARγ-activation***: Complex of ligand-PPARγ forms a heterodimer with RXR and binds to PPARγ-response elements (PPREs) which lead to transcription. PPARγ ligands display direct and dependent effects with or without implication of the PPARγ/RXR heterodimer. Direct effects may contain PPARγ protein interacting with ligands or may be completely independent of PPARγ [6].

V-2-2- PPARγ ligands

PPARs are present in the nucleus of the cells in the presence and/or absence of ligands. Interestingly, PPARs are capable to interact with more than one ligand. Their potential of specific ligand binding properties makes them interesting therapeutic targets for wide variety of disorders. There are various compounds that bind selectively to PPAR-RXR hetero-dimer and act as ligands. PPARγ ligands are divided into natural and synthetic ligands (Table-iii).

Natural Ligands		Synthetic ligands	
Fatty acids	Eicosanoids	Antidiabetic drugs	NSAIDs
Arachidonic acid Eicosapentaenoic Docosahexaenoic 9-HODE 13-HODE	15d-PGJ2 PGJ2 15-HETE	Rosiglitazone (BRL-49653) Pioglitazone Troglitazone Ciglitazone	Indomethacin Ibuprofen Fenoprofen Flufenamic acid

Table-iii*: PPARγ ligands:* PPARγ has two different types of ligands: natural and synthetic. ABBREVIATION; NSAIDS: Nonsteroidal anti-inflammatory drugs 9- HODE: 9-Hydroxy-10,12-octadecadienoic acid, 13- HODE: 13-hydroxy-9,11-octadecadienoic acid, 15d-PGJ2: 15-Deoxy-Delta-12,14-prostaglandin J2, PGJ2: Prostaglandin J2, 15- HETE: 15-Hydroxyeicosatetraenoic acid,.

V-2-2-1- Natural Ligands

15-deoxy-Δ12,14-prostaglandinJ2 (15d-PGJ2) and a derivative of arachidonic acid called hydroxyl-eicosatetraenoic acid (15-HETE) work as natural ligands of PPARγ [215]. 15d-PGJ2 is the first endogenous/natural ligand of PPARγ. It activates PPARγ in low concentrations and induces adipocyte differentiation [215]. Although, this prostanoid is the most widely utilized natural ligand of PPARγ, it mediates some of its effects in cell through PPARγ independent signaling pathways [216]. Polyunsaturated fatty acids including arachidonic and eicosanpentaenoic acids (exogenous agonist of PPARγ in food) can bind to PPARγ as natural agonists. Fatty acids such as linoleic acid need intracellular conversion to eicosanoids via increasing the expression of 15-lipoxygenase. Thus, 9-HODE and 13-HODE (the 15-lipoxygenase metabolites of linoleic acid) can be effective natural ligands for PPARγ to enhance PPARγ-mediated transactivation [217].

V-2-2-2- Synthetic Ligands

Among PPARγ synthetic ligands, the class of anti-diabetic drugs belonging to the thiazolidinedione (TZDs) family is the most common group. These include troglitazone, rosiglitazone, pioglitazone, and ciglitazone [215, 218]. Muraglitazar, tesaglitazar, and farglitazar, belong to the glitazar family and act as dual agonists on both PPARγ and PPARα [219]. Nonsteroidal anti-inflammatory drugs such as indomethacin, ibuprofen, fenoprofen, and flufenamic acid have also been reported to activate PPARγ [220]. TZDs induce gene expression in adipocytes and increase adipocytes differentiation in cell culture [221]. Although TZDs work as synthetic ligands of PPARγ, they exhibit less affinity for PPARγ compared to its natural ligands. Amongst TZDs, rosiglitazone is more efficient than pioglitazone or piglitazone [222].

Jiang *et al.* have demonstrated that troglitazone, one of the TZDs families, inhibits monocyte elaboration of inflammatory cytokines such as TNF-α, IL-1, and IL-6. This PPARγ synthetic agonist suppresses gene expression by antagonizing the activities of AP-1 and NF-κB [223]. Another study has shown that, pioglitazone or rosiglitazone can block iNOS expression in both the ankle and temporomandibular joints through suppression of the NF-κB pathway in rat adjuvant arthritis model [224]. Recently, Kobayashi *et al*. have demonstrated that pioglitazone reduces the progression of OA via decreased the expression of MMP-13 and IL-1 in Guinea pigs model of OA[225]. Although these evidence [225, 226] have demonstrated that PPARγ synthetic ligands have positively effects OA,TZDs have also been associated with several side effects including weight gain, fluid retention, liver toxicity, and congestive heart failure [227].

Studies demonstrate that some NSAIDs like indomethacin, ibuprofen, fenoprofen, and flufenamic acid have roles in PPARγ activation and the promotion of adipocytes differentiation [220]. Coxibs such as celecoxib (selective Cox-2 inhibitor) are the newest class of NSAIDs and have also been implicated in PPARγ activation. The affinity of NSAIDs for PPARγ activation is still debatable as NSAIDs can activate both PPARα and PPARγ [220].

Despite different chemical structures of PPARγ ligands, they bind to PPARγ in the same shape and configuration [228] and can down regulate catabolic and pro-inflammatory gene transcription by antagonizing activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Nuclear factor of activated T-cells (NF-AT) and signal transducer and activator of transcription (STATs) signaling pathways using CBP/p300 and steroid receptor coactivator-1 (SRC-1) cofactors [229]. For example, 15-d-PGJ2 binds to PPARγ-RXR complex and interacts with STAT3 to inhibit the binding of STAT3 to the promoter of the target genes and repress IL-6 signal transduction [230].

V-2-3-PPARγ in cartilage development

Recent studies suggest that PPARγ plays a key role in cartilage and bone growth and development. PPARγ KO mouse in hematopoetic tissues including bone marrow, spleen and thymus have shown that PPARγ plays a role in promoting osteoclasto-genesis in mice. PPARγ elevates osteoclast differentiation via controlling c-fos pathway. PPARγ KO mice exhibit osteopetrosis associated with enhanced bone volume, and diminished medullary cavity space (central cavity of bone shaft) and bone resorption (bone break down by osteoclast and release of minerals, specifically calcium from the bone to blood) [231]. *In vivo* study using cartilage specific PPARγ germline KO mice has shown that PPARγ is necessary for normal cartilage

growth and development, deficiency of which can lead to severe impairement in the process of endochondral ossification [232]. Specific ablation of PPARγ in the cartilage results in decreased body size, skeletal size, length of long bone, bone density and trabecular bone thickness associated with delayed primary and secondary ossification (Figure-5) and growth plate defects. In addition, PPARγ deficient chondrocytes exhibit reduced production of ECM components including collagen type II and aggrecan in contrast with enhanced expression of degradation product such as MMP13 [232].

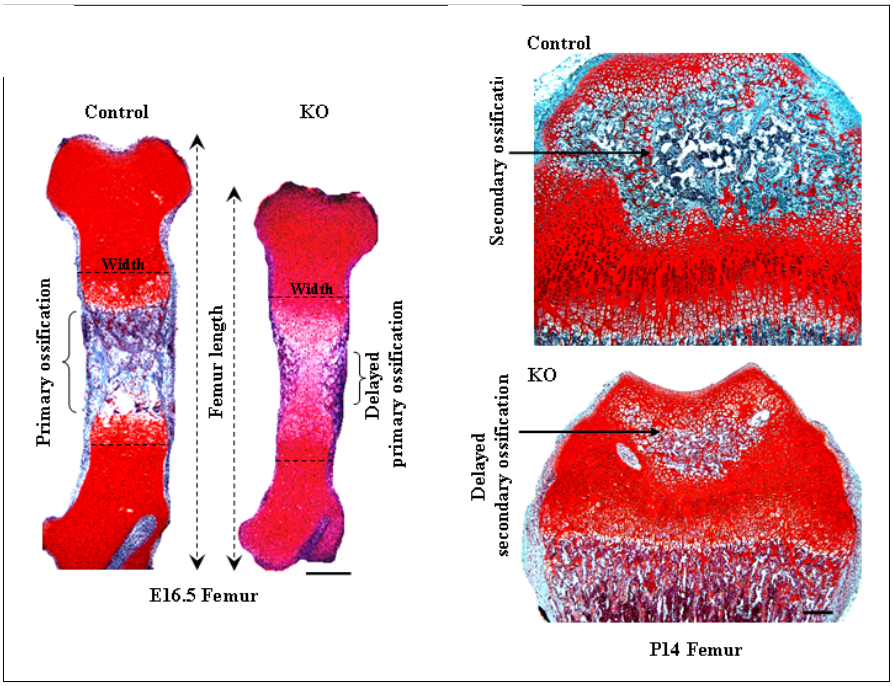

Figure-5: ***Role of PPARγ in primary and secondary ossification. Monemdjou et al.;2012, Arthritis and Rheumatism:*** Cartilage-specific germline PPARγ KO mice exhibit delayed primary as well as secondary ossification process associated with reduced length of long bones as shown in E16.5 femurs.

V-2-4- Role of PPARγ in OA

Extensive research has been performed to investigate the role of PPARγ in a variety of diseases. Studies have shown that PPARγ is expressed in many inflammatory and immune

cells including macrophages, osteoclasts, articular chondrocytes and synovial fibroblasts [233]. It is well established that PPARγ exhibits potent anti-inflammatory properties. PPARγ acts as trans-repressor of macrophage inflammatory gene expression through ligand dependent manner [234]. In macrophages, PPARγ activation can provoke NF-kB action resulting in the reduction of pro-inflammatory cytokines [223]. Recent studies suggest that activation of PPARγ is a therapeutic target for OA.

V-2-4-1- Expression of PPARγ in OA

Bordji *et al.* first reported the expression of PPARγ in the cartilage [235]. Dumond *et al.* have shown that PPARγ expression is down-regulated in the cartilage of rat induced mono-idoacetate model of OA (chondrocyte metabolism is disrupted of by mono-iodoacetate (MIA), vitamin A or steroids injection) [236]. They found that despite up-regulated expression of iNOS, COX2 and IL1β in the superficial layer of cartilage after 2 days MIA injection, basal expression of PPARγ was down-regulated in MIA rats model of OA from day 2 to day 20 of MIA injection [236]. In addition, it has been reported by Afif *et al.* that PPARγ production is reduced in human OA cartilage compared to normal human cartilage [237]. They have shown that IL-1-induced down-regulation of PPARγ1 expression in human OA chondrocytes. Further they believe that the (Mitogen activated protein kinases) MAPKs, JNK (c-Jun N-terminal kinase) and p38 are implicated in the down regulation of PPARγ1 via IL-1β [237]. Several other studies confirm that not only PPARγ is expressed in macrophages, synoviocytes and chondrocytes [49], but also its activators control the expression of different genes that are implicated in the pathogenesis of OA [200, 233, 238]. Reduction of the PPARγ protein expression in chondrocytes extracted from human OA treated with IL-1β, TNF-α and PGE2

suggests that decreased PPARγ expression in OA may result in up-regulation of inflammatory and catabolic factors [237], suggesting a critical role of PPARγ in maintaining the balance between catabolic and anabolic processes within the articular cartilage. It has also been suggested that reduced expression of PPARγ in the cartilage during OA is associated with MAPK, AP-1 and NF-κB signaling pathway [233, 237]. Fahmi *et al*. have suggested that PPARγ natural ligand, 15d-PGJ2, may attenuate the transcription of iNOS and MMP-13 through interfering with the NF-κB and AP-1 signaling pathways [233]. Furthur Afif *et al.* have shown that PPARγ down-regulation is induced by IL-1β which is activated via MAKP, JNK and P38 signaling pathway [237]. Recently, Nebakki *et al.* have revealed that down regulation of PPARγ expression in human OA via IL-1 is connected with the overexpression of Egr-1(Early growth response protein 1). Increased expression of IL-1 induced the recruitment of Egr-1 that contributed to the down regulation in the expression of PPARγ [239]. It appears that PPARγ ligands decrease inflammatory responses in chondrocytes and synovium fibroblasts [238, 240]. Farrajota *et al.* have shown that PPARγ natural ligand (15d-PGJ2) suppresses the expression of IL-1β–induced COX-2 in human OA synovial fibroblast. They suggested that 15d-PGJ2 inhibits COX-2 expression in human OA fibroblasts via IL-1β by a histone deacetylase–independent mechanism (HDAC) [238].

Effects of PPARγ	Cells	Involved factors
Anti-inflammatory	Chondrocytes, Synovial cells, Osteoclasts	IL-1β, TNF-α, iNOS, COX-2, mPGES-1, IL-6, IL-8, RANKL
Anti- catabolic	Chondrocytes	MMP-13, MMP-1, MMP-3, ADAMTS-5, AP-1, NF-κB
Anti- fibrotic	Synovial cells	TGF-β, Smad signaling, Collagen type I

Table-iv: ***Effects of PPARγ in joint tissue.*** PPARγ imparts anti-inflammatory effects by decreasing the expression of IL-1β and TNF-α-induced inflammatory markers such as iNOS and COX-2. It exhibits anti-catabolic properties by reducing the expression of catabolic markers including MMPs MMPs and ADAMTS-5. It imparts anti-fibrotic role by downregulating TGF-β/smad signaling pathway in synovial membrane ABBREVIATIONS; ADAMTS: a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs, AP-1: activated protein-1, COX-2 : cyclooxygenase-2, iNOS: induced nitric oxide synthase, IL: interleukin, MMP: matrix metalloproteinase, mPGES-1: microsomal prostaglandin E synthase-1, NF-κB: nuclear factor-kappa B, RANKL: receptor activator of nuclear factor kappa-B ligand, TGF: transforming growth factor, TNF: tumor necrosis factor.

V-2-4-2- Anti-inflammatory role of PPARγ

Synthetic agonists of PPARγ including roziglitazone, poiglitazone and troglitazone as well as 15d-PGJ2, the most well-known natural ligand of PPARγ, exhibit anti-inflammatory effects on joint cells [241-243]. *In vitro* studies, using human OA chondrocytes show that iNOS and COX-2 expression can be blocked in human chondrocytes treated with 15-d-PGJ2 or troziglitazone through IL-1β inhibition [244]. Similarly, 15-d-PGJ2 prevents iNOS, COX2, and PGE2 expression and production in IL-1β-treated rat chondrocytes [235]. In guinea pig animal model of OA, treatment with pioglitazone results in decreased OA progression through reduction in the production of IL-1β and MMP-13 [225]. Another published data has revealed that the DNA binding activity of NF-kB is decreased by troglitazone in response to TNF-α or IL-1ß in synovial tissue specimens from patients with rheumatoid arthritis (RA) [245]. It has

been shown that rosiglitazone, the synthetic PPARγ ligand, has the ability to limit inflammation and protect cartilage and bone destruction in Collagen induced arthritis (CIA) and Adjuvant-induced arthritis (AIA) mice [224, 246].

In human OA synovial fibroblasts, the endogenous expression of several inflammatory and catabolic factors such as TNF-α, IL-6, IL-8 and MMP-3 can be blocked by 15d-PGJ2 and troglitazone [245]. Moreover, mPGES-1, MMP-1 and COX-2 expressions are blocked by troglitazone or 15-dPGJ2 in human OA synovial fibroblasts treated with IL-1β [212, 238, 247]. In rat synovial cells a reduction in expression of PPARγ induced by lipopolysaccharide (LPS) was detected. In this interesting study, 15d-PGJ2 has been shown to block IL-1β induced inflammatory responses through reduction of LPS-induced COX-2 and iNOS mRNA expression. Also, 15d-PGJ2 controlled LPS-induced IL-1β and TNF-α mRNA expression. Furthermore, 15d-PGJ2 made a suppression of DNA binding activity of NF-κB and AP-1. They suggested that antiinflammatory effects of PPARγ can be a result of antagonized PPARγ activity with AP-1 and NF-κB activities [248].

Osteoclasts play a significant role in bone damage of inflamed joints in arthritis. Recently the protective effects of THR0921, an active synthetic ligand of PPARγ, on osteoclastogenesis in a collagen-induced arthritis (CIA) mice model of RA have been investigated. In this study, bone marrow cells were cultured from the femur and tibia parts in the presence or absence of THR0921. They found that THR0921 reduce proinflammatory cytokines production including TNF-α, IL-1β, monocyte chemotactic protein-1 and receptor activator of NF-κB ligand (RANKL), suppress T-cell proliferation, and inhibit osteoclastic bone resorption. They believe that possibly THR0921 act directly via a family of monocytes/macrophages that differentiate into osteoclastic bone resorption [249]. In addition,

Sugiyama *et al.* have reported that PPARγ agonists may have protective effect on inflammatory bone resorption via suppression of RANKL or TNF-α-induced osteoclastogenesis [250].

These studies suggest new visions in respect to the antiinflammatory role of the PPARγ and its possible signaling pathways in chondrocytes, synovial cells and bone cells of the joint.

V-2-4-3- Anti-catabolic role of PPARγ

In normal cartilage, a balance between catabolic and anabolic factors is maintained to preserve the structural integrity of the articular cartilage ECM. However, during initiation and progression of OA, the imbalance in expression of anabolic and catabolic factors results in cartilage damage and deterioration.

MMPs especially MMP-1 (fibroblast collagenase), MMP-3 (stromelysin-1) and MMP-13 (Collagenase-3) are important proteases implicated in cartilage and bone destruction during OA [233]. PPARγ agonists can reduce the synthesis of various catabolic factors involved in OA pathophysiology. PPARγ activators can prevent articular cartilage ECM degradation by blocking MMPs production in chondrocytes. Rosiglitazone inhibits IL-1β induced production of NO and MMP-13 in human chondrocytes, *in vitro*. It appears that rosiglitazone works by interfering with AP-1binding activity [233]. Similarly, 15-d-PGJ2 blocks IL-1β mediated induction of MMP-13 and NO genes expression in human OA chondrocytes through the inhibition of a number of signaling pathways including the AP-1 and NF-κB and the inhibition of the expression of MMP-1 gene by reducing the AP-1binding activity [232, 250].

Kobayashi *et al.* created guinea pig model of OA using the partial medial meniscectomy surgery. Pioglitazone treatment in this experimental model of OA has demonstrated that

pioglitazone can reduce the percentage of MMP-13 expression in articular chondrocytes [225]. Furthermore, *in vivo*, pioglitazone treatment results in reduced severity of cartilage lesions associated with significant reduction in the levels of MMP-13 and IL-1β in guinea pig model of OA [225]. This study also suggested that the decreased expression of MMP-13 is caused by interference with IL-1β expression in chondrocytes. Boileau *et al*. have shown that pioglitazone significantly decreases cartilage lesions in dog model of OA by reducing the synthesis of the key OA catabolic mediators MMP-1 and ADAMTS-5 [226]. Recently, it has been shown that lack of PPARγ expression in the mouse cartilage results in decreased expression of collagen type II and aggrecan with increased expression of MMP-13 [232]. These studies show that PPARγ agonists in *in vivo* animal models of OA as well as *in vitro* human OA chondrocytes impart anti-catabolic properties and protect cartilage from degradation. There is a possibility that loss of PPARγ in OA may down-regulate the synthesis of anabolic markers with increased synthesis of catabolic markers resulting in cartilage damage.

V-2-4-4- PPARγ and Fibrosis

In OA, continuous loading and mechanical forces on the joints lead to the deposition of damaged matrix molecules in the cartilage, resulting in synovial inflammation and ultimately can lead to synovial fibrosis. In OA, FLS activation and proliferation, as well as synovial hyperplasia all represent reactive changes within the joint aimed at clearing molecular debris deposited into the synovial fluid from cartilage destruction. Activated synovial lining macrophages and fibroblast-like synoviocytes (FLS) are largely responsible for the production of growth factors that drive fibrogenesis and osteophyte formation.

Fibrosis is characterized by the excessive production of the ECM resulting in impaired organ function. The pathophysiologic alterations underlying fibrotic responses are typically characterized by prolonged and exaggerated activation of fibroblasts and persistent maintenance of fibroblast-mediated effects. Role of PPARγ in synovial fibrosis is yet unknown. However, studies in other models of fibrosis do suggest that PPARγ exhibits anti-fibrotic properties. TGF-β is the major pro-fibrotic cytokine involved in fibroblast activation and differentiation. Activation of PPARγ by synthetic or natural ligands results in the down-regulation of TGFβ-induced pro-fibrotic mechanisms. Rosiglitazone, troglitazone, and 15d-PGJ_2 have been shown to suppress TGFβ-induced collagen synthesis, collagen type I α2 promoter activity, and α-SMA expression, as well as to directly antagonize the activation and function of SMAD3 in fibroblasts [251-253] (Figure-6). Mouse model of skin sclerosis with lack of PPARγ in fibroblasts has revealed that PPARγ deficiency in fibroblasts enhanced collagen production with increased expression of Smad3 phosphorylation and with increase in the expression of α-SMA and type I collagen in TGF-β-induced fibroblasts[253].

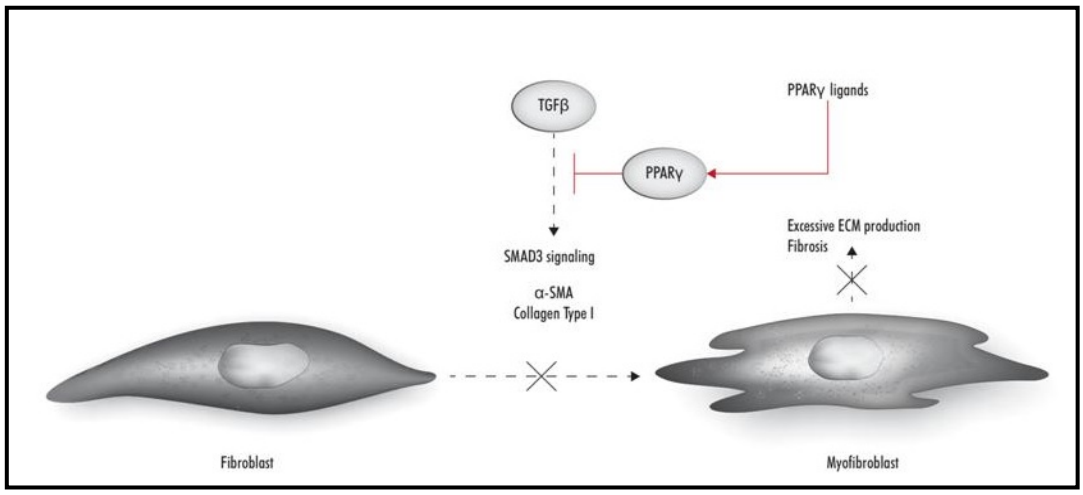

Figure-6: ***Emerging antifibrotic role of PPARγ. Lagares D, Ghassemi P, Vasheghani, F and Kapoor M.; 2011, Future Medicine***. Activation of PPARγ suppresses TGFβ-induced collagen synthesis, collagen type I α2 promoter activity, and α-SMA expression, as well as to directly antagonize the activation and function of SMAD3 in fibroblasts.

VI-1- Autophagy

Autophagy is an essential, homeostatic process by which cells break down their own components and is essential for survival, differentiation, development and homeostasis [254]. It is a process engaged in by all cellular organisms in which a portion of the cell contents becomes enclosed by lipid membranes to form the autophagosome and then fuses with lysosomes to form a digestive organelle (autolysosome) [255]. The process is classically triggered by nutrient stress and is induced when the major repressor of autophagy, the nutrient sensing kinase, mammalian target of rapamycin (mTOR) is inhibited [256] (Figure-7). This permits the cell to digest enclosed harmful proteins, lipids and DNA to liberate nutrients for survival of the cells [257].

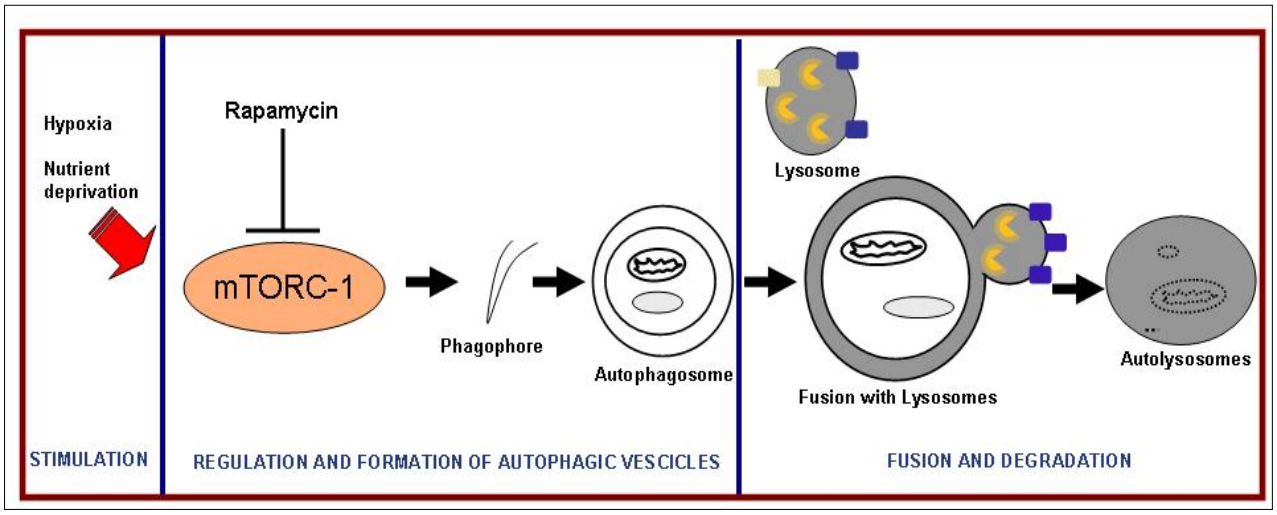

Figure-7: ***Process of autophagy:*** ABBREVIATION; mTORC: mammalian target of rapamycin complex

Autophagy results in degradation of cytoplasmic soluble macro-molecules such as damaged organelles (mitochondria, peroxisomes and endoplasmic reticulum), protein aggregates, and pathogens [254, 258, 259]. Autophagy can be triggered by wide variety of stimuli including stress, starvation, reactive oxygen species (ROS), hypoxia, lack of glucose,

energy and growth factor deficiency which initiate nutrient-sensing signaling pathways that stimulate autophagy [260-263]. Failure of regulated autophagy results in accumulation of damaged and disposal macromolecules in the cell resulting in enhanced ROS production, and abnormal gene expression resulting in cell death.

Deficiency of autophagy is associated with wide variety of disorders including cardiomyopthies, neurodegeneration and abnormalities in skeletal development [264, 265]. In early embryonic development, autophagy has an essential key role. Deficiency of Beclin 1, one of the key autophagy markers, leads to suppression of autophagy, apoptosis and embryonic lethality [266].

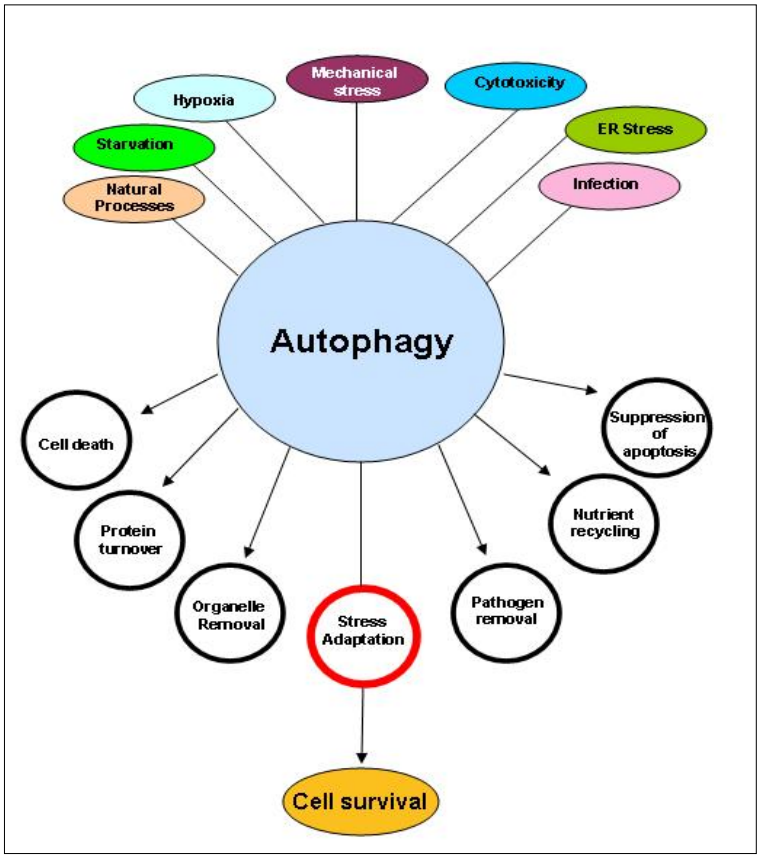

Figure-8: ***Various initiators and outcomes of autophagy:*** Autophagy can be induced by hypoxia, mechanical stress, starvation *etc.* and can perform several biological and pathological functions including cell survival, cell death, pathogen removal and stress adaptation.

Although autophagy works as a recycling system in cells by removing unwanted cytosolic proteins and organelles and maintains cell quality and nutrient supply during harsh conditions; excessive autophagy leads to vacuolization of autophagosome and massive accumulation of autophagic vacuoles in the cytoplasm causing excessive cell death [267, 268].

Autophagy is classified into macro-autophagy, micro-autophagy and chaperone-mediated autophagy that protect cells during stress responses. Autophagy classification is dependent on the choice of the pathway by which the cellular material is delivered to lysosome.

During macro-autophagy cellular constituents are sequestered into a double-membrane selective vesicle named an autophagosome. In macro- autophagy, autophaosome is formed by a portion of cytoplasm, containing organelles, which is enclosed by a phagophore or isolation membrane. Consequently, the outer membrane of the completed autophagosome fuses with the endosome and then lysosome and subsequently, the vesicle is released into the lumen for degradation of the internal material [47, 269].

Micro-autophagy is a non-selective lysosomal degradation process. In micro-autophagy, cytosolic components transfer to the lysosomes through direct invagination of lysosomal membrane via autophagic tubes. Vesicles are created on the top of the tube, fuse and bud into the lysosome lumen [270].

In the third type of autophagy, chaperone-mediated autophagy (CMA), cytosolic components are directly delivered into the lysosomal lumen by specific chaperone complex receptors. CMA targets only single proteins containing a specific peptide sequence for selective degradation. In this type of autophagy, the specific protein substrates are recognized

by chaperons and translocated to the lysosome through interaction with the lysosome receptor and lysosomal chaperones on the lumenal side [271].

VI-2- Autophagy Markers

Autophagy markers were identified in Sacharomyces Cerevisiae and were termed Atg genes [272]. In mammalian cells, most of the ATG genes are strictly conserved. The autophagy pathway is controlled by Atg genes in almost all the steps including the induction and nucleation of autophagic vesicles, expansion and fusion with lysosomes, degradation, and recycling [273]. Among the Atg autophagy markers, ULK1 (Unc-51–like kinase 1), Beclin1 and LC3 (microtubule-associated protein 1 light chain 3) in mammals, (Atg1, Atg6 and Atg8 in yeast) are three key regulators in the process of autophagy.

ULK1 and ULK2 are mammalian homologs of Atg1 [274]. They were first recognized as homologs of Caenorhabditis elegans uncoordinated-51 (Unc-51) [275]. Although the function of ULK2 is vague, a critical role for ULK1 in autophagy mechanism has been reported. ATG1 is represented by ULK1 in mammalian cells [276]. ULK1 is the most upstream mediator in autophagy process and induces autophagy. It plays a key role in autophagosome formation via transduction of proautophagic signals [277]. UKL1 is an essential regulator of cofactors including mATG13, FIP200 (focal adhesion kinase family-interacting protein 200 kDa) and ATG101 [276]. It has been shown that, mTORC1 complex negatively regulates autophagy downstream of ULK1 complex [276]. Hosokawa *et al.* reported that upon a high nutrient availability mTORC1 associates ULK1-Atg13-FIP200 complex through ULK1 [278]. In this manner mTOR phosphorylates ULK1 and Atg13 and this phosphorylation of ULK1 results in inhibition of autophagy [275, 278]. It has been

suggested that when mTOR is suppressed, ULK1 and ULK2 is activated. Activated ULK1 phosphorylates Atg13 and FIP200 (essential co-factors in autophagy induction) [278]. De-phosphorylation of ULK1 occurs by starvation or rapamycin treatment [278]. Upon this condition, activation of ULK1 through inhibition of mTORC1 creates a complex with FIP200 and Atg13 resulting in the initiation of autophagosomes formation [279].

Beclin 1 is the mammalian equivalent of the yeast Atg6 and has a central role in autophagy mechanism and dysfunction of Beclin 1 has been associated with variety of disorders such as cancer and neurodegeneration [280]. Beclin 1 is essential for localization of autophagic protein to a pre-autophagosomal structure (PAS). It participates in autophagosome formation by creating type III PI3 kinase and Vps34-Vps15 (class III PI 3-kinase) complex [281]. Beclin 1 regulates autophagy by formation of autophagic vesicle nucleation [282]. Interestingly, anti-apoptotic markers including Bcl-2 (B-cell lymphoma 2) and Bcl-XL (B-cell lymphoma-extra-large) can suppress the interaction between Beclin 1 and Vps34 [268]. In fact, Beclin 1 is a BH3-only protein that interacts with the BH3 receptor domain of anti-apoptotic factors including Bcl-2, Bcl-xL and Bcl-w [283] . Published data suggest that NF-κB and E2F transcription factors (E2F) are implicated in Beclin 1 expression during autophagy [284, 285]. For instance, in T cells, NF-κB binds directly to the Beclin 1 promoter through its p65 subunit and up-regulates the mRNA and protein expression of Beclin1 resulting in autophagy [284]. Also, transcription factor E2F1 directly activates Beclin 1 promoter and regulates autophagy [285].

Microtubule-associated protein Light Chain (LC3) is among the well known initiators of autophagy process [261]. The ATG (Autophagy-related protein) proteins analysis has shown that two ubiquitination-like conjugations Atg12 to Atg5 and Atg8 (LC3 in mammalian) to

phosphatidylethanolamine (PE) are required for autophagosome formation [273, 286]. The conjugation form of Atg12-Atg5 exists on the outer part of the isolation membrane and is involved in the elongation of the isolation membrane [287]. LC3 has an important role in selective autophagy to remove protein aggregates, organelles and pathogens [288]. LC3 exists in two forms; LC3-I is 18 k-Da in weight and is located in the cytoplasm in un-conjugated form. Whereas, LC3-II is present in the conjugated form and is bound to the autophagosome membrane. LC3-II is product of proteolytic maturation of LC3-I during autophagy [268].

BNIP3 belongs to the BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) family which contains a transmembrane domain and a BH3 domain. Alongside its role in autophagy as an inter-actor of LC3, BNIP3 also exhibits pro-apoptotic function [268]. It has been shown that the third 20-mer BH3 peptides of BNIP3 are responsible for initiating autophagy during normoxia. The BH3 domains of hypoxia-induced BNIP3 can induce autophagy by disrupting the Bcl-2-Beclin1 complex without inducing cell death [289].

VI-3- Initiation of autophagy

Mizushima classified autophagy in two subtypes; basal autophagy and induced autophagy [290]. Basal autophagy is activated in the cell constitutively to maintain cell hemostasis whereas during starvation, autophagy is induced [290]. Autophagy comprises of several steps including induction, autophagosome formation, autophagosome fusion, autophagosome breakdown (degradation), and recycling (amino acid/peptide generation) [291].

Amino acids, hormones and growth factors are the most well know autophagy stimulators in mammalian cells [292]. Upon the lack of nutrient and starvation, the AMP/ATP

ratio in the cells increases resulting in enhanced adenosine 5-monophosphate (AMP)-activated protein kinase (AMPK) and activation of most upstream of autophagy inducer, ULK1. Autophagy in mammalian cells starts with formation of phagophore, an isolation membrane. This membrane is believed to originate from the Golgi complex, endosomes, the endoplasmic reticulum (ER), mitochondria and the plasma membrane [291].

During induction by stimulator, a portion of cytoplasm with organelles or proteins is sequestered by a cisternal membrane, designated by phagophore or isolation membrane [272]. Sequestration is completed by the phagophore elongation and autophagosome formation, a double-membraned organelle. This processing and recruitment is essential for the extension, curvature, and closure of the isolation membrane to form autophagosomes. In yeast, autophagosomes are generated from the site called as PAS (preautophagosomal structure) [293].

ATG proteins including ATG 1-10, 12-14, 16-18 and atg 29 are involved in autophagosome formation in yeast [294-296]. Although several ATG proteins are common between yeast and animal, there are some specific mediators in mammalian autophagy including ULK-1, Beclin1, LC3, and BNIP3. Beclin 1, an orthologue of the Atg6 in yeast, is vital for localization of autophagic proteins to a PAS [277]. Beclin 1 function is controlled by Bcl-2. Upon the nutrient rich condition, Bcl-2 binds and sequesters Beclin 1. Detachment of Beclin 1 from Bcl-2 is compulsory for autophagy induction. In mammalian cells, autophagosome forms in presence of LC3 (homologue of Atg8 in yeast), atg5 and BNIP3.

After autophosome formation, the outer membrane of autophagosome fuses with the lysosome and the inner membrane and cytoplasm derived materials of autophagosome are degraded by lysosome. This process is named autolysosome or phagoautolysosome. The last

step in autophagy is recycling. In this phase, amino acids, yield of marco-molecules degradation, are transferred to the cytosol for reuse. Atg22 in yeast cooperates with vacuolar permeases, Avt3, Avt4 to export monomeric units in to the cytoplasm [286]. Re-utilization of other macro-molecules, carbohydrates and lipids, is still unknown (Figure-9).

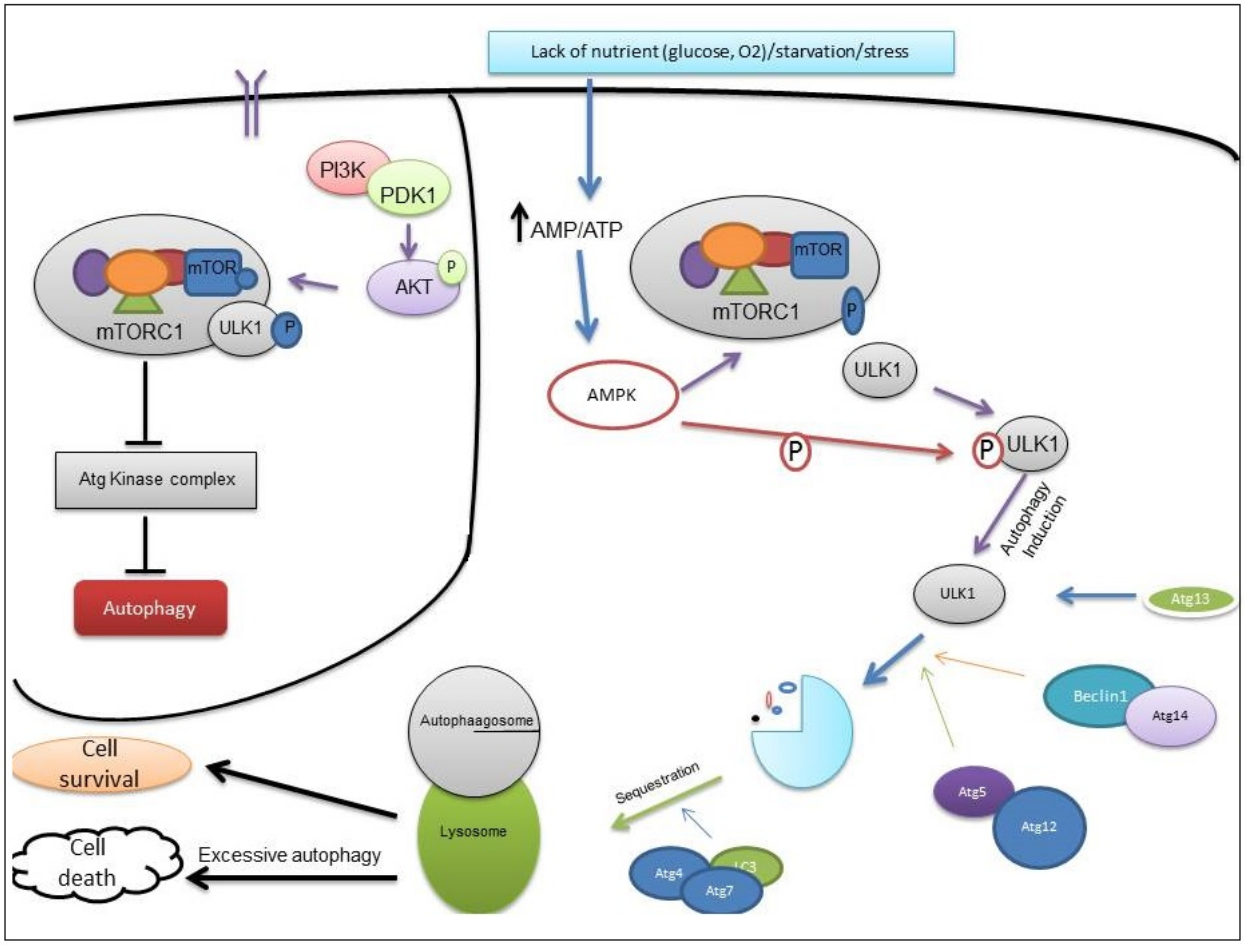

Figure-9: ***Initiation of autophagy:*** Autophagy initiation can prevent by the mTOR. mTORC complex phosphorylates ULK-1 leads to inactivation of ATG kinase complex and aoutophagy inhibition. However, lack of nutriet, glucose, oxygen or stress can activate autophagy. Phosphorylated ULK1 induce autophagy in the presence of Atg13. The Atg-complex and LC3 are indispensable for elongation of the double membrane. Lysosomes fuse with the autophagosome and create the autolysosome. Finally, the degradation products are transported to the cytosol and recycled [5]. Exessive autophagy results in cell death.ABBREVIATIONS; Atgs: autophagy related genes, ULK-1: Unc-51–like kinase 1, LC3: light chain 3, mTOR: mammalian target of rapamycin.

VI-4- mTOR: Master regulator of autophagy

The mammalian target of rapamycin (mTOR) is the major negative regulator of autophagy. One of the key functions of mTOR is the suppression of autophagy process. In mammalian cells it has been shown that activation of mTORC-1 results in phosphorylation of ULK-1 resulting in disruption of interaction between adenosine monophosphate-activated protein kinase and ULK-1, thus inhibiting AMPK-mediated ULK1 activation and hence loss of autophagy [297, 298].

mTOR is a serine/ threonine protein kinase encoded by the FRAP1 gene and located on human chromosome 1p36. It belongs to the phosphatidylinositol 3-kinase-related kinase protein family and modulates growth, proliferation, motility and survival in cells. It is named also, as mechanistic target of rapamycin or FK506 binding protein 12-rapamycin associated protein 1(FRAP1) [289] as well as the mammalian target of rapamycin. mTOR was identified and cloned [299-301] shortly after the discovery of the two yeast genes, *TOR1* and *TOR2*, in the budding yeast *Saccharomyces Cerevisiae* during a screen for resistance to the immunosuppressant drug rapamycin [302, 303].

mTOR comprises of two complexes mTOR Complex1 and mTORC2. mTORC1 complex consists of mTOR, Rapator (regulatory-associated protein of mTOR), and mLST8/GβL (mammalian LST8/G-protein β-subunit like protein) protein, It is characterized by the classic role of mTOR as a nutrient/energy/redox sensor and controlling protein synthesis [304]. mTORC2 is composed of mTOR, Rictor (rapamycin-insensitive companion of mTOR), and mSIN1 (mammalian stress-activated protein kinase interacting protein 1). It plays a key role in regulation of cytoskeleton as well as phosphorylation of a serine residue in the serine/threonine protein kinase Akt/PKB [305]. mTOR signaling is in the downstream of

PI3K/Akt pathway and is involved in the cell growth, metabolism, survival and lifespan in different organisms [255]. It has been shown that loss of TOR in C.elegans doubles the lifespan of these organisms [306]. Likewise, treatment with rapamycin (mTORC1 inhibitor), results in increased lifespan in both genders of mice [307]. This function of mTOR signaling in aging control represents a link between nutrition, metabolism, induction of autophagy and longevity, suggesting mTOR as a key mediator of lifespan regulation.

Under normal conditions, phosphorylation of ULK1 by mTORC1 complex results in inactivation of ULK1-ATG13-FUP200 complex. This phosphorylated ULK1 is unable to interact with AMPK. However, during starvation AMPK (second cellular energy sensor) represses mTORC1 complex and decreases the level of phosphorylated ULK1. This function of AMPK releases ULK1 from mTORC1complex. Subsequently, ULK1 is re-phosphorylated again by AMPK in another residue. Re-phosphorylated ULK1 transfers to the region of autophagosome formation and induces autophagy [297]. Rapamycin is a lipophilic macrolide antibiotic used as an immunosuppressive drug during transplantation. Rapamycin has been proposed as a potential mTOR signaling inhibitor through inhibition of mTOR complex 1 (mTORC1) and induction of autophagy in different cell types [308].

VI-5- Role of autophagy in aging

Autophagy, a vital mechanism to maintain cell homeostasis has role in pathogenesis of multiple diseases and regulation of aging [261]. In aging, accumulations of toxic protein products in aged cells within slow clearance of autophagosome are as a result of dysregulation of autophagy function which is associated to mis-function of lysosomal hydrolase enzymes [261].

Several studies report that autophagy mechanism is decreased during aging as a consequence of reduction in expression of autophagy regulatory markers such as ATGs in aged tissues. For example the expression of Atg5, Atg7 and Beclin 1 are reduced in normal human brain during aging [309]. Likewise, Carames *et al.* have been reported that the expression of key autophagy markers including Beclin 1 and LC3 are reduced in aging-related cartilage degradation with an increased apoptotic cell death in mouse articular cartilage [261].

Paradoxically, autophagy deficiency is related with amplified tumorigenesis in cancer [310, 311]. Zhou *et al.* have shown that PPARγ activation is associated with autophagy in breast cancer cells They found that PPARγ activation induces autophagy by increased expression of HIF1α and BNIP3 [310]. In addition, the emerging evidence suggests that altered autophagy is involved in the pathogenesis of age related disorders [310, 312, 313]. For instance, Meng *et al.* have revealed that hypothalamic autophagy deficiency play a crucial role in obesity through hypothalamic inflammation, including the activation of proinflammatory nuclear factor kappa-B kinase subunit beta (IKK-β)/NF-κB pathway. They found that defective hypothalamic autophagy increased energy intake leading to obesity [313]. These findings suggest that the diminished autophagy mechanism may contribute to the aging phenotype. However, prolonged activation of the autophagy mechanism can ultimately lead to cell death independent of apoptosis.

VI-6- Role of Autophagy in cartilage development and degradation

Direct *in vivo* role of mTOR in cartilage development is unknown but studies using rapamycin do suggest that mTOR could be essential for normal cartilage growth and development. In rabbits, direct infusion of rapamycin into proximal tibial growth plates

resulted in significantly smaller hypertrophic and proliferative zones and overall reduction in size of the growth plate long bone growth [314]. Organ culture studies using E 15.5 mice tibiae treated with rapamycin show multiple growth plate defects including reduced mineralization, reduced length of the hypertrophic layers, reduction in the extracellular matrix area in the resting and proliferating layers and reduced chondrocyte proliferation within the growth plate[315]. In rats, using fetal rat metatarsal explant culture system and ATDC5 chondrogenic cell line, it has been shown that treatment with rapamycin results in inhibition of proteoglycan accumulation and Indian Hedgehog (Ihh) accumulation, a regulator of chondrocyte differentiation [316]. Overall, these results suggest that mTOR is required for normal chondrocyte differentiation, proliferation and organization of growth plates. Chondrocytes, only cell type present in the articular cartilage, are responsible for maintaining the dynamic equilibrium of the cartilage environment. One of the critical events during OA is the loss of chondrocyte cellularity within the articular cartilage that can disrupt the balance between catabolic and anabolic processes resulting in destruction of the cartilage. However, the mechanisms that control the fate of the chondrocytes in the articular cartilage during normal versus OA conditions remain to be elucidated. Articular cartilage is a postmitotic tissue with a very low rate of cell turnover [9] and requires endogenous mechanisms such as autophagy to preserve cellular integrity and enable cells to adapt to the harsh conditions for cell survival. It has been shown that autophagy is a protective process required for maintaining the articular cartilage homeostasis [317]. Recent studies by Carames *et al*. and Sasaki *et al.* have suggested that autophagy process is dys-regulated during OA [261, 263, 308, 318]. Carames *et al.* reported that the expression of some of the key autophagy markers including ULK1, LC3, and Beclin 1 is significantly reduced in human OA articular cartilage

compared to normal human cartilage [261]. Further, they subjected C57Bl/6J mice to model of OA using transection of medial colateral ligament (MCL) and medial meniscotibial ligament (MMTL) surgery and found that the expression of autophagy markers including ULK1 and Beclin 1 was decreased during OA condition [261]. This study also examined the expression level of LC3 and Beclin 1 in aged mouse articular cartilage and found that the expression of both LC3 and Beclin 1 was reduced in 9 and 12 months old mouse cartilage compared to 2 months old articular cartilage [261]. This loss of autophagy inducers and regulators in the OA cartilage was related to an increase in the rate of chondrocyte apoptosis and cartilage degradation [261]. Studies also suggest a key relationship between mechanical loading and autophagy process. Bovine and human cartilage explants subjected to mechanical injury induces cell death in association with the decrease in the expression of ULK1, LC3 and Beclin 1 in cartilage explants [318]. Contrastingly, Sasaki *et al.* have reported that the expression of autophagy markers LC3 and Beclin 1 were up-regulated in human OA chondrocytes and cartilage compared to non-OA cartilage [263].

The contrasting data obtained from these studies is not surprising based on the fact that the articular cartilage from distinct OA patients during various phases of OA may exhibit different thresholds of stress stimuli resulting in increased or decreased autophagy signaling. It is also critical to understand that within the degrading cartilage, the functional ability of articular chondrocytes to exhibit decreased or increased autophagy may not be dependent on one or two factors but could be regulated by a cocktail of genes working together or in opposition to decide the fate of autophagy signaling within the cell. Rapamycin has also shown to exhibit chondro-protective effects via induction of autophagy *in vitro* and *in vivo* model of OA [308]. It has been reported that rapamycin not only protects cell death induced

by mechanical injury but also increase the expression of autophagy regulators [318], and reduces the severity of OA in experimental mice model of OA [308].

Dr. Zhang Y. from my group determined the expression of known autophagy genes in human OA cartilage compared to normal human cartilage using human autophagy PCR arrays. Results obtained from the PCR arrays showed that the key autophagy-related genes such as ATG3, ATG5, ATG12, LC3B, Beclin-1 and GABA(A)receptor-associated protein like 1 (GABARAPL1) involved in autophagic vacuole formation and phagophore expansion are down-regulated in OA cartilage compared to normal human cartilage. Also, co-regulators of autophagy and apoptosis including BNIP3, cyclin-dependent kinase inhibitor 1b (CDKN1B), Fas (TNF receptor superfamily member 6 (FAS) are down-regulated in OA cartilage. Chaperone mediated autophagy-related genes, such as HSP90AA1, HSPA8 were also significantly down-regulated in OA cartilage. Array data also revealed that critical regulators of cell death/apoptosis mechanisms including APP, CTSB, BCL2 and BCL2-associated agonist of cell death (BAD) were up-regulated in OA cartilage compared to normal cartilage (Zhang, Y. *et al.* accepted manuscript in Journal of Annals of Rheumatic Diseases).

These results do suggest a critical role of mTOR/autophagy in chondroprotection and maintaining the articular cartilage homeostsis. However, mechanisms and signaling pathways that control mTOR and subsequently autophagy in articular cartilage are unknown, Identifying exact mediator and signaling pathway that controls mTOR/autophagy signaling and ultimately the fate of chondrocytes in the articular cartilage will lead to promising therapeutic strategies to counteract cartilage destruction associated with OA pathogenesis.

CHAPTER II:

RATIONALE & SPECIFIC AIMS

RATIONALE AND AIMS OF MY THESIS

Recent studies suggest that the activation of the transcription factor PPARγ is a therapeutic target for OA. Agonists of PPARγ inhibit inflammation and reduce the synthesis of cartilage degradation products both *in vitro* and *in vivo*, and reduce the development/progression of cartilage lesions in OA animal models [225, 226, 237, 319]. However, studies using agonists of PPARγ do not elucidate the exact effects mediated by this complex gene. Indeed, some of these agonists (for example, rosiglitazone, an FDA approved anti-diabetic drug) have the ability to regulate, *in vivo*, various other signaling pathways independent of PPARγ, resulting in serious side effects [72, 320-322]. Current studies suggest that PPARγ is involved in the maintenance of bone homeostasis by stimulating and inhibiting the osteoclastogenic and osteoblastogenic pathways, respectively [203]. **However, the specific *in vivo* function of PPARγ in articular cartilage homeostasis and in OA pathophysiology remains largely unknown**. It is therefore vital, in order to achieve therapeutic efficacy with potentially less safety concerns, my PhD research is focused on elucidating the specific *in vivo* role of PPARγ in cartilage homeostasis and OA pathophysiology using genetically modified mice and murine model of OA.

OVERALL HYPOTHESIS: PPARγ is required for the normal maintenance of cartilage health and homeostasis. Loss of PPARγ and its downstream signalling in the articular cartilage contributes to increased inflammatory and catabolic responses resulting in enhanced degradation of the cartilage during OA.

To test this hypothesis, I generated two separate PPARγ KO mice harboring a (a) constitutive cartilage-specific germ-line deletion of PPARγ gene for developmental and age-related OA

study and (b) inducible cartilage-specific deletion of PPARγ in adult mouse specifically for OA studies using LoxP Cre system.

First, I generated mice harbouring a germ-line cartilage-specific deletion of the PPARγ gene using the LoxP/Cre system in which Cre is under the control of collagen 2 promoter to achieve specific deletion of PPARγ in the chondrocytes. Study performed in my laboratory has previously shown that constitutive cartilage-specific germ-line PPARγ KO mice exhibit serious growth and developmental defects associated with reduced length of long bone, growth plate abnormalities, delayed primary and secondary ossification process (***Monemjou R., Vasheghani F., et al. 2012; Journal of Arthritis Rheum*** [232]). To further explore the role of PPARγ in OA pathophysiology *in vivo*, the first aim of my PhD research was:

Specific Aim-1: To investigate the *in vivo* role of PPARγ in age-related OA using constitutive cartilage-specific germ line PPARγ KO mice.

Hypothesis-1: *Genetic ablation of PPARγ in the cartilage will result in spontaneous OA phenotype.*

Specific Objectives of Aim-1: Using constitutive cartilage-specific germ-line PPARγ KO mice, I determined the effect of specific ablation of PPARγ in the cartilage on:

1. ***Integrity of the articular cartilage at 6 and 14 months to ascertain any changes on cartilage integrity during aging.***
2. ***Integrity of synovium to determine any signs of inflammation or synovial fibrosis.***
3. ***Expression of catabolic and inflammatory mediators in the articular cartilage during aging.***

This part of my thesis showed that constitutive cartilage-specific germ-line PPARγ KO mice exhibited a spontaneous OA phenotype associated with enhanced cartilage degradation,

hypocellularity, synovial fibrosis, and increased expression of catabolic and inflammatory factors in adulthood **(*Vasheghani F.,et al., 2013; American Journal of Pathology*** [323]**)**. From this aim of the study, I could not ascertain if constitutive cartilage-specific germ-line PPARγ KO mice exhibited spontaneous OA because of developmental defects or as a result of PPARγ deficiency. To bypass the developmental defects, I then generated inducible cartilage-specific PPARγ KO mice using Col2rTACre system in which Cre is under the control of doxycycline to achieve specific inactivation of PPARγ in adult mouse. 6 weeks old mice were fed doxycycline for a week. rtTA requires interaction with doxycycline to permit interaction with the TetO sequence and so drives Cre expression and as the result both floxed alleles of PPARγ were inactivated to generate a cartilage-specific PPARγ KO mice. Using these mice, the next aim of my PhD thesis was:

Specific Aim-2: To investigate *in vivo* contribution of PPARγ to OA using inducible cartilage-specific PPARγ KO mice and subjecting these mice to DMM model of OA:

Hypothesis: *cartilage specific ablation of PPARγ in mice results in enhanced production of pro-inflammatory and catabolic markers resulting in accelerated cartilage degeneration, proteoglycan loss and ultimately causing severe OA.*

Specific Objectives of Aim-2: I subjected inducible cartilage-specific PPARγ KO mice to OA surgery and during the time course of OA (0, 5 and 10 weeks post-surgery) I determined, in comparison with the control mice, the effect of cartilage specific ablation of PPARγ on the:

1. ***Kinetics of cartilage degradation and joint changes during the time course of OA***
2. ***Expression/production profile of cartilage ECM anabolic factors***
3. ***Expression/production profile of cartilage catabolic factors***
4. ***Expression/production profile of inflammatory factors***

5. ***Expression of cell death/survival operative mediators in the articular cartilage during OA***

This part of my thesis showed that PPARγ KO mice subjected to OA surgery exhibited accelerated OA phenotype associated with accelerated cartilage degradation, hypocellularity, synovial fibrosis and increased expression of inflammatory and catabolic factors. This part of my thesis also identified that PPARγ KO mice which exhibit accelerated OA also exhibited increased chondrocyte apoptosis associated with compromised autophagy signaling. Since autophagy is a key cell survival mechanism that is suppressed by its master negative regulator called mTOR; my results further showed that mTOR expression was up-regulated in PPARγ KO mice. These results led to my next hypothesis that PPARγ signaling in the articular cartilage is mediated via the mTOR/autophagy signaling and deficiency of PPARγ in the articular cartilage up-regulates mTOR signaling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA.

Specific Aim-3: Identify the exact PPARγ signaling pathway operative in the articular cartilage during OA.

Hypothesis: PPARγ signaling in the articular cartilage is mediated by mTOR/autophagy pathway.

To determine the exact PPARγ signaling pathway operative in articular cartilage, I undertook: **(1)** *In vitro* approach using PPARγ expression vector to examine if restoration of PPARγ expression in PPARγ KO can alter the signaling of mTOR/autophagy pathway and rescue the phenotype of PPARγ KO cells *in vitro*. **(2)** *In vivo* approach by generating inducible PPARγ-mTOR cartilage-specific double KO mice to determine if genetic loss of mTOR in

PPARγ KO mice (double KO mice) can protect articular cartilage from excessive chondrocyte cell death and rescue mice from accelerated OA phenotype observed.

Specific Objectives of Aim-3:

1: PPARγ KO chondrocytes were transfected with PPARγ expression vector and the effect of restoring PPARγ expression in PPARγ KO cells was determined on:

(a) The expression of mTOR and autophagy genes.

(b) The expression of anabolic, catabolic and inflammatory factors in vitro.

2: I subjected inducible cartilage-specific PPARγ-mTOR double KO mice to OA surgery and during the time course of OA (0, 5 and 10 weeks post-surgery) I determined the effect of genetic deletion of mTOR in PPARγ KO mice (double KO mice) on:

(a) *Kinetics of cartilage degradation and joint changes during the time course of OA.*

(b) Chondrocyte cell death/survival mechanisms in the articular cartilage during OA.

(c) Expression/production profile of autophagy genes in the articular cartilage.

(d) Expression/production profile of inflammatory and catabolic factors

Using *in vitro* rescue studies (PPARγ expression vector) and *in vivo* studies (PPARγ-mTOR double KO mice), this part of my thesis clearly showed that PPARγ is involved in the regulation of mTOR/autophagy signaling in the articular cartilage. Specifically, deficiency of PPARγ in the articular cartilage up-regulates mTOR signaling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA (**Vasheghani *et al.*; Submitted to Journa of Annals Rheumatoid Diseases**).

This project comprehensively defines the *in vivo* role of PPARγ and its downstream signalling in OA pathophysiology. Fulfill the specific aims outlined above have shown that

PPARγ is required for the normal maintenance of cartilage health and homeostasis (Monemjou R., Vasheghani F., *et al.* [232]) and deficiency of PPARγ in the articular cartilage contributes to increased inflammatory and catabolic responses resulting in enhanced degradation of the cartilage during OA (Vasheghani F., *et al.* [323]). Further, this study for the first time provides direct evidences on the role of PPARγ in chondroprotection by modulation of mTOR/autophagy signalling in the articular cartilage. PPARγ signaling in the articular cartilage is mediated via the mTOR/autophagy signaling and deficiency of PPARγ in the articular cartilage up-regulates mTOR signaling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA (Vasheghani F., *et al.* submitted to Journal of Annals Rheumatoid Diseases). These findings outline PPARγ and its signalling by mTOR/autophagy as a potential therapeutic target for the treatment of OA thus avoiding side effects caused by using non-specific agonists.

CHAPTER III:

METHODOLOGY

Experimental Animal

C57BL/6-PPARγ$^{fl/fl}$ mice were obtained from Jackson Laboratory (Bar Harbor, Maine). C57BL/6 Col2-Cre transgenic mice were obtained from Shriners Hospital for Children, Montreal, QC, Canada.

1- Generation of germ-line cartilage-specific PPARγ KO mice

Genetically modified mice harboring a cartilage-specific deletion of PPARγ were generated using the Cre Lox methodology in which mice carrying Cre recombinase under the control of the collagen type II promoter were used to induce specific recombination in chondrocytes as previously established [72]. Mice containing a PPARγ gene flanked by LoxP sites (C57BL/6-PPARγ$^{fl/fl}$,Jackson Laboratory) were mated with C57BL/6 Col2-Cre transgenic mice to generate mice bearing Col2-Cre and a floxed allele in their germ-line (genotype: PPARγ$^{fl/+}$-Cre). These mice were backcrossed to homozygote floxed mice in the following cross: PPARγ $^{fl/+}$-Cre × PPARγ$^{fl/fl}$ to generate mice with both alleles inactivated in chondrocytes (genotype: PPARγ$^{fl/fl}$-Cre). PPARγ$^{fl/fl}$ -Cre mice are referred to as homozygote PPARγ KO mice, PPARγ$^{fl/+}$-Cre mice are referred to as heterozygote PPARγ KO mice, and PPARγ$^{fl/fl}$ mice without Cre transgene are referred to as control mice (Figure-10).

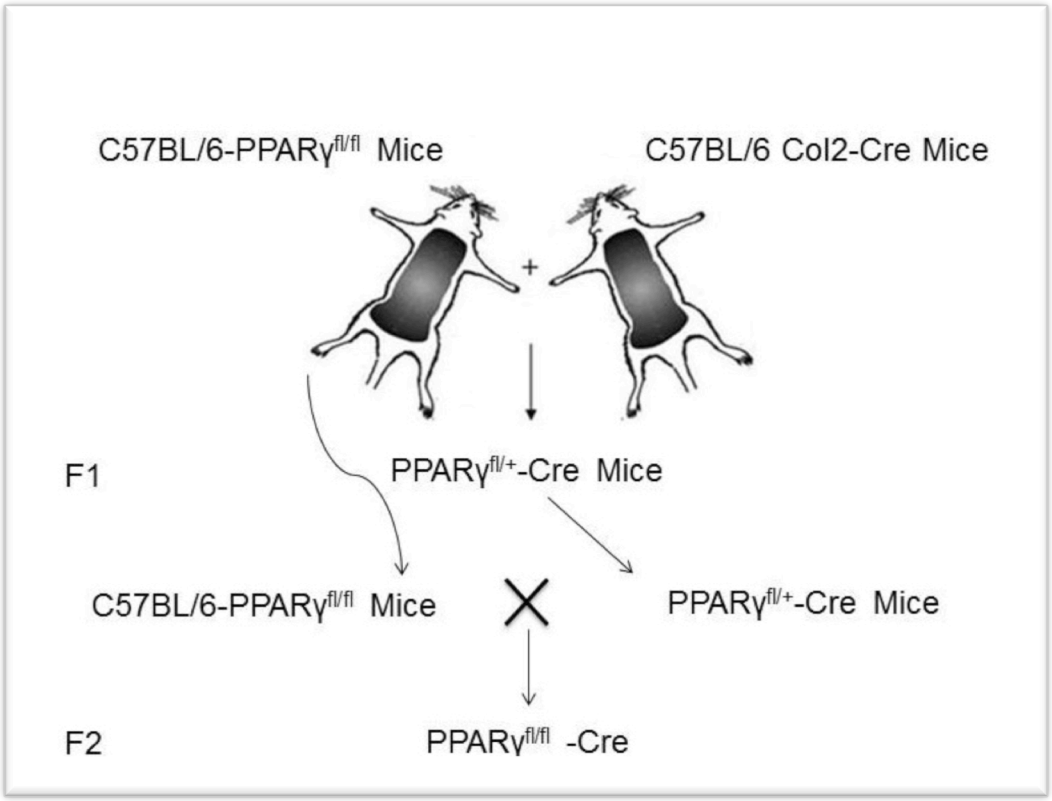

Figure-10: ***Schematic depiction of generation of germ-line cartilage-specific PPARγ KO mice.*** Mice containing a PPARγ gene flanked by LoxP sites (C57BL/6-PPARγ$^{fl/fl}$, Jackson Laboratory) were crossed with C57BL/6 Col2-Cre mice to generate mice carrying Col2-Cre and a floxed allele in their germ-line (PPARγ$^{fl/+}$-Cre). These mice were backcrossed to homozygote PPARγ$^{fl/fl}$ mice to generate PPARγ$^{fl/fl}$-Cre mice.

2- Generation of Inducible Cartilage-specific PPARγ KO Mice

Inducible cartilage-specific PPARγ KO mice were generated by mating mice containing a PPARγ gene flanked by LoxP sites [C57BL/6- PPARγ$^{fl/fl}$, Jackson Laboratory] with C57BL/6 Col2-rt-TA-Cre transgenic mice (obtained from Dr. Peter Roughley, McGill University, Montreal). The purpose of this generation was to create a transgenic mouse in which Cre production was under control of the collagen type II promoter and doxycycline (rt-TA promotor), and to validate its ability to create cartilage-specific PPARγ KO [324] (Figure-11).

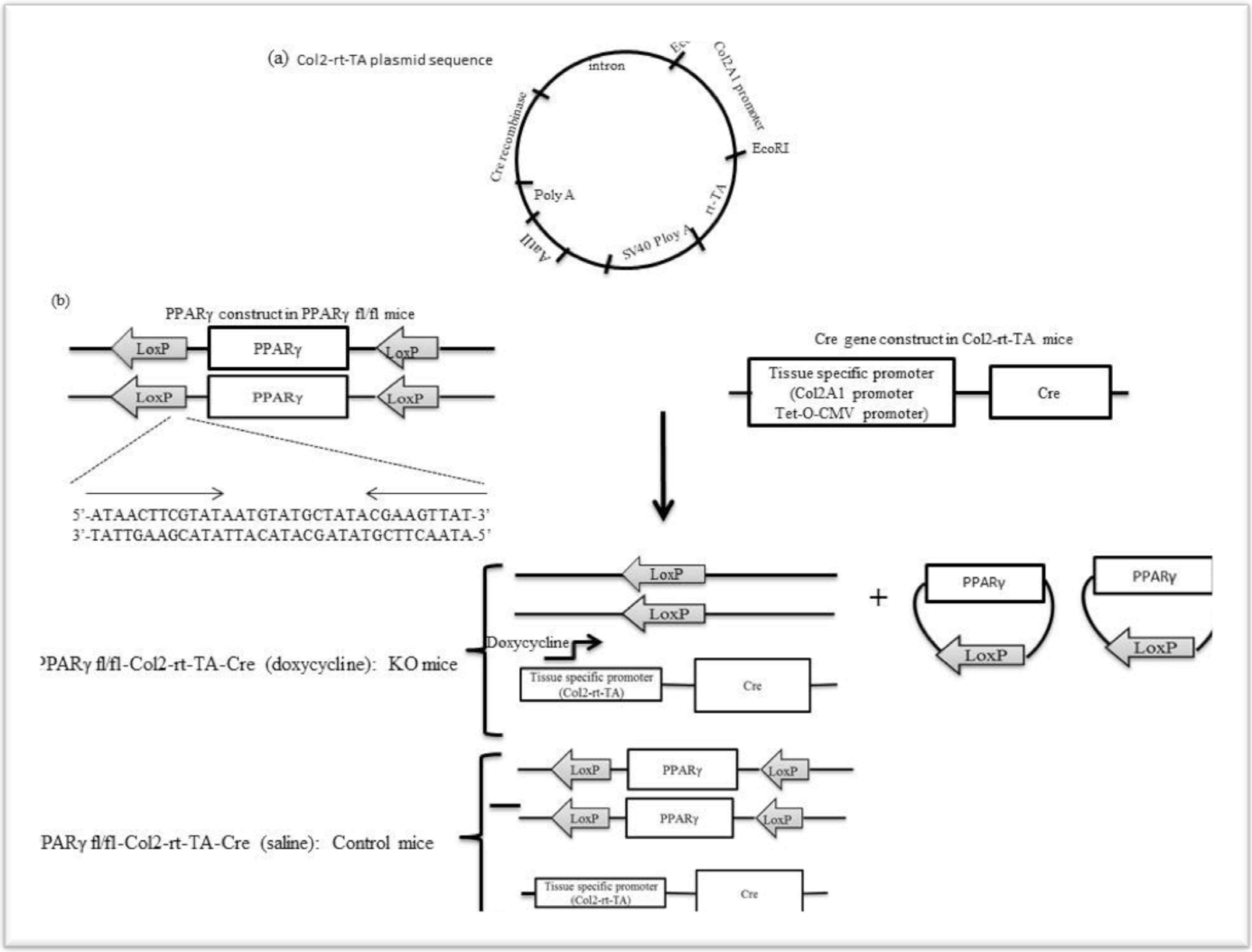

Figure-11: ***Schematic diagram of inducible conditional PPARγ KO mice:*** (a) The Col2-rtTA-Cre plasmids construct. In this construct Cre recombinase is under control of a CMV promoter regulated by a tetO operon responsive to rtTA. Also rtTA is under control of the Col2A1 (type II collagen) promoter. The EcoR1enzyme used for insertion of the Col2A1 promoter and the AatII site used for linearizing the construct[324]. (b)C57BL/6 Col2-rt-TA-Cre transgenic mice were mated with C57BL/6 mice containing PPARγfl/fl to generate PPARγfl/fl mice containg Col2-rt-TA-Cre. Cre recombinase in these mice was activated in the presence of doxycycline. Thus, mice treated with doxycycline are referred as KO mice while mice treated with saline were referred as control mice.

6 weeks old PPARγfl/fl-Cre mice were fed doxycycline (Sigma) dissolved at 10 µg/ml in Phosphate Buffer Saline (PBS), pH 7.4 by oral gavage with the dose of 80µl/g body weight for 7 days. rtTA requires interaction with doxycycline(Sigma-Aldrich Inc., Oakville, ON) to permit interaction with the TetO sequence to drive Cre expression resulting in inactivation of PPARγ

floxed alleles to generate a cartilage-specific PPARγ KO mice. Mice without doxycycline (only PBS) treatment were used as control mice (Figure-12).

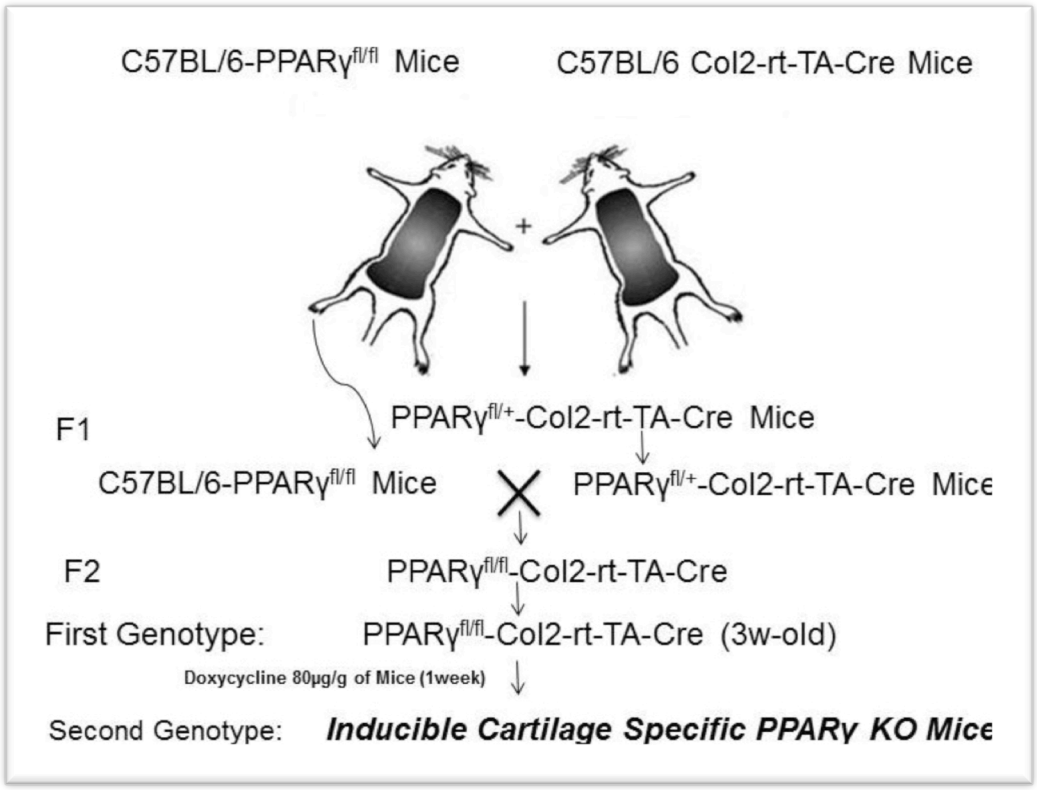

Figure-12: ***Schematic depiction of Inducible cartilage-specific PPARγ KO generation:*** These KO mice were generated by crossing mice containing a PPARγ gene flanked by LoxP sites with C57BL/6 Col2-rt-TA-Cre transgenic mice. 6 weeks old PPARγ$^{fl/fl}$-Cre mice were fed doxycycline by oral gavage. Doxycycline treated mice were used as KO mice and mice with only PBS treatment were used as control mice.

3- Generation of Inducible Cartilage-specific PPARγ-mTOR Double KO Mice

Generation of inducible cartilage-specific PPARγ-mTOR double KO mice was performed by using the Cre-Lox methodology. mTOR$^{fl/fl}$-Col2-rt-TA-Cre mice generated by mating mice containing a mTOR gene flanked by LoxP sites [C57BL/6-mTOR$^{fl/fl}$, Jackson Laboratory] with C57BL/6-Col2-rt-TA-Cre transgenic mice [324] (obtained from Dr. Peter Roughley, McGill University, Montreal; see letter of collaboration). Subsequently, PPARγ$^{fl/fl}$-Col2-rt-TA-Cre

mice were crossed with mTOR$^{fl/fl}$-Col2-rt-TA-Cre mice to create PPARγ$^{fl/fl}$-mTOR$^{fl/fl}$-Col2-rtTA-Cre animals. Next, 6 weeks old PPARγ$^{fl/fl}$ -mTOR$^{fl/fl}$-Col2-rtTA-Cre mice were fed doxycycline (Sigma) dissolved at 10 µg/ml in PBS by oral gavage with the dose of 80µl/g body weight for 7 days. PPARγ$^{fl/fl}$ -mTOR$^{fl/fl}$-Col2-rtTA-Cre treated with doxycycline are refereed as PPARγ-mTOR Double KO Mice whereas PPARγ$^{fl/fl}$-mTOR$^{fl/fl}$-Col2-rtTA-Cre treated with PBS (Control) are refereed as Control mice.

All procedures involving animals were approved by the Comité institutionnel de protection des animaux of the CRCHUM, and the Animal Use Subcommittee of the Canadian Council on Animal Care at the University of Western Ontario. All animal studies including housing and breeding were performed as approved by the aforementioned committees. All mice were kept in a 12 hour light/dark cycle. Food and water were available ad libitum.

Genotyping

Routine genotyping of ear clips DNA was performed using specific forward and reverse primers for Cre, PPARγ and mTOR.

- **DNA extraction**

50µl NaoH 25mM was added in the 0.5 ml eppendorf tube which contains ear clip and keep 30-60 min at 96 °C. Then 50µl TrisHcl 40mM was added to the sample and spin for 5 min, with 13000 rpm at room temperature. In final step, samples were kept in -20 °C for PCR.

- **Polymerase Chain Reaction (PCR)**

The samples were prepared for PCR using PPARγ F:5'-TGGCTTCCAGTGC-ATAAGTT-3', and R:5'-TGTAATGGAAGGGCAAAAGG-3' primers, Cre F:5'-ATCCGAA-AAGAAACGTG-3', and R:5'-ATCCAGGTTACGGATATAGT-3' primers and mTOR F:5'-

TTATGTTTGATAATTGAGTTTTGGCTAGCAGT3' and R:5'TTTAGGACTCCTTCTGT-GTGACATACATTTCCT-3' primers.The master mix (Invitrogen) was prepared as followed for each sample as shown underneath in Table-v:

MgCl2 50mM	dNTP 10mM	10x Buffer	ddH2O	Primer (F-R)	Tag polymerase
0.75 µl	1 µl	2.5 µl	11.55 µl	2 µl	0.2 µl

Table-v: ***PCR materials:*** For PCR reaction, MgCl2, dNTP, 10xPCR Buffer, Primers (Forward & Reverse), Tagpolymerase Enzyme and ddH2O were mixed ABBEREVIATIONS; MgCl2: Magnesium chloride, dNTP: deoxyribonucleotide solution mix dd H2O: double- distillation water, Primer (F-R): Forward & Reverse primers.

Then 2 µl of DNA sample was added in tube which contained 18 µl of master mix and samples were spun. The prepared samples were placed in PCR machine and standard PCR program was followed as shown in Table-vi.

Step	Temperature(°C)	Time
1	94	5 min
2	94	20 sec
3	60	20sec
4	72	20sec
5	Go to step3, 34x	
6	72	5 min
7	4	forever

Table-vi: ***Standard PCR program,*** these steps were repeated for 35 cycles by PCR machine to amplify DNA.

Surgically Induced OA Mouse Model

Wild type control mice and PPARγ KO mice were subjected to surgically induced OA by DMM model in the right knee of 10 weeks old animals as we have previously described [325]. Briefly, after induction of anesthesia with isoflurance in O2, the mouse was placed in dorsal recumbancy and the right limb prepared for aseptic surgery. After establishing a sterile surgical field using a fenestrated drape isolating the stifle, a medial approach to the stifle was performed. An 8 mm skin incision centered 3 mm medially to the patella was performed. The

subcutaneous tissues were dissected to the joint capsule. A medial arthrotomy was performed with Vannas microsurgical scissors. The patella was luxated laterally. The cranial pole of the medial meniscus was visualized; the cranial medial meniscotibial ligament identified and transacted at its insertion to the meniscus using a # 12 blade. Instability was documented by observation of the caudomedial retraction of the medial meniscus. The arthrotomy was closed with a simple continuous pattern using 8-0 polyglactin 910 (Vicryl, Ethibond, Somerville NJ, USA). The subcutaneous tissues were closed using simple intradermal sutures (8-0 polyglactin 910) with the knot buried. Skin closure was performed using a Ford interlocking pattern (8-0 polyglactin 910) to maintain apposition. A broad spectrum antibiotic ointment (silver sulfadiazine) (Smith & Nephew, St-Laurent Qc, Canada) was applied to the sutured wound. Sham surgery consisted of the same procedures with the exception of the cranial medial menisco-tibial ligament transaction (Figure-13).

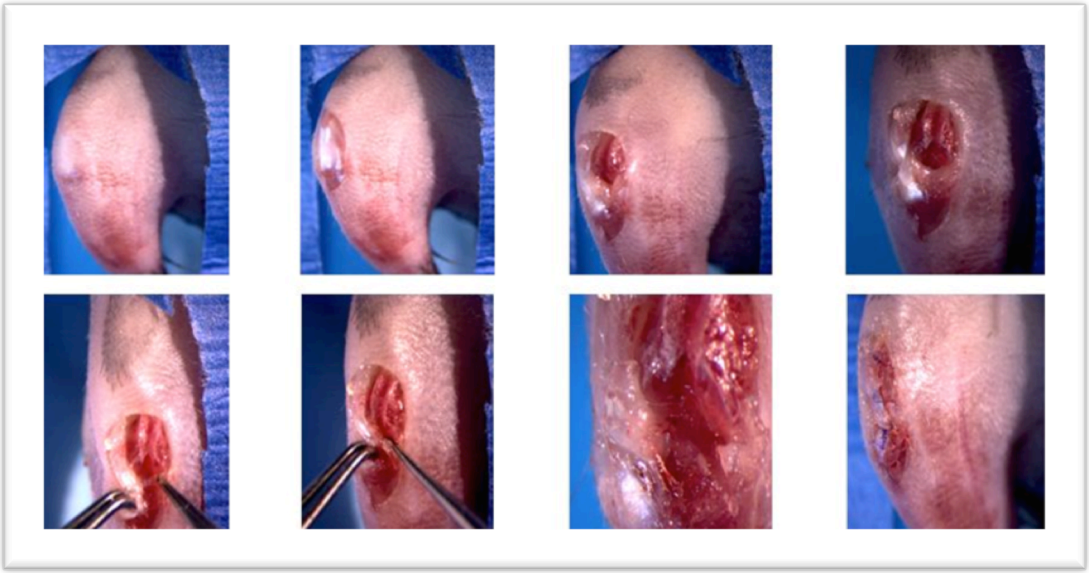

Figure-13: ***DMM model.*** In DMM surgery a small skin incision to the patella is performed. The subcutaneous tissues are dissected to the joint capsule. A medial arthrotomy is performed. Then the cranial medial meniscotibial ligament is identified and transacted at its insertion to the meniscus.The subcutaneous tissues were closed using simple intradermal sutures. Surgeries were performed by Dr. Bertrand Lussier DMV MSc Dipl.ACVS, University of Montreal. Photo credit goes to Glidys Valverde-Franco PhD, Arthrose unit of University of Montreal.

Histological Evaluation

Freshly dissected mouse knee joints were fixed overnight in TissuFix (Chaptec, Montreal, QC, Canada), decalcified for 1.5 hours in RDO Rapid Decalcifier (Apex Engineering, Plainfield, IL, USA), further fixed in TissuFix overnight, followed by embedding in paraffin and sectioned. 5 µm sections were deparaffinised in xylene followed by a graded series of alcohol washes. Sections were stained with Safranin-O/Fast Green (Sigma-Aldrich, Oakville, Ontario) according to the manufacturer's recommendations. Slides were evaluated by two independent readers in a blinded fashion. To determine the extent of cartilage deterioration, joint sections were stained with Safranin-O/Fast Green and histological scoring method issued by Osteoarthritis Research Society International (OARSI) was used for analysis as previously described [326] (Table-vii).

Grade	Score
0	Normal
0.5	Loss of safranin-O without structural changes
1	Roughened articular surface and small fibrillations
2	Fibrillation down to the layer immediately below the superficial layer and some loss of surface lamina
3	Fibrillation/erosion to the calcified cartilage extending to <25% of the articular surface
4	Fibrillation/erosion to the calcified cartilage extending to <25-50% of the articular surface
5	Fibrillation/erosion to the calcified cartilage extending to 50-75% of the articular surface
6	Fibrillation/erosion to the calcified cartilage extending to >75% of the articular surface

Table-vii: ***OARSI scoring criteria.*** ABBREVIATION; OARSI: Osteoarthritis Research Society International

ImmunoHistoChemistry (IHC)

IHC studies were performed using specific antibodies for target genes. For IHC analysis, Dakocytomation (Dako) labelled streptavidin biotin + System-horseradish peroxidase kit was

used following manufacturer's recommended protocol. Briefly, freshly dissected mice joint areas were fixed with 10% neutral buffered formalin, overnight at 4°c and decalcified with 0.1 M EDTA at room temperature before paraffin embedding and sectioning at the Notre-Dame Hospital Pathology Department (Montreal, QC). 5 µm sections were deparaffinised in xylene 3 times; each 5 min followed by a graded series of 100%, 95% and 70% alcohol washes. Endogenous peroxide was blocked for 5 minutes using 3% H2O2. Nonspecific IgG binding was blocked by incubating sections with BSA (0.1%) in PBS for 1 hour. Sections were then incubated with primary antibody in a humidified chamber and left overnight at 4 °C. Next, sections were incubated with biotinylated secondary antibody for 30 minutes followed by streptavidin for 1 hour. The diaminobenzidine tetrahydrochloride chromogen substrate solution was then added until sufficient color developed (Table-viii).

Material	Period	Condition
Xylene	5 min	
Xylene	5min	
Xylene	5min	
100%Ethanol	3min	
95% Ethanol	3min	
70% Ethanol	3min	
Distilled Water	2min	Washing, very slowly up & down to avoid washing sections
Hydrogen peroxide (Dako cytomation kit)	5min	Humidified chamber, 1 drop per sample
Distilled Water	2min	Washing
1%BSA in PBS	60 min	4 ºC
Primary Anti body	Overnight	Humidified chamber, 50µl per each sample
Distilled Water	2min	Washing
Biotinlated secondary antibody (Dako cytomation kit)	30min	Humidified chamber, room temperature, 1drop per sample
Distilled Water	2min	Washing
Sterpto avidin (Dako cytomation kit)	60min,	Humidified chamber, room temperature, 1drop per sample
Distilled Water	2min	Washing
DAB solution (Dako cytomation kit)	Till color develop	Under microscope, 1 drop per sample
Distilled Water		

Table-viii: ***IHC step by step.*** ABBREVIATION; IHC: immunohistochemistry

Degree of Inflammation

Enhanced synovial inflammation was shown by Hematoxylin & Eosin (H & E) staining according to the Sigma-Aldrich manufacturer's recommendations. Briefly, slides were taken through 3 changes of xylene each 5 minutes followed by graded series of 100%, 95% and 70% alcohol washes 5 minutes each. After washing the sections with H2O, the slides were stained with the nuclear dye (hematoxylin) and rinsed, then stained in the counterstain (eosin). Next, sections were washed with H2O and run in the reverse manner (water, alcohol, and xylene) and were cover-slipped. Stained sections were blindly scored for number of mononuclear cells on a scale from 0 to 3: 0, no mononuclear cells; 1, few mononuclear cells; 2, a moderate number of mononuclear cells; and 3, a high number of mononuclear cells.

Immunofluorescence

Immunofluorescence using rat anti-mouse macrophage/monocyte monoclonal antibody (MAB1852) recognizes an intracellular antigen of mouse macrophages and monocytes in the synovium of homozygote PPARγ KO mice compared to control mice. Briefly, sections were deparaffinized and dehydrated with incubation in three washes of xylene for 5 minutes each followed by graded series of 100%, 95% and 70% alcohol washes 5 minutes each. After rinsing sections twice in dH2O for 5 min each, specimen were quenched with 10% NH4Cl in PBS for 10 minutes and blocked by 0.13% Triton in PBS for 10 minutes followed by incubating sections with BSA (0.1%) in PBS for 1 hour. Sections were then incubated with MAB1852 (Millipore, Billerica, MA) antibody in a humidified chamber and left overnight at 4°C. Next, sections were incubated with secondary Texas-Red anti rabbit IgG antibody for 1 hour. The blue-fluorescent

DAPI nucleic acid stain solution (Invitrogen) was then added for 2 minutes at room temperature and followed by mount cover slip.

Trichrome Staining

To evaluate fibrosis in the articular cartilage and in the synovium, sections were stained with Masson's trichrome stain kit (Sigma-Aldrich, Oakville, Ontario) according to the manufacturer's recommendations. Briefly, slides were passed through 100% ethanol twice followed by staining with Hematoxylin for 10 minutes, Ferric chloride for 1 minute, Fuchsine acid 5 minutes, Phosphomolybdic acid for 5 minutes and Fast Green for 10 minutes. Slides were washed by tap water and Acetic acid. In the last step, samples were passed through 95% and 100% Ethanol for a minute and kept in xylene for 2 minutes. Stained sections were blindly scored for amount of fibrosis on a scale from 0 to 3: 0, no fibrosis; 1, few fibrosis; 2, a moderate fibrosis; and 3, a high amount of fibrosis.

TUNEL Assay

The ApopTag ® Plus Proxidase In situ Apoptosis Detection Kit from Millipore Company was used to performed TUNEL assay. This kit detects apoptotic cells in samples by modifying DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT) by specific staining. Briefly, 5 µm cartilage sections were washed in 3 times changes xylene for 5 minutes each and followed by 2 times wash in absolute 100% ethanol for 5 minutes each. Then the specimen were washed by a graded series of 95% and 70% alcohol 3 min each time and kept in one change of PBS for 5 minutes. Hydrogen peroxide was blocked for 5 minutes using 3% H_2O_2 in PBS at room temperature. 75µl per 5 cm^2 of equilibration buffer applied immediately on each

sample for minimum 10 seconds followed by 1hour incubation with Working Strength TdT Enzyme in a humidified at 37°C. Sections were then agitated in a coplin jar with Stop/Wash buffer for 15 seconds and incubated for 10 minutes. Specimen were washed in 3 changes of PBS for 1 minute each wash and incubated again with Anti-Digoxigenin Conjugate in humidified chamber for 30 minutes at room temperature. After 4 times each 2 minutes PBS washing, Working Strength Peroxidase Substrate was applied until sufficient color developed.

Chondrocyte Primary Cell Culture

Primary chondrocytes were prepared from the articular cartilage of Control and PPARγ KO mice at the time point as well as age-matched control as previously described [232, 327]. Articular cartilage was dissected, rinsed in phosphate buffered saline (PBS), and incubated at 37 °C for 15 minutes in trypsin-EDTA followed by digestion with 2 mg/ml collagenase P at 37 °C for 2 hours in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin under an atmosphere of 5% CO2. The cell suspension was filtered through a 70-µm cell strainer (Falcon, Fort Worth, Texas), washed, counted and plated. At confluence, the cells were detached and plated for experiments. To retain the phenotype, only first-passage cultured chondrocytes were used throughout the study.

Transient Transfection

Transient transfection experiments were performed using the Transfectine TMLipid Reagent according to the manufacture's recommended protocol (Biorad Inc., Missisuga, Canada). Briefly, chondrocytes were seeded 24 hours prior to transfection at a density of $2x10^5$

cells/well in 6- well plates. In a 1.5 µl eppendorf tube 2–4 µl Transfectin™lipid reagent per 50 µl serum-free medium was prepared. In another 1.5 µl eppendorf tube 1 µg of the PPARγ expression vector or pcDNA empty vector as a control per 50 µl serum-free medium was prepared. Then, equal volumes of TransFectin and DNA solutions were mixed and incubated for 20 minutes. After 20 minutes incubation, the mix was added directly to cells. The amount of transfected DNA was kept constant by using the corresponding empty vector. After 5 hours, medium was changed with DMEM containing 1% FCS and samples were incubated at 37°C incubator containing 5% CO2 for 48 hours. PPARγ expression and pcDNA empty vectors were donated by Dr. R. Evans (The Salk Institute, San Diego, CA). Cells were then harvested for RNA and protein extractions as previously described (Monemdjou *et al.*, 2012). Briefly, after aspiration the media from the cell cultures, plated cells were washed with 20ml 1x PBS. Then, plates were kept on ice and 1ml TRIzol was added on each plate. After 5 minutes, detached cells in TRIzol were collected and stored at -80 °C till the time of RNA extraction or kept on ice for instant RNA extraction. For protein extraction, cells were first washed with 20ml 1x PBS, and peletes then cells harvested with scraping on ice in precence of 1 ml 1x PBS. Harvested cells were collected in tubes, centrifuged, and palets were lysed in Tris-buffered saline containing 0.1% SDS, sonicated and stored at -80 °C till the time of use.

RNA Isolation

Total RNA was isolated from the chondrocytes using TRIzol (Invitrogen, Burlington, Ontario) and RNeasy (QIAGEN, Toronto, Ontario). First the media were aspirated off from the cell culture. After washing the cell culture surface with 20ml 1xPBS, 1ml TRIzol was added. Cells were kept on ice for 5 minutes and then 200µl of Chloroform was added to each

eppendorf tube which contains the cells and the tubes were shaken upside down for 15 sec followed with cold centrifuge (4 °C)/12000 rpm for 15 min. Upper side of white layer of each tube was separated in a new 1.5 µl tube very carefully and added 500 µl of Isopropyl – Alcohol, followed by shaking completely and kept on ice for 20-30 min. Another cold centrifuge (4 °C)/12000 rpm for 10 min were performed and then, 1000 µl of 70% Ethanol was added to each pellets tube and vortex. Again, cold centrifuge at 4 °C / 5 min /7500 rpm was done followed by removing all supernatant. Then the RNeasy Mini kit (QIAGEN) was used including on-column DNase digestion to eliminate DNA as mentioned above (RNase-Free DNase Set, QIAGEN).Samples were kept at -20 °C till RNA quantification.

cDNA Synthesis and RT-PCR

cDNAs were synthesized using Invitrogen Multi-Script Reverse Transcriptase kit (Table-ix). Then 25µl of prepared RNA was added in each Master Mix, and kept at room temperature for 10 minutes. Normal PCR machine was used to synthesize cDNA with performed program.

Material	µl/sample
PCR Buffer 10x	5
MgCl2-25mM	10
dNTPs Mix-10mM	5
Ramd.Hexam-2.5µM	2.5
RNase inhibitor-0.2µ/µl	0.125
MultiScri RevT-2.5 µ/µl	0.625
ddH2O	1.75
Total	25

Table-ix: ***cDNA synthesis materials.*** We used RNA reverse transcriptase to cDNA synthesis from RNA samples.ABBREVIATIONS: MgCl2: Magnesium chloride, dNTP: Deoxyribonucleotide triphosphate (A, T, G, C) mix, Ramd.Hexam: Random Hexam, MultiScri RevT: multi script reverse transcriptase, ddH2O: double distilled water

Real-time PCR

Samples were reverse transcribed and amplified using the TaqMan Assays-on-Demand (Applied Biosystems, Streetsville, Ontario) in a reaction solution containing two unlabeled primers and a 6-carboxyfluorescein-labeled TaqMan MGB probe. Samples were combined with One-Step MasterMix (Eurogentec, San Diego, California) (Table-x).

Then this 15 µl volume of Master Mix was mixed to 10µl of prepared cDNA, to reach total volume of 25µl. Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems) according to the manufacturer's instructions. The expression values were standardized to values obtained with control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA primers using the ΔCt method (Table-xi). All the primers and probe sets were obtained from Applied Biosystems, and represent averages and SEM from direct comparison of KO and control littermates. The statistical significance of real-time PCR results was determined by two-way analysis of variance with the Bonferroni posttest using GraphPad Prism, version 3.00 for Windows, software (GraphPad Software Inc., San Diego, CA).

Materials	µl/sample
ddH2O	1.75
Master-Mix-Q	12.5
Primer-F	0.25
Primer-R	0.25
UDG	0.25
Total volume	15

Table-x: ***Real-Time PCR master mix.*** I used SYBR Green Master Mix, forward & reverse primers, UDG and water in Real-Time PCR master mix. ABBREVIATIONS; Primer-F: forward primer, Primer R: reverse primer, UDG: uracil DNA glycosylase, ddH2O: double distilled water.

Western Blotting

Cells were lysed in Tris-buffered saline containing 0.1% SDS, and the protein content of the lysates was determined using bicinchoninic acid protein assay reagent (Pierce Rockford) with BSA as the standard. Cell lysates were adjusted to identical equals of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10 mM Tris Buffered Saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with the respective antibodies in TBS-T. After washing the membranes with TBS-T, the membranes were incubated overnight with horseradish peroxidase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse IgG (1:10,000 dilution in TBS-T containing 5% skim milk) at 4°C. Subsequently by further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad Chemidoc Apparatus.

Primer	Sense (5'-3')	Antisense (5'-3')
GAPDH	GTGCAGTGCCAGCCTCGTCC	GCCACTGCAAATGGCAGCCC
PPARγ	AGGGCGATCTTGACAGGAAAGACA	AAATTCGGATGGCCACCTCTTTGC
Cox-2	GCCAGCAAAGCCTAGAGCAACAAA	TACTGAGTACCAGGCCAGCACAAA
iNOS-2	TACATGGGCACCGAGATTGGAGTT	TCTGGAAACTATGGAGCACAGCCA
MMP-13	TGGAGTGCCTGATGTGGGTGAATA	TGGTGTCACATCAGACCAGACCTT
ADAMTS-5	AGGATCTGCGTGTGTCTGGTCATT	ACAATAACTTGGCTTGGCTGCCTG
Aggrecan	TGAAGACGACATCACCATCCAGAC	GACAGGTCAAAGATGGGCTTTGCT
Col2a1	ACATGTCAGCCTTTGCTGGCTTAG	TGCGGATGCTCTCAATCTGGTTGT
mTOR	TCCCACGGGACACCCACG	ACCATGGCCCCATAGGCTC
ULK1	ACCTTGCCAAGTCCCAAACA	TGCGCATAGTGTGCAGGTAG
LC3B	GCAGCGCCGGAGCTTTGAAC	GCTGCAAGCGCCGTCTGATT
ATG5	AGCAGCTCTGGATGGGACTGC	GCCGCTCCGTCGTGGTCTGA
BNIP3	ACCATGTCGCAGAGCGGGGA	GGGAGCGAGGTGGGCTGTCAC

Table xi- ***Primers list.*** The sequence of the mouse specific sense and antisense primers.

Statistical analysis

All images were taken at room temperature with a Retiga OEM Fast camera connected to a Leica Diaplan fluorescence microscope using either 4X numerical aperture (NA) 0.12, 6.3X NA 0.20, 10X NA 0.30, 25X NA 0.60, and 40X NA 0.70 objective lenses. All primary images were acquired and analyzed using Bioquant Osteo II software. Blinded observers were used for each histomorphometric analysis. Results are expressed as mean ± SEM for all counts. Statistical analysis unless stated otherwise was performed using the two-tailed Student's t-test. $P < 0.05$ was considered statically significant.

CHAPTER IV:
DISCUSSION, PERSPECTIVE & CONCLUSION

DISCUSSION

Osteoarthritis (OA) is the most common form of arthritis and aging-related joint pathology associated with degradation of the articular cartilage, reduced chondrocyte cellularity, inflammation, synovial fibrosis, subchondral bone remodelling and osteophyte formation [57, 328-331]. Loss of chondrocyte cellularity within the articular cartilage is one of the critical events that initiate the degradation of articular cartilage during OA. However, it is still uncertain as to what mechanisms control the fate of chondrocytes in the articular cartilage during OA. Secondly, it also uncertain as to what endogenous mediators control the production of anabolic, catabolic and inflammatory factors implicated in the pathogenesis of OA. My PhD research thesis is focussed on answering two major research questions:

(a). How can we control the process of accelerated chondrocyte cell death in the articular cartilage during OA?

(b). How can we stop the production of harmful inflammatory and catabolic factors involved in the destruction of articular cartilage during OA?

It is therefore essential to find answers to these two key questions in order to understand the exact pathogenesis and identify potential therapeutic targets to prevent/stop the progression of OA disease.

Published data have well established that PPARγ, a transcription factor, plays crucial roles in the regulation of insulin sensitivity, glucose homeostasis, and lipid metabolism [194]. It has been shown that the effect of PPARγ on lipid and glucose metabolism is via direct activation of target genes which contain PPRE motifs [332]. Recent studies by using reporter gene assays, gel shift experiments and ChIP methods have provided clear evidence that direct target genes of PPARγ contain PPRE sequences on their promoter region. For example, Zhou *et*

al. have revealed that FAM3A (Family with sequence similarity 3) is one of the direct target gene of PPARγ. They have revealed that PPARγ bounds to the PPRE-like motif in FAM3A promoter [332]. Furthur to the role of PPARγ on lipid and glucose metabolism, studies suggest that PPARγ possesses potent anti-inflammatory, anti-catabolic and anti-fibrotic activities [232, 253, 319, 323]. The ability of PPARγ in repression of several proinflammatory genes and cytokines expression is due to the activation of anti-inflammatory genes and ligand-dependent trans-repression by which PPARγ inhibits partially the activity of pro-inflammatory transcription factors like NF-κB, AP-1 and STAT-1 [48, 49]. Due to generation of the ROS hydrogen peroxide(H2O2) in the inflammatory process. Girnun *et al.* have proven that the antioxidant enzyme catalase is one of the direct target genes of PPARγ. They identified a putative PPRE element containing the canonical direct repeat 1 motif in the rat catalase promoter [46].

Prevoius studies suggest that PPARγ, a transcription factor, possesses potent anti-inflammatory, anti-catabolic and anti-fibrotic activities. Afif *et al*. showed that PPARγ expression is diminished in human OA cartilage [237] and it was postulated that down-regulation of PPARγ expression in OA cartilage may in part be responsible for increased expression of inflammatory and catabolic factors in the cartilage during OA. Indeed, treatment of human OA chondrocytes with proinflammatory factors such as IL-1β, TNF-α, IL-17, and PGE2 suppresses PPARγ expression [237]. Simultaneously, *in vitro* and *in vivo* studies using PPARγ natural/synthetic ligands have shown that PPARγ agonists can reduce the synthesis of various catabolic and inflammatory factors such as PGE2, IL-1β, TNF-α, IL-6, NO, and MMPs such as MMP-1 and MMP-13 involved in OA pathophysiology. Kobayashi *et al*. [225] and Boileau *et al.* [226] have shown that pioglitazone, a PPARγ agonist, diminishes the

development of experimental OA by reduction in expression of inflammatory and catabolic factors in animal model of OA. Kobayashi and his colleagues have suggested that pioglitazone decelerates the progression of OA via decreased synthesis of MMP-13 and IL-1β in the cartilage [225]. Likewise, pioglitazone has been shown to reduce the development of cartilage lesions and synthesis of the OA catabolic mediators including MMP-1, ADAMTS-5, and iNOS in a dog model of experimental OA [226]. Several *in vivo* and *in vitro* studies believe that the activation of PPARγ by its ligands can block the production of several inflammatory markers such as IL-1, NO and iNOS and diminish the synthesis of major cartilage catabolic mediators including MMP-13, MMP-1 and ADAMTS-5 which are responsible for the degradation of articular cartilage during OA.

Studies using agonists of PPARγ have been helpful but do not elucidate the exact effects mediated by this complex gene. Indeed, some of these agonists (for example, rosiglitazone, an FDA approved anti-diabetic drug) have the ability to regulate, *in vivo*, various other signaling pathways independent of PPARγ, resulting in serious side effects [72, 320-322]. Since exact specific *in vivo* role of PPARγ in articular cartilage homeostasis and in OA pathophysiology is not fully understood; my PhD thesis is directed towards elucidating the specific *in vivo* role of PPARγ in cartilage homeostasis and OA pathophysiology using genetically modified mice and murine model of OA.

Recently we showed that PPARγ is essential for normal cartilage growth and development and loss of PPARγ in the articular cartilage results in developmental defects associated with reduced length of long bone, growth plate abnormalities, delayed primary and secondary ossification process (Monemjou R.,Vasheghani F. *et al.* Arth & Rheum.; [232]). The first aim of my PhD research project was to determine if early developmental defects observed

in constitutive cartilage-specific germ-line PPARγ KO mice lead to any phenotypic changes in the articular cartilage during aging. Indeed, my results showed that constitutive cartilage-specific PPARγ germ line KO mice exhibited spontaneous OA phenotype during adulthood associated with enhanced cartilage degradation, synovial inflammation, synovial and cartilage fibrosis, increased expression of MMP-generated neoepitopes (VDIPEN and C1-2C), increased expression of catabolic markers including MMP13, ADAMTS-5, HIF-2α, and syndecan-4, and increased expression of COX-2 and inducible NO synthase as inflammatory factors (Vasheghani F. *et al.* Am J Pathol; [323]). Although, this study supports the role of PPARγ as an anti-catabolic, anti-inflammatory and anti-fibrotic factor; we could not ascertain if PPARγ was directly involved in OA pathogenesis or the spontaneous OA phenotype observed was due to early developmental defects.

To elucidate the specific *in vivo* role of PPARγ in OA pathophysiology and to bypass the developmental defects associated with germ-line KO mice, I generated an inducible cartilage-specific PPARγ KO mice using Cre-doxycycline system (Cre is under the control of doxycycline to achieve specific inactivation of PPARγ in adult mouse) and subjected these mice to OA surgery. My results showed that inducible cartilage-specific PPARγ KO mice exhibit accelerated OA phenotype compared to control wild type (WT) mice at 5 and 10 week post OA surgery. My histology results using safranin-O-fast green staining show that in comparison to WT mice at 5 and 10 weeks post OA surgery, KO mice exhibit greater cartilage degradation, increased proteoglycan loss and significant loss of chondrocytes in the articular cartilage. Previous studies have shown that PPARγ possesses potent anti-inflammatory and anti-catabolic properties [232, 253, 319, 323]. Indeed, my qPCR results revealed that PPARγ KO mice OA chondrocytes produced significantly increased levels of catabolic and

inflammatory mediators and significantly reduced expression of anabolic factors. It has also been shown that PPARγ possesses potent anti-fibrotic properties [253]. My results also showed that PPARγ KO mice not only exhibited increased cartilage destruction but also showed increased synovial fibrosis during OA *in vivo*, thus confirming a key role of PPARγ in controlling fibrosis *in vivo*.

In this study, I also observed that PPARγ KO mice which show accelerated OA and accelerated loss of chondrocyte cellularity and apoptosis, also exhibited increased expression of serine/threonine protein kinase called mTOR. In addition to increase in mTOR expression, I also observed a significant reduction in the expression of autophagy-inducing factors including ULK1 (most upstream autophagy inducer) [333], LC3B (an autophagy structural and functional factor), ATG5 (an autophagy regulator) [334], and BNIP3 (an interactor of LC3 in autophagy) [335] in chondrocytes isolated from PPARγ KO mice compared to control mice at 5 weeks post OA surgery. Recent studies suggest that autophagy, a form of programmed cell survival/cell adaption process is impaired during OA and may contribute towards increased chondrocyte cell death and degradation of the articular cartilage [261, 263, 308]. It has been shown that the expression of the key autophagy genes involved in initiating autophagy process such as UNC-51-like kinase 1 (ULK1) and Light Chain 3B (LC3B) is dys-regulated during OA [261]. mTOR (a serine threonine kinase) is the major negative regulator of autophagy and inhibition of mTOR complex I by rapamycin has been shown to activate autophagy and protect cartilage damage and chondrocyte cell death in mice model of OA [308]. Since mTOR is a repressor of autophagy and autophagy is essential for cell-survival, these results suggested a possibility that upregulation of mTOR and down-regulation of autophagy in PPARγ KO mice could be responsible for accelerated chondrocyte cell death and cartilage degradation observed. I then

isolated chondrocytes from PPARγ KO mice and performed rescue experiments using PPARγ expression vector and observed that restoration of PPARγ in KO chondrocytes resulted in a significant reduction in mTOR expression and recovery of autophagy markers including ULK1, BINP3, LC3B and ATG5 associated with inhibition of catabolic mediators including MMP-13 and ADAMTS-5. These results suggested a direct relationship between PPARγ signaling and mTOR autophagy signaling pathway. Role of PPARγ in autophagy signaling in the articular cartilage has never been reported. However, Zhou *et al.* have published that PPARγ activation by its ligands induces autophagy in breast cancer. They showed that BNIP3, an autophagy marker is the major target of PPARγ in autophagy induction [310]. Our studies also confirm that PPARγ KO mice exhibit decreased expression of BNIP3 and PPARγ expression vector results in increased expression of BNIP3.

Since my *in vitro* rescue studies using PPARγ expression vector showed that PPARγ could be involved in the regulation of mTOR/autophagy signaling in the articular cartilage, I then generated inducible cartilage-specific PPARγ-mTOR double KO mice to determine if genetic deletion of mTOR in PPARγ KO mice (double KO mice) could rescue the accelerated OA phenotype *in vivo*. Indeed, my results showed that PPARγ-mTOR double KO mice were significantly protected from DMM-induced OA associated with significant protection from cartilage destruction, proteoglycan loss and loss of chondro-cellularity compared with control mice. Since mTOR is a major repressor of autophagy, I found that the expression of two critical autophagy markers (ULK1 and LC3B) was significantly elevated in chondrocytes extracted from PPARγ- mTOR double KO mice compared to control mice, thus rescuing the accelerated OA phenotype *in vivo*. Although, it has been revealed that PPARγ regulates its downstream genes that contain PPRE motifs directly [46], we still do not know if mTOR is a direct target of

PPARγ. But, these results clearly showed that PPARγ is involved in the regulation of mTOR/autophagy signaling in the articular cartilage. Therefore, deficiency of PPARγ in the articular cartilage up-regulates mTOR signaling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA. See Figure-13 for the proposed PPARγ signaling in the articular cartilage via mTOR/autophagy pathway.

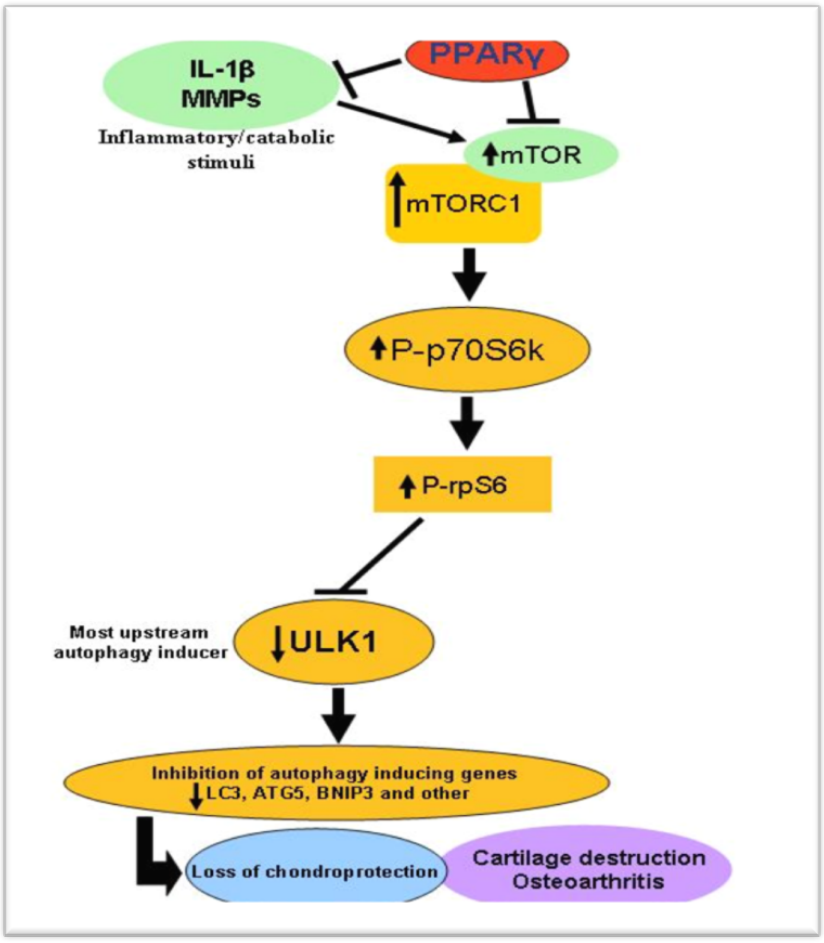

Figure-13: ***Proposed PPARγ signaling pathway in articular cartilage:*** PPARγ is required for normal regulation of mTOR/autophagy signaling in the articular cartilage. Loss of PPARγ results in increased mTOR signaling and subsequent decrease in autophagy. This results in reduced chondroprotection and imbalance between catabolic and anabolic activities in the cartilage leading to accelerated cartilage destruction.

PERSPECTIVE

Limitation of the study & potential approaches

OA is a joint disease with a complex pathophysiology. Etiology and pathogenesis underlying OA is poorly understood. Hence, there is not yet a satisfactory treatment for this disorder. Therefore, a better understanding of the pathophysiological pathways involved in progression of OA could have important implications in the development of new drug therapies. Recent studies suggest that the activation of the transcription factor, PPARγ, is a therapeutic target for OA. Agonists of PPARγ inhibit inflammation and reduce the synthesis of cartilage degradation products both *in vitro* and *in vivo*, and reduce the development/progression of cartilage lesions in OA animal models [201, 225, 226, 237]. However, studies using agonists of PPARγ do not elucidate the exact effects mediated by this complex gene. Indeed, some of these agonists like rosiglitazone have the ability to regulate, *in vivo*, various other signalling pathways independent of PPARγ, resulting in serious side effects [12, 72, 321, 322]. It is therefore vital, in order to achieve therapeutic efficacy with potentially less safety concerns, to elucidate the exact *in vivo* role of PPARγ in OA pathophysiology.

In this perspective, we wanted to investigate more about the specific role of PPARγ in the degradation of cartilage and the loss of chondrocyte cellularity. Thus, our project determines the specific *in vivo* role of PPARγ and its downstream signalling in OA pathophysiology using cartilage-specific PPARγ KO mice and subjecting these mice to DMM surgical model of OA. In this project, we have shown that this transcription factor, in collaboration with other factors involved in the degradation of cartilage and hypocellularity during OA. Despite the importance of our experimental results, additional data should be

obtained to better target its involvement in the whole joint tissues including subchondral bone, and synovial membrane and supporting connective tissue elements.

Although PPARγ has been shown to play a key role in lipid and glucose homeostasis, recent studies have suggested that it also plays a role in bone homeostasis by contributing to osteoclastogenic and osteoblastogenic pathways. It has been shown that, PPARγ plays a role in promoting osteoclast differentiation and bone resorption [203]. In our project we did not investigate the effect of cartilage specific deletion of PPARγ on subchondral bone changes during OA. We used surgical DMM mouse model of OA, as the most widely used surgically-induced model of OA in mice [322], to investigate specific *in vivo* role of PPARγ in pathophysiology of this degenerative disease. OA characteristics in DMM model closely resemble the slowly-progressive human OA manifestations. In comparison with ACL model, DMM surgery is much easier to perform. Also, lesions in the DMM model progress from mild-to-moderate OA at 4 weeks and from moderate-to-severe OA at 8 weeks post-surgery. However, this model has a specific limitation which is that the subchondral bone changes are rarely observed in the DMM model [322, 336]. Therefore, limitation of using the DMM surgery could not allow us to explore the effect of cartilage specific PPARγ deficiency on osteoblast and osteoclast changes as well as subchondroral bone remodeling during OA. Although ACLT surgery requires the high surgical skill, it is not recommended in the mouse because of development of severe OA that may destruct subchondral bone. It has been suggested that the DMM model has adequate sensitivity to show disease modification in mouse. Therefore, this model could be first choice to mice model of OA [336].

In this perspective, we were interested in the functional consequences of specific deletion of PPARγ in the cartilage using cartilage specific PPARγ KO mice. Indeed, among other tissues involved in the development of osteoarthritis, we partially focused on the effect of cartilage specific PPARγ deficiency on fibrotic and inflammation of the synoviocytes. We were not able to focus deeply on the PPARγ involved molecular pathways which lead to fibrosis and inflammation in the synovial membrane. This was a delicate step because to perform the study of the synovium is difficult in small animals such as mice. Extraction of synoviocytes not only requires surgical/anatomical proficiency, but also is an expensive time consuming process. Although, this study supports the role of PPARγ as an anti-inflammatory and anti-fibrotic factor; we could not determine the exact molecular pathway that PPARγ involves to exhibit these effects. For example, we could not answer if PPARγ implicates in synovial fibrosis through antagonising the major pro-fibrotic factor TGF-β? Investigate the exact mechanism of anti-fibrotic role of PPARγ in synoviocytes would allow us to be one step closer in achieving therapeutic efficacy for OA target drugs.

Regarding to the *in vivo* part of the project, we could continue our work by histological examinations of the fibrosis and inflammation levels in synovial membrane of the PPARγ/mTOR double KO mice to explore if restoration of the PPARγ can rescue its anti-fibrotic/anti-inflammatory effects in synovial membrane? If anti-fibrosis/anti-inflammation effects of PPARγ in the synovial membrane are involved in the regulation of mTOR/autophagy signalling? In addition, Zhou *et al.* have shown that PPARγ activation is associated with autophagy in breast cancer cells. They found that PPARγ activation induces autophagy by increased expression of HIF1α and BNIP3 [310]. We could investigate the expression level of

both HIF1α and BNIP3 in PPARγ/mTOR double KO mice to investigate if PPARγ restoration could induce the expression of HIF1α via mTOR/autophagy pathway?

Also, to confirm our hypothesis that PPARγ controls mTOR/autophagy signalling in the articular cartilage in human OA, we could use siRNA approach to silencing PPARγ expression in human chondrocytes and treat cells with mTOR inhibitor, rapamycin.

It has been shown that the effect of PPARγ on its down stream pathways can be directly via activation of target genes which contain PPRE sequences [46, 332]. Thus, we could investigate if mTOR has PPRE motif and is regulated directly by PPARγ. Further, to explore the effect of PPARγ activation/inhibition on promoter activity of target genes/proteins, shift assay or Chromatin immunoperecipitaton assay (CHIP) could be used, to determine if the target gene/protein is a direct PPARγ target. Indeed, supernatants of chondrocytes culture could be collected for Enzyme linked immunosorbent assay (ELISA) to determine the concentration level of inflammatory cytokines, chemokines and other markers using ELISA kits, according to the standard protocol.

Although determining the specific *in vivo* function of PPARγ and its downstream signaling pathways activity in pathophysiology of OA is complex, by beginning to gain an understanding of the role of PPARγ, specifically its role in cartilage, this study has created advances in the field, thus allowing us to be one step closer in achieving therapeutic efficacy for OA with potentially less side effects.

CONCLUSION

Previous clinical studies to treat established OA by inhibiting cartilage ECM degradative factors have met with disappointing results associated with low efficacy and serious side effects, as a result there is no disease-modifying OA drugs (DMOADs) currently available. Moreover, the drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors prescribed for OA treatment only reduce inflammatory symptoms but don't cure the underlying pathology of the disease. This study for the first time provides direct evidence on the role of PPARγ in chondroprotection by modulation of mTOR/autophagy signaling in the articular cartilage. These findings outline PPARγ and its signaling by mTOR/autophagy as a potential therapeutic target for the treatment of OA. In addition, data presented in this thesis will lead to a better understanding on catabolic and inflammatory mechanisms as well as cell death/survival mechanisms associated with the pathophysiology of OA and related diseases.

CHAPTER V:

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APPENDIX

RESEARCH ARTICLES

ARTICLES CONTRIBUTIONS

Article 1: *Adult cartilage-specific peroxisome proliferator-activated receptor gamma knockout mice exhibit the spontaneous osteoarthritis phenotype*

Experimental Procedures: For this article, germ line PPARg KO mice have been generated already so, I prepared optimal condition for these mice until aging. I did major experiments including DNA extraction, genotyping PCR, joint dissection, histology, staining, IHC, mice chondrocytes isolation and culture, cDNA preparation, q-PCR.

Data analysis & statistics: I participated in results analysis such as histomorphometric analysis and statistical analysis with our graduated student R. Monemdjou.

Manuscript: I revised the article and some photos under supervision of my director. This article was accepted and published in American Journal of Pathology.

Article 2: *PPARγ controls mTOR/autophagy signaling in the articular cartilage*

Experimental Procedures: In this article, I did all experiments including generation of inducible PPARg KO mice and inducible PPARg/mTOR double KO mice, DNA extraction and genotyping PCRI prepared all necessary material for surgery and participated in mice preparation during surgery, and knee measuring after surgery. Dr. Bertrand Lussier operated surgeries. I scarified mice with assist of Meryem Blati. Then I did joint dissection, mice chondrocytes isolation and culture, cDNA preparation, q-PCR and western blot. Dr. You Zhang assisted me in the procedure of RNA extraction and q-PCR of the autophagy markers. I stained the slides and did IHC in assist of Feredrique Pare.

Data analysis & statistics: I analyzed all the data and did statistics.

Manuscript: I wrote the whole article and prepared photos under supervision of my director. This article has been submitted to the journal of Annals of the Rheumatic Diseases.

Article 3: *Association of cartilage-specific deletion of peroxisome proliferator-activated receptor γ with abnormal endochondral ossification and impaired cartilage growth and development in a murine model*

Experimental Procedures: For this article, I did some experiments including PCR-genotyping (DNA extraction, PCR), IHC, chondrocytes isolation from E16.5 control and homozygote PPARγ KO mice, RNA extraction, q-PCR, and I did the whole part of explant study including extraction of the femur head cartilage explants from 3-week-old control and homozygote PPARγ KO mice, tissue homogenising and RNA extraction and q-PCR.

Data analysis & statistics: I participated in some parts of data analysis such as histomorphometric analysis and q-PCR analysis.

This article was published in the journal of Arthritis Rheum.

Article 4: *Cartilage-specific deletion of mTOR up-regulates autophagy and protects mice from Osteoarthritis*

Experimental Procedures: In this article, I did some experiments such as OA mice's joints dissection, OA mice cell culture, RNA and cDNA preparation of OA mice. Further, I did some IHC. I assisted in the first mice surgery. I dissected mTOR joints for the first experiment to extract chondrocytes, and cultured them.

Data analysis & statistics: I participated in some parts of histomorphometric analysis.

This article was accepted in the journal of Annals of the Rheumatic Diseases.

ARTICLE 1

Adult cartilage-specific peroxisome proliferator-activated receptor gamma knockout mice exhibit the spontaneous osteoarthritis phenotype

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SHORT COMMUNICATION

Adult Cartilage-Specific Peroxisome Proliferator–Activated Receptor Gamma Knockout Mice Exhibit the Spontaneous Osteoarthritis Phenotype

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Osteoarthritis (OA) is an age-related progressive degenerative joint disease. Peroxisome proliferator–activated receptor gamma (PPARγ), a transcription factor, is suggested as an attractive therapeutic target to counteract degradative mechanisms associated with OA. Studies suggest that activation of PPARγ by its agonists can reduce the synthesis of OA catabolic and inflammatory factors and the development of cartilage lesions in OA animal models. Because these agonists impart several PPARγ-independent effects, the specific *in vivo* function of PPARγ in cartilage homeostasis and OA remains largely unknown. Herein, we describe the *in vivo* role of PPARγ in OA using cartilage-specific PPARγ knockout (KO) mice generated using the Cre-lox system. Adult PPARγ KO mice exhibited a spontaneous OA phenotype associated with enhanced cartilage degradation, hypocellularity, synovial and cartilage fibrosis, synovial inflammation, mononuclear cell influx in the synovium, and increased expression of catabolic factors, including matrix metalloproteinase-13, accompanied by an increase in staining for matrix metalloproteinase–generated aggrecan and type II collagen neoepitopes (VDIPEN and C1-2C). We demonstrate that PPARγ-deficient articular cartilage exhibits elevated expression of the additional catabolic factors hypoxia-inducible factor-2α, syndecan-4, and a disintegrin and metalloproteinase with thrombospondin motifs 5 and of the inflammatory factors cyclooxygenase-2 and inducible nitric oxide synthase. In conclusion, PPARγ is a critical regulator of cartilage health, the lack of which leads to an accelerated spontaneous OA phenotype. *(Am J Pathol 2013, 182: 1099–1106; http://dx.doi.org/10.1016/j.ajpath.2012.12.012)*

Osteoarthritis (OA) is a progressive degenerative joint disease and the most common form of arthritis. It is an age-related disease in which the effects of aging contribute to disease progression.[1–3] OA is characterized by cartilage deterioration, hypocellularity, synovitis, and remodeling of the subchondral bone. Symptoms usually appear in middle age, and >50% of Americans 65 years and older show radiologic signs of the disease.[4] As age is a major contributor to the disease and individuals are living longer owing to increased life expectancy, OA is a growing socioeconomic and clinical concern.

Peroxisome proliferator–activated receptor gamma (PPARγ) is a ligand-activated transcription factor and a member of the nuclear receptor superfamily. PPARγ was originally identified as a key regulator of adipocyte differentiation and lipid metabolism.[5] Synthetic agonists of PPARγ, such as thiazolidinediones, are clinically used for the treatment of type 2 diabetes mellitus. Apart from its role in adipogenesis and insulin sensitization, it is now known that PPARγ plays a key role in a variety of pathophysiologic processes. Mouse studies using agonists of PPARγ and targeted deletion of PPARγ in specific cells/tissues

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have been useful in dissecting the key pathophysiologic role of PPARγ in a variety of disorders, including wound healing/fibrosis,[6–8] tumorigenesis in the breast,[9] oxidative damage to the myocardium,[10] intraepithelial neoplasia in the prostate,[11] pancreatic inflammation,[12] and T-cell–mediated gut inflammation.[13]

PPARγ is also believed to be a potential therapeutic target for the treatment of OA based on the fact that PPARγ agonists can reduce the synthesis of various catabolic and inflammatory factors involved in the pathogenesis of OA. For example, it can reduce inflammatory cytokines such as IL-1β, tumor necrosis factor-α, prostaglandin E2, IL-6, nitric oxide (NO), and matrix metalloproteinases (MMPs) such as MMP-1 and MMP-13 (reviewed in the study by Fahmi et al[14]). Moreover, PPARγ agonists can reduce the development of cartilage lesions *in vivo* in experimental dog[15] and guinea pig[16] models of OA and have also been shown to inhibit OA-involved signaling pathways, such as the mitogen-activated protein kinases extracellular signal–regulated kinase 1/2 and p38 and NF-κB. Apart from its anticatabolic and anti-inflammatory effects, we and others also showed that PPARγ exhibits antifibrotic properties.[6,7] Loss of PPARγ in fibroblasts results in excessive inflammation and fibrosis in a mouse model,[7] and PPARγ attenuates fibrosis by blocking activation of the transforming growth factor β/Smad signaling pathway.[8,17–21]

In vitro and *in vivo* studies using agonists of PPARγ have been helpful yet unable to define the specific *in vivo* role of PPARγ in the pathogenesis of OA. In fact, it is now understood that some of these agonists may also act through mechanisms independent of PPARγ activation. To devise OA therapeutic strategies targeting PPARγ, it is essential to first determine the role of this complex transcription factor in cartilage health and physiologic function *in vivo*. PPARγ knockout (KO) mice exhibit embryonic lethality due to placental defects.[22] Therefore, to specifically elucidate the *in vivo* role of PPARγ in cartilage health and OA, we generated cartilage-specific PPARγ KO mice using the Cre-lox system. Using these mice, we previously showed that cartilage-specific deletion of PPARγ results in delayed endochondral ossification and cartilage growth and developmental defects.[23] In the present study, for the first time, to our knowledge, we show that loss of PPARγ in the cartilage results in the OA phenotype in adult PPARγ KO mice.

Materials and Methods

Generation of Cartilage-Specific PPARγ KO Mice

Genetically modified mice harboring a cartilage-specific deletion of PPARγ were generated using the Cre-lox method in which mice carrying Cre recombinase under the control of the collagen type II promoter were used to induce specific recombination in chondrocytes as previously established.[24] Briefly, mice containing a PPARγ gene flanked by loxP sites (C57BL/6-PPARγ$^{fl/fl}$; The Jackson Laboratory, Bar Harbor, ME) were mated with C57BL/6 Col2-Cre transgenic mice to generate mice bearing Col2-Cre and a floxed allele in their germline (genotype: *PPARγ*$^{fl/+}$; *Cre*). These mice were backcrossed to homozygote floxed mice in the following cross: *PPARγ*$^{fl/+}$; *Cre* × *PPARγ*$^{fl/fl}$ to generate mice with both alleles inactivated in chondrocytes (genotype: *PPARγ*$^{fl/fl}$;*Cre*). *PPARγ*$^{fl/fl}$;*Cre* mice are referred to as homozygote PPARγ KO mice, *PPARγ*$^{fl/+}$;*Cre* mice are referred to as heterozygote PPARγ KO mice, and *PPARγ*$^{fl/fl}$ mice without Cre transgene are referred to as control mice. All procedures involving animals were performed according to the approved protocol by the Comité Institutionnel de protection des animaux (Institutional Animal Protection Committee) of the University of Montreal Hospital Research Centre, Montreal, QC, Canada. All the mice were kept in a 12-hour light/dark cycle. Food and water were available ad libitum.

Histomorphometric Assessment of OA and Immunohistochemical Studies

Freshly dissected mouse knee joints from 6- and 14-month-old mice were fixed overnight in TissuFix (Chaptec, Montreal, QC, Canada), decalcified for 1.5 hours in RDO rapid decalcifier (Apex Engineering Products Corp., Plainfield, IL), and further fixed in TissuFix overnight, followed by embedding in paraffin and sectioning, which was performed at the Centre for Bone and Periodontal Research at McGill University (Montreal, QC, Canada). Sections (5 μm) were deparaffinized in xylene, followed by a graded series of alcohol washes.

Sections were stained with safranin O/fast green (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's recommendations. Slides were evaluated by two independent readers in a blinded manner. To determine the extent of cartilage deterioration, the medial tibial plateau and medial femoral condyle were histologically scored using the method issued by the Osteoarthritis Research Society International as we have previously described.[25] To evaluate the degree of inflammation in the synovium, sections were stained with H&E (Sigma-Aldrich) according to the manufacturer's recommendations. Stained sections were blindly scored for number of mononuclear cells on a scale from 0 to 3: 0, no mononuclear cells; 1, few mononuclear cells; 2, a moderate number of mononuclear cells; and 3, a high number of mononuclear cells. To evaluate fibrosis in the articular cartilage and in the synovium, sections were stained with Masson's trichrome stain (Sigma-Aldrich) according to the manufacturer's recommendations.

Immunohistochemical (IHC) analysis and immunofluorescence were performed on the sections obtained from 14-month-old mouse knee joints using the following antibodies: a rat anti-mouse macrophage/monocyte monoclonal antibody (MAB1852; Millipore, Billerica, MA) that recognizes an intracellular antigen of mouse macrophages and monocytes, a rabbit polyclonal anti–C-terminal peptide of aggrecan G1 domain (VDIPEN, a gift from Dr. John S. Mort,

Shriners Hospital for Children, Montreal, QC, Canada), a rabbit polyclonal that represents a type II collagen primary cleavage site [COLII-3/4C short peptide (C1-2C), a gift from Dr. Robin Poole, McGill University, Montreal, QC, Canada], a rabbit anti−MMP-13 antibody (Sigma-Aldrich), and a rabbit anti−hypoxia-inducing factor-2 α (HIF-2α; AbCam Inc., Cambridge, MA). IHC analysis was performed using the LSAB+ system horseradish peroxidase kit (Dako, Burlington, ON, Canada) following the manufacturer-recommended protocol as previously reported.[23]

RNA Isolation and Real-Time PCR

Articular cartilage was microdissected from the knee joints of 6-month-old PPARγ-deficient and control mice. Microdissection of articular cartilage was performed under a high-power surgical microscope (Motic SMZ-168; Fisher Scientific Canada, Ottawa, ON, Canada) to carefully dissect only articular cartilage and avoid subchondral bone. Total RNA was then isolated from dissected cartilage using TRIzol (Invitrogen, Burlington, ON, Canada) and RNeasy (Qiagen, Toronto, ON, Canada) reagents according to the manufacturers' recommendations, reverse transcribed, and amplified using the TaqMan Assays-on-Demand (Applied Biosystems, Streetsville, ON, Canada) in a reaction solution containing two unlabeled primers and a 6-carboxyfluorescein−labeled TaqMan MGB probe. Samples were combined with one-step master mix (Eurogentec, San Diego, CA). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems) according to the manufacturer's instructions. The expression values were standardized to values obtained with control glyceraldehyde-3-phosphate dehydrogenase RNA primers using the ΔC_T method. All the primers and probe sets were obtained from Applied Biosystems, and the data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and represent averages and SEM from direct comparison of KO and control littermates. The statistical significance of real-time PCR results was determined by two-way analysis of variance with the Bonferroni posttest using GraphPad Prism, version 3.00 for Windows, software (GraphPad Software Inc., San Diego, CA).

Statistical Analysis

Statistical analysis, unless stated otherwise, was performed using the two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Characterization of Cartilage-Specific PPARγ KO Mice

The generation of cartilage-specific PPARγ conditional KO mice was determined by tail DNA genotyping, which confirmed the presence of the Cre transgene in heterozygote (PPARγ F/W Cre) and homozygote (PPARγ F/F Cre) PPARγ KO mice and its absence in wild-type (control) mice (Figure 1A), as previously reported.[23]

Cartilage-Specific PPARγ KO Mice Develop Spontaneous OA Phenotypic Changes

We examined the effects of cartilage-specific ablation of PPARγ on the integrity of knee joints in 6- and 14-month-old mice (Figure 1B). Safranin O/fast green staining demonstrated that the articular cartilage (medial femoral condyle and medial tibial plateau) of 6-month-old control mouse knee joints was intact, with no signs of cartilage deterioration. Similarly, in 14-month-old control mouse

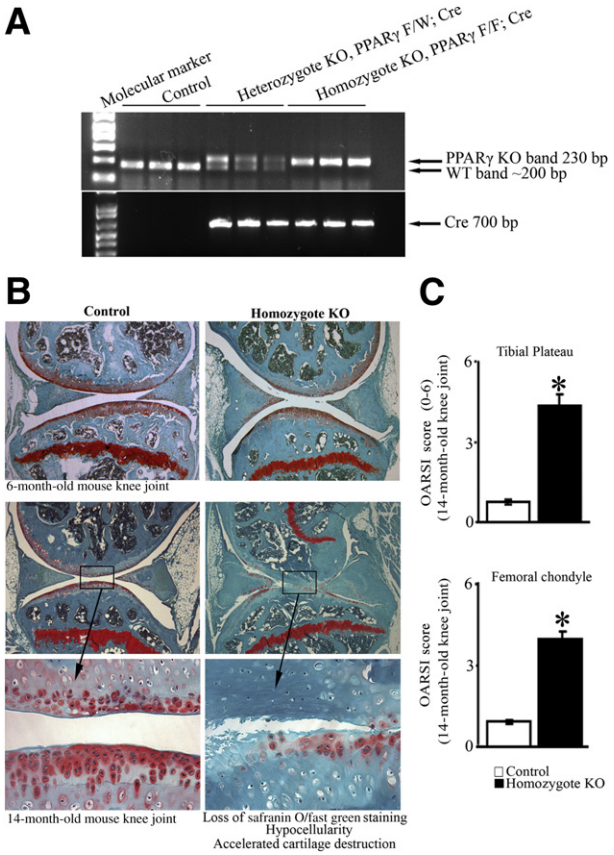

Figure 1 Cartilage-specific deletion of PPARγ results in spontaneous OA-like characteristics during aging. **A:** Genotyping confirms the presence of the Cre transgene in PPARγ heterozygote and homozygote KO mice and its absence in control mice. The PPARγ KO band is detected at 230 bp, the wild-type (WT) band at approximately 200 bp, and the Cre band at 700 bp. **B:** Histologic analysis using safranin O/fast green staining demonstrates that 6- and 14-month-old homozygote PPARγ KO mice exhibit the spontaneous OA phenotype associated with greater cartilage degradation, loss of safranin O staining, and hypocellularity compared with age-matched control littermates. Images show one representative specimen of at least five independent sections per genotype per timepoint. **C:** Osteoarthritis Research Society International (OARSI) scores are significantly higher in the medial tibial plateaus and medial femoral condyles of 14-month-old homozygote PPARγ KO mice compared with age-matched control littermates. Bar graphs show the means ± SEM scores of each group. $*P < 0.05$.

knee joints, no cartilage degradation was observed; however, as expected, some loss of articular chondrocyte cellularity and proteoglycan loss (loss of safranin O/fast green staining) was observed. In 6-month-old homozygote PPARγ KO mice, we observed early signs of cartilage deterioration in terms of roughening of the articular surface, reduced cellularity, and increased proteoglycan loss (loss of safranin O staining). In 14-month-old homozygote PPARγ KO mice, we observed more profound spontaneous OA phenotypic changes, such as accelerated cartilage degradation, loss of chondrocytes, and calcified cartilage fibrillation on ≥50% of the articular surface. The average Osteoarthritis Research Society International score, to account for OA severity,[25] was significantly higher in the medial tibial plateaus and medial femoral condyles of 14-month-old homozygote PPARγ KO mice compared with control mice (Figure 1C). The OA phenotype was observed in both sexes in PPARγ KO mice.

Loss of proteoglycans (loss of safranin O/fast green staining) in control wild-type mice (14 months old) and PPARγ KO mice (6 months old) was mostly observed in the deep zone/calcified cartilage zone rather than in the superficial zone. This is an indication that proteoglycan loss in spontaneous OA models may not necessarily begin from the superficial layer as opposed to injury-induced OA mouse models, such as the destabilization of medial meniscus model, where we usually observe loss of proteoglycans beginning from the superficial layer.[26]

Note that a previous study has shown that C57Bl/6 mice exhibit spontaneous OA characteristics during aging.[27] In this study, lateral and medial compartments of joints were analyzed, and OA lesions were observed mainly on the lateral side of the tibiofemoral joint. In the present study, control wild-type and PPARγ KO mice were also developed on the C57Bl/6 background. Histopathologic scoring, which was performed on the medial compartment, also showed no substantial cartilage degradation apart from some degree of hypocellularity and proteoglycan loss (loss of safranin O/fast green staining) in the articular cartilage of 14-month-old control mice.

Cartilage-Specific PPARγ KO Mice Exhibit Enhanced Synovial Inflammation and Fibrosis

To examine synovial inflammation, we performed H&E staining on 14-month-old mouse knee joints, which revealed an increased amount of mononuclear cell infiltration in homozygote PPARγ KO mice compared with control mice (Figure 2A). Quantification of the degree of inflammation (amount of mononuclear cells) by two blinded observers further showed significantly increased inflammatory scores in homozygote PPARγ KO mice versus control mice (Figure 2B). In addition, immunofluorescence using antibody to detect mouse monocytes/macrophages showed an increased number of monocytes/macrophages in the synovium of homozygote PPARγ KO mice compared with the synovium of control mice (Figure 2C). Fourteen-month-old mouse knee joints were also stained with trichrome stain to assess joint fibrosis. Trichrome staining clearly showed increased synovial fibrosis in homozygote PPARγ KO mice

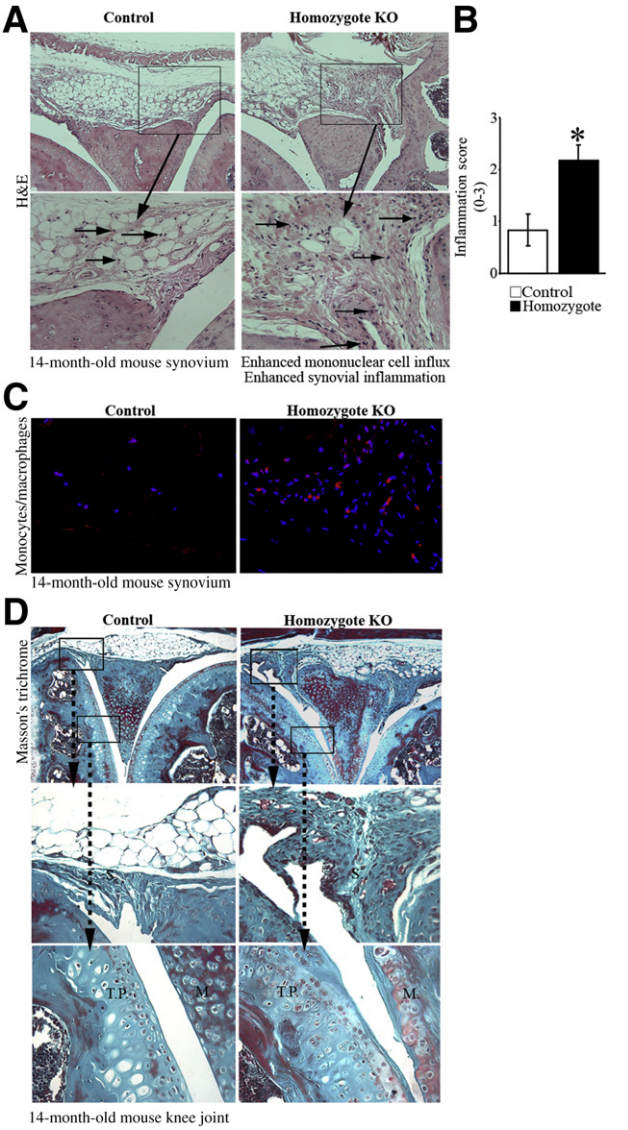

Figure 2 PPARγ-deficient mice exhibit enhanced synovial inflammation and fibrosis. **A:** Histologic analysis using H&E staining demonstrates that knee joint synovium of 14-month-old homozygote PPARγ KO mice exhibits greater influx of mononuclear cells compared with age-matched control littermates. Images show one representative specimen of at least five independent sections per genotype per timepoint. **B:** Synovial inflammation scores are significantly higher in the knee joints of 14-month-old homozygote PPARγ KO mice compared with age-matched control littermates. Bar graphs show the means ± SEM scores of each group. $^*P < 0.05$. **C:** Immunofluorescence using MAB1852 antibody that recognizes an intracellular antigen of mouse macrophages and monocytes shows a greater number of monocytes/macrophages in the synovium of homozygote PPARγ KO mice than in the synovium of control mice. **D:** Trichrome staining showed increased synovial fibrosis in homozygote PPARγ KO mice compared with control mice. Fibrotic deposits were also observed in the superficial zone of the articular cartilage and in some regions of the menisci (meniscal attachments) in homozygote PPARγ KO mice compared with control mice. S, synovium; M, meniscus; TP, tibial plateau. Figures show one representative specimen of at least four independent sections per genotype per timepoint.

compared with the synovium of control mice (Figure 2D). Furthermore, fibrotic deposits were also observed in the superficial zone of the articular cartilage and in some regions of the menisci (meniscal attachments) in homozygote PPARγ KO mice compared with control mice.

PPARγ-Deficient Cartilage Exhibits Increased Expression of Cartilage Degradation Markers

To further assess the extent of cartilage degradation in PPARγ KO mice versus control mice, we performed IHC studies for VDIPEN and C1-2C. IHC studies for VDIPEN, an MMP-generated neoepitope on aggrecan, demonstrated that tibial plateaus of 14-month-old homozygote PPARγ KO mice exhibited enhanced VDIPEN staining compared with control mice (Figure 3A). IHC analysis for the type II collagen breakdown product C1-2C demonstrated that tibial plateaus of 14-month-old homozygote PPARγ KO mice exhibited increased C1-2C staining compared with control mice. Because MMP-13 is one of the major catabolic enzymes associated with the pathogenesis of OA, we performed IHC studies for MMP-13. Tibial plateaus of 14-month-old homozygote PPARγ KO mice exhibited greater MMP-13 expression than those of age-matched control mice.

In addition, we determined the expression of HIF-2α, a recently identified catabolic factor in OA.[28] HIF-2α expression was higher in tibial plateaus of 14-month-old homozygote PPARγ KO mice compared with those of control mice.

PPARγ-Deficient Cartilage Exhibits Increased Expression of Catabolic and Inflammatory Mediators

We next microdissected cartilage from knee joints of 6-month-old control and homozygote PPARγ KO mice and determined the expression of key markers implicated in mediating destructive mechanisms associated with the pathogenesis of OA. By quantitative PCR, we determined the expression of MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) 4, ADAMTS-5, HIF-2α, and syndecan-4. PPARγ-deficient cartilage exhibited significantly increased expression of ADAMTS-5, MMP-13, syndecan-4, and HIF-2α but not ADAMTS-4 compared with cartilage isolated from age-matched control littermates (Figure 3B).

In addition to catabolic markers, we observed significantly elevated expression of key inducible inflammatory enzymes implicated in OA, including cyclooxygenase (COX)-2 and inducible NO synthase, in PPARγ-deficient cartilage compared with control mouse cartilage (Figure 3B).

Discussion

We recently reported that cartilage-specific PPARγ KO mice exhibit cartilage/bone growth and developmental

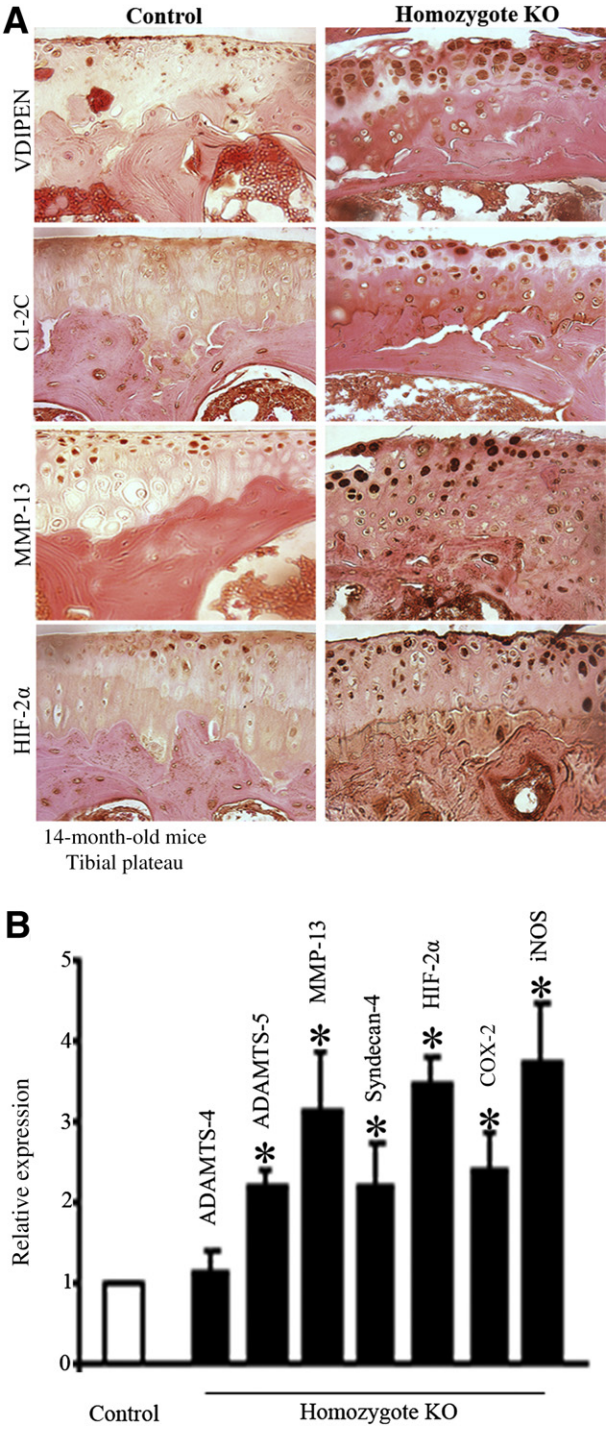

Figure 3 PPARγ-deficient cartilage exhibits greater expression of catabolic and inflammatory mediators. **A**: IHC staining demonstrates that the tibial plateaus of 14-month-old homozygote PPARγ KO mice exhibit enhanced expression of VDIPEN, C1-2C, MMP-13, and HIF-2α compared with control mice. Figures show one representative specimen of at least four independent sections per genotype per timepoint. **B**: Real-time PCR results show that PPARγ-deficient cartilage exhibits greater expression of ADAMTS-5, MMP-13, syndecan-4, HIF-2α, COX-2, and inducible NO synthase (iNOS) but not ADAMTS-4 compared with cartilage isolated from age-matched control mice (set at a value of 1 for each gene). Representative data from at least five independently isolated cartilages per genotype are shown. Bar graphs show the means ± SEM values of each group. $^*P < 0.05$.

defects associated with delayed endochondral ossification process, growth plate abnormalities, and reduced length of long bones.[23] In the present study, we further demonstrate, for the first time, an important *in vivo* role of PPARγ in OA through the use of cartilage-specific PPARγ KO mice. We show that adult cartilage-specific PPARγ KO mice develop a spontaneous OA phenotype associated with enhanced cartilage degradation, synovial inflammation, synovial and cartilage fibrosis, increased expression of MMP-generated neoepitopes (VDIPEN and C1-2C), increased expression of catabolic markers (including MMP-13, ADAMTS-5, HIF-2α, and syndecan-4), and increased expression of inflammatory enzymes (including COX-2 and inducible NO synthase).

Recent studies have shown that HIF-2α expression is markedly increased in human and mouse OA cartilage and that it causes cartilage destruction by regulating crucial catabolic genes, including MMP-1, MMP-3, MMP-9, MMP-12, MMP-13, ADAMTS-4, NO synthase 2, and COX-2.[28] In addition to HIF-2α, other key catabolic factors involved in cartilage degradation are ADAMTS-5 and syndecan-4. Syndecan-4 has been shown to regulate ADAMTS-5 activation and cartilage breakdown in OA.[29] The present study shows that PPARγ-deficient cartilage exhibits elevated expression of HIF-2α, which may, in part, be responsible for the increased expression of MMP-13, inducible NO synthase, and COX-2 observed. In addition, increased expression of syndecan-4 in PPARγ-deficient cartilage could contribute toward the increased expression of ADAMTS-5 observed. The up-regulation of these catabolic mediators in the articular cartilage of PPARγ-deficient mice could be a contributing factor resulting in cartilage destruction and the OA phenotype observed.

Note that PPARγ expression is reduced in human OA cartilage compared with normal cartilage.[30] This finding suggests that reduced PPARγ expression in OA cartilage may reflect increased expression of inflammatory and catabolic factors. Indeed, treatment of human OA chondrocytes with pro-inflammatory factors, including IL-1β, tumor necrosis factor-α, IL-17, and prostaglandin E2, suppresses PPARγ expression.[30] Also, PPARγ agonists can reduce the expression of inflammatory/catabolic mediators and protect cartilage from degradation.[15,16] In normal articular cartilage, there seems to be an optimum equilibrium between catabolic/inflammatory factors and PPARγ. In a catabolic and inflammatory event in the cartilage, PPARγ expression is down-regulated and, as a result, cartilage destruction is promoted. Consequently, increased PPARγ can reduce the expression of critical catabolic/inflammatory mediators, thus preventing cartilage damage.

One can also speculate that early developmental defects observed in PPARγ KO mice[23] could also be a contributing factor toward enhanced cartilage degradation observed during aging. For example, in KO animal models for integrin α1 and Smad3, it has been shown that defects in early cartilage development and endochondral ossification are associated with spontaneous OA during aging.[31,32] It is hypothesized that one of the reasons for spontaneous OA-like characteristics during aging is that defects in early cartilage development cause skeletal malformations that affect joint geometry and biochemical properties predisposing to OA. As a result, cartilage degradation occurs at a higher rate, as does the incidence of OA. Stattin et al[33] supported this finding by demonstrating that patients with familial dysplasia develop joint malformations and, subsequently, secondary OA. The present data clearly demonstrate that disruption of PPARγ function leads to earlier OA. However, at this moment, we cannot determine whether accelerated OA in the present mice is secondary to the observed developmental defects or whether it is caused by the independent roles of PPARγ in articular cartilage. Specific inactivation of the PPARγ gene in adult articular cartilage (using an inducible Cre system) will be required to resolve this question. In addition, future studies should also be directed toward understanding the role of PPARγ in chondrocyte hypertrophy as studies show that chondrocyte hypertrophy can play a vital role in the development of OA.[34]

Apart from cartilage destruction, another key phenotypic characteristic associated with the development of OA is synovial inflammation and fibrosis.[35] In cartilage-specific PPARγ KO mice, we also observed increased synovial inflammation (increased presence of monocytes/macrophages) and increased synovial and cartilage fibrosis. This is probably due to the fact that PPARγ is a key anti-inflammatory and antifibrotic agent. We previously showed that loss of PPARγ in fibroblasts results in excessive skin inflammation and fibrosis in a bleomycin-induced fibrosis model.[7] Similarly, agonists of PPARγ attenuate bleomycin-induced skin inflammation and dermal fibrosis and block the activation of transforming growth factor β/Smad signaling.[8] In addition, agonism of PPARγ has been shown to attenuate cardiac fibrosis by inhibiting myocardial macrophage infiltration.[36] Thus, in the present study, it seems that loss of PPARγ results in not only loss of chondroprotective effects but also loss of anti-inflammatory and antifibrogenic effects, resulting in increased synovial inflammation (accumulation of macrophages) and increased synovial and cartilage fibrosis.

In closing, these results clearly demonstrate, for the first time, to our knowledge, that PPARγ is a critical regulator of cartilage health and integrity. Cartilage-specific PPARγ KO mice, which exhibit a slow-progressing spontaneous form of OA, provide further insights into endogenous mechanisms associated with the pathology of OA. For example, PPARγ KO mice provide evidence that loss of proteoglycans in spontaneous OA as opposed to injury-induced OA can begin from the deep zone of the articular cartilage and not necessarily from the superficial zone. This model also clearly defines synovial and cartilage fibrosis as key spontaneous OA characteristics in addition to cartilage degradation. Furthermore, this study further supports the role of PPARγ as an anticatabolic, anti-inflammatory, and antifibrotic factor.

Acknowledgments

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ARTICLE 2

PPARγ controls mTOR/autophagy signaling in the articular cartilage

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Brief Definitive Report

PPARγ controls mTOR/autophagy signalling in the articular cartilage

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ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis whose exact pathogenesis is unknown. In the present study, we for the first time show that cartilage-specific deletion of peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor, results in aberrant mammalian target of rapamycin (mTOR)/autophagy signalling resulting in accelerated OA in mice model. We created inducible cartilage-specific PPARγ knockout (KO) mice and subjected them to surgery-induced OA. Compared to control mice, PPARγ KO mice exhibit accelerated cartilage destruction, chondrocyte apoptosis, synovial fibrosis and overproduction of OA inflammatory/catabolic factors. PPARγ deficiency also resulted in increased expression of mTOR and suppression of key autophagy inducing genes. *In vitro* rescue experiments using PPARγ expression vector reduced mTOR expression, increased expression of autophagy genes and reduced the expression of OA inflammatory/catabolic factors, thus reversing the phenotype of PPARγ KO mice chondrocytes. To validate our *in vitro* findings *in vivo*, we created cartilage-specific PPARγ-mTOR double KO mice. Loss of mTOR in PPARγ KO mice resulted in increased autophagy signalling and significant protection from OA in mice. This study clearly shows that PPARγ directly regulates mTOR/autophagy pathway in the articular cartilage and loss of PPARγ results in aberrant mTOR/autophagy signalling resulting in accelerated OA.

INTRODUCTION

Osteoarthritis (OA) is among the most prevalent chronic human health disorders and the most common form of arthritis. Typical characteristics of OA include cartilage deterioration/damage, inflammation, synovial fibrosis, subchondral bone remodelling and osteophyte formation (Blaney Davidson et al., 2006; Kuettner and Cole, 2005; Monemdjou et al., 2010a; Monemdjou et al., 2010b). Chondrocytes are the only cell types present in the adult articular cartilage and are essential for maintaining homeostasis as well as integrity of the extra cellular matrix (ECM) within the articular cartilage. During OA initiation and progression, chondrocytes can be stimulated by catabolic cytokines (interleukin-1β [IL-1β] and tumor necrosis factor-α [TNF-α]) and pro-inflammatory enzymes (such as cyclooxygenase-2 [COX-2], inducible nitric oxide synthase [iNOS] and microsomal prostaglandin E synthase-1 [mPGES-1]) to stimulate cartilage degradative molecules such as matrix metalloproteinases (MMPs) and A Disintegrin-Like and Metalloproteinase with Thrombospondin motif (ADAMTS) which degrade the ECM components (aggrecan and collagen) resulting in enhanced articular chondrocyte cell death and cartilage deterioration/damage (Kapoor et al., 2011). The mechanisms leading to accelerated chondrocyte cell death and subsequent cartilage degeneration during OA are not well characterized. Studies suggest that the process of autophagy, a form of programmed cell survival (Levine and Klionsky, 2004), is impaired during OA and may contribute to decreased chondroprotection and degradation of the articular cartilage (Carames et al., 2010) (Carames et al., 2012b) (Carames et al., 2012a). Since mammalian target of rapamycin (mTOR) is a major negative regulator of autophagy; studies have also shown that treatment with rapamycin (mTOR Complex I Inhibitor) upregulates autophagy and reduces the severity of experimental OA in mice (Carames et al., 2012a). Thus identifying endogenous mediators that control mTOR/autophagy signalling and ultimately chondrocyte cell death/survival mechanisms could lead to several promising OA therapeutic strategies.

Peroxisome proliferator activated receptor gamma (PPARγ) is a ligand-activated transcription factor, originally identified to play a key role in lipid homeostasis. We and others have shown that PPARγ possesses potent anti-inflammatory, anti-catabolic and anti-fibrotic properties and is a potential therapeutic target for OA disease (Afif et al., 2007; Boileau et al., 2007; Kapoor et al., 2006; Kapoor et al., 2007; Kapoor et al., 2009; Kobayashi et al., 2005; Monemdjou et al., 2012; Vasheghani et al., 2013). Since global PPARγ knockout (KO) mice are not viable (Barak et al.,

1999) and cartilage-specific PPARγ germ-line KO mice exhibit serious growth and developmental defects (Monemdjou et al., 2012) (Vasheghani et al., 2013), we created inducible cartilage-specific PPARγ KO mice using Col2-Cre-doxycycline system to bypass the early developmental defects and to specifically elucidate the *in vivo* role of PPARγ in OA pathophysiology.

This study first explored the role of PPARγ in chondro-protection by determining the effect of PPARγ deletion on mTOR and autophagy signalling pathway and its subsequent effect on the kinetics of OA progression and severity using mice model of OA. In addition to cartilage-specific PPARγ KO mice, we also generated inducible cartilage specific PPARγ-mTOR double KO mice to specifically dissect the *in vivo* role of mTOR/autophagy pathway in PPARγ signalling during OA. This study is the first to show that PPARγ controls mTOR/autophagy signalling in the articular cartilage and loss of PPARγ results in aberrant mTOR/autophagy signalling resulting in accelerated degradation of the articular cartilage.

RESULTS & DISSCUTION

To determine the specific *in vivo* role of PPARγ in OA pathophysiology, we first generated inducible cartilage-specific PPARγ KO mice using LoxP/Cre system since global PPARγ KO mice are not viable (Barak et al., 1999) and germ-line cartilage-specific PPARγ KO mice exhibit developmental defects (Monemdjou et al., 2012) (Vasheghani et al., 2013). $PPAR\gamma^{fl/fl}$ mice were mated with Col2-rt-TA-Cre (Cre is under the control of doxycycline) transgenic mice (Grover and Roughley, 2006) and the presence of Cre transgene in $PPAR\gamma^{fl/fl}$ mice was confirmed by genotyping (Monemdjou et al., 2012) (Figure 1A). 6 weeks old $PPAR\gamma^{fl/fl}$ Cre mice were fed doxycycline (or saline for controls) for 1 week by oral gavage and loss of PPARγ expression in the articular cartilage chondrocytes isolated from $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre (with doxycycline treatment) was confirmed by qPCR and western blotting (Figure 1B and C). Further, immunohistochemistry using specific antibody for PPARγ showed loss of PPARγ expression in the articular cartilage of doxycycline-treated $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre mice compared to saline-treated $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre mice, confirming the generation of inducible cartilage-specific PPARγ KO mice (Figure 1 D). Doxycycline-treated $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre mice are referred as PPARγ KO mice and saline-treated $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre mice are referred as control mice throughout the manuscript. Assessment of articular cartilage by histology at 10 weeks post birth

and measurements of weight and size showed no significant phenotypic differences between control and PPARγ KO mice. We then subjected 10 weeks old male control and PPARγ KO mice to destabilization of medial meniscus (DMM) model of OA or sham surgery. 10 weeks post OA or sham surgery, mice knee joints were extracted. Safranin-O/Fast Green staining in conjunction with the OARSI scoring on the medial tibial plateau and femoral chondyle of non-surgery and sham-surgery mice showed no signs of cartilage deterioration in both PPARγ KO mice compared to control mice (Figure 1 E). As expected, histological analysis at 5 weeks post OA surgery, control mice knee joints showed some loss of proteoglycans (loss of safranin O staining), roughening of the articular cartilage and some loss of articular chondrocyte cellularity. However, PPARγ KO mice showed greater loss of proteoglycans, loss of cellularity and destruction in some regions of the articular cartilage at 5 weeks post OA surgery. This phenotype became more profound at 10 weeks post OA surgery where PPARγ KO mice, in comparison to control mice, showed significant and severe destruction of the articular cartilage (in both medial tibial plateau and medial femoral condyle) associated with greater loss of proteoglycans and chondrocyte cellularity. These results were confirmed by significant increase in the OARSI scores (assessed by two blinded observers) in PPARγ KO mice compared to control mice at 5 weeks and 10 weeks post OA surgery in both medial tibial plateau and femoral condyle (Figure 1 F). In addition to cartilage destruction, Masson's Trichrome staining on 10 weeks post-OA surgery mice joints showed increased synovial fibrosis in PPARγ KO mice compared to control mice (Figure 1 G). Quantification of the degree of fibrosis by blinded observers showed significantly increased fibrosis scores in PPARγ KO mice compared to control mice (Figure 1 H).

Loss of chondrocyte cellularity within the articular cartilage is a critical feature of OA pathogenesis (van der Kraan and van den Berg, 2012) resulting in imbalance between catabolic and anabolic processes leading to the destruction of the articular cartilage. Interestingly, our results showed that PPARγ KO mice that exhibit accelerated OA phenotype also exhibit increased articular chondrocyte apoptosis. TUNEL assay performed on 10 weeks post OA mice knee joints showed increased number of apoptotic chondrocytes in the tibial plateau of PPARγ KO mice compared to control mice (Figure 2 A), suggesting a possible relationship between PPARγ and cell death/survival mechanisms.

Recent studies suggest that a form of programmed cell survival/cell adaption process called autophagy is impaired during OA and may contribute towards increased chondrocyte cell death and degradation of the articular cartilage (Carames et al., 2012a; Carames et al., 2010; Sasaki et al., 2012). It has been shown that the expression of the key autophagy genes involved in initiating autophagy process such as UNC-51-like kinase 1 (ULK1) and Light Chain 3B (LC3B) is dysregulated during OA (Carames et al., 2010). mTOR (a serine threonine kinase) is the major negative regulator of autophagy and inhibition of mTOR complex I by rapamycin has been shown to activate autophagy and protect cartilage damage and chondrocyte cell death in mice model of OA (Carames et al., 2012a). Since PPARγ KO mice exhibit accelerated OA and increased chondrocyte apoptosis, we determined the effect of PPARγ on the expression of mTOR and autophagy markers in articular chondrocytes isolated from 5 weeks post OA surgery PPARγ KO and control mice. Interestingly, our results revealed that PPARγ-deficient chondrocytes exhibit enhanced RNA and protein expression of mTOR compared to chondrocytes extracted from control OA mice (Figure 2 B and C). Furthermore, qPCR analysis further showed that PPARγ-deficient OA chondrocytes exhibit lower expression of key autophagy genes including ULK1 (most upstream autophagy inducer)(Inoki et al., 2012), LC3B (an autophagy structural and functional factor), ATG5 (an autophagy regulator)(Kuma et al., 2004), and BNIP3 (an interactor of LC3 in autophagy)(Hanna et al., 2012) compared to chondrocytes extracted from control OA mice (Figure 2D). We further measured the effect of PPARγ deficiency on the expression of collagen type II and aggrecan (two major anabolic components of the ECM of the articular cartilage), as well as the expression level of catabolic factors (MMP13 and ADAMTS5) and inflammatory inducible enzymes (COX-2 and iNOS). The expression level of anabolic markers including type II collagen and aggrecan was significantly reduced, however a significant increase in the expression of catabolic factors (MMP-13 and ADAMTS-5) and inflammatory enzymes (iNOS and COX-2) was observed in PPARγ-deficient chondrocytes compared to control chondrocytes (Figure 2 E).

Since PPARγ deficiency in the chondrocytes resulted in aberrant expression of catabolic, anabolic and inflammatory markers associated with dys-regulated expression of mTOR/autophagy genes, we hypothesized that PPARγ is involved in the regulation of mTOR/autophagy pathway and may in part be responsible for maintaining the balance between catabolic and anabolic processes in the articular cartilage. To test this hypothesis, we transfected

PPARγ-deficient OA chondrocytes with PPARγ expression vector to determine if restoration of PPARγ expression can rescue the phenotype of PPARγ KO OA cells. We observed that restoration of PPARγ expression in KO cells by PPARγ expression vector was able to significantly down-regulate the expression of mTOR and significantly up-regulate the expression of autophagy genes including ULK-1, ATG5, LC3B and BNIP3 in PPARγ KO OA cells (Figure 3 A, B and C). Further, our findings revealed that PPARγ expression vector was able to significantly rescue the expression of collagen type II and aggrecan and significantly down-regulate the expression of critical catabolic markers (MMP13 and ADAMTS-5) and inflammatory markers (iNOS and COX-2) in PPARγ KO OA chondrocytes (Figure 3 D).

These results suggest that PPARγ could be responsible for increased mTOR signalling, resulting in inhibition of critical autophagy genes, ultimately resulting in increased catabolic/inflammatory activity (and reduced anabolic activity) within the articular cartilage leading to severe/acclerated OA. To prove our hypothesis that enhanced mTOR signalling and resultant decrease in autophagy is responsible for accelerated OA phenotype observed in PPARγ KO mice, we generated inducible cartilage-specific PPARγ-mTOR double KO mice. For this, we first created $mTOR^{fl/fl}$Col2-rtTA-Cre mice and mated $PPAR\gamma^{fl/fl}$Col2-rtTA-Cre mice with $mTOR^{fl/fl}$Col2-rtTA-Cre mice. The presence of the Cre transgene in heterozygote (PPARγ-$mTOR^{fl/W}$-Cre) and homozygote (PPARγ-$mTOR^{fl/fl}$Col2-rtTA-Cre) was confirmed by routine genotyping PCR (Figure 4 A). 6 weeks old PPARγ-$mTOR^{fl/fl}$Cre mice were fed doxycycline (or saline for controls) for 1 week by oral gavage and loss of PPARγ and mTOR expression in the articular cartilage chondrocytes isolated from PPARγ-$mTOR^{fl/fl}$Col2-rtTA-Cre (with doxycycline treatment) and PPARγ-$mTOR^{fl/fl}$Col2-rtTA-Cre (with saline treatment) were confirmed by qPCR and western blotting (Figure 4 B and C). PPARγ-$mTOR^{fl/fl}$Col2-rtTA-Cre with doxycycline treatment are referred as PPARγ-mTOR double KO mice and PPARγ-$mTOR^{fl/fl}$Col2-rtTA-Cre with saline treatment are referred as control. These mice were subjected to DMM surgery. Histological analysis using Safranin-O/Fast Green staining in 10 weeks post OA surgery demonstrated that of PPARγ-mTOR double KO mice were significantly protected from DMM-induced OA associated with significant protection from cartilage destruction, proteoglycan loss and loss of chondro-cellularity compared with control mice (Figure 4 D). OARSI assessment further confirmed that the average OARSI score at 10 weeks post OA surgery was significantly lower in both medial tibial plateau and femoral chondyle of PPARγ-mTOR double KO mice

compared to control mice (Figure 4 E). Since mTOR is a major repressor of autophagy, we found that the expression of two critical autophagy markers (ULK1 and LC3B) was significantly elevated in chondrocytes extracted from PPARγ- mTOR double KO mice compared to control mice (Figure 4 F).

Our *in vitro* rescue studies using PPARγ expression vector and *in vivo* studies using PPARγ-mTOR double KO mice clearly show that PPARγ is involved in the regulation of mTOR/autophagy signalling in the articular cartilage. Therefore, deficiency of PPARγ in the articular cartilage upregulates mTOR signalling resulting in the suppression of autophagy and decreased chondroprotection and increased catabolic activity leading to accelerated severe OA.. The relationship between PPARγ and autophagy has been previously reported in breast cancer. Zhou *et al*. showed that PPARγ activation by its ligands induces autophagy in breast cancer (Zhou et al., 2009). They specifically showed that BNIP3 is the major target of PPARγ in autophagy induction. In addition, Carames *et al*. have suggested that dys-regulation of autophagy with a reduction in expression of autophagy key markers including ULK1, Beclin1, and LC3 could lead to increase in apoptosis and chondrocyte death during OA (Carames et al., 2010). It has also been shown that autophagy activation by rapamycin attenuates the severity of OA in animal model of OA (Carames et al., 2012a). Our study for the first time, provides a direct evidence on the role of PPARγ in chondroprotection by modulation of mTOR/autophagy signalling in the articular cartilage. These findings outline PPARγ and its signalling by mTOR/autophagy as a potential therapeutic target for the treatment of OA.

METHODS

Generation of Inducible Cartilage-specific PPARγ KO Mice

Inducible cartilage-specific PPARγ KO mice were generated by mating mice containing a PPARγ gene flanked by LoxP sites [C57BL/6- PPARγfl/fl, Jackson Laboratory] with C57BL/6 Col2-rt-TA-Cre transgenic mice (Grover and Roughley, 2006) (obtained from Dr. Peter Roughley, McGill University, Montreal). 6 weeks old $PPAR\gamma^{fl/fl}$ Cre mice were fed doxycycline (Sigma) dissolved at 10 µg/ml in Phosphate Buffer Saline (PBS), pH 7.4 by oral gavage with the dose of 80µl/g body weight for 7 days. rtTA requires interaction with doxycycline (Sigma-Aldrich Inc., Oakville, ON) to permit interaction with the TetO sequence to drive Cre expression resulting in inactivation of PPARγ floxed alleles to generate a cartilage-specific PPARγ KO

mice. PPARγ$^{fl/fl}$ Cre mice without doxycycline (saline) treatment were used as control mice. Routine genotyping of tail DNA followed by confirmation of loss of PPARγ expression in chondrocytes by RT-PCR, western blotting and immunohistochemistry was performed as described before (Grover and Roughley, 2006) All animals' procedure protocols were approved by the Comité Institutionnel de protection des animaux (Institutional Animal Protection Committee) of the University of Montreal Hospital Research Centre CRCHUM.

Surgically Induced OA Mouse Model

Wild type control mice and PPARγ KO mice were subjected to surgically induced OA by destabilization of the medial meniscus (DMM model) in the right knee of 10 weeks old animals as we have previously described (Valverde-Franco et al., 2012). Briefly, after anesthesia with isoflurance in O2, the cranial attachment of the medial meniscus to the tibial plateau (menisco-tibial ligament) of the right knee was transected. A sham operation, consisting of an arthrotomy without transaction of the cranial menisco-tibial ligament, was also performed.

Histology

Freshly dissected mouse knee joints (10 weeks post OA surgery) were fixed overnight in TissuFix (Chaptec, Montreal, QC, Canada), decalcified for 1.5 hours in RDO Rapid Decalcifier (Apex Engineering, Plainfield, IL, USA), further fixed in TissuFix overnight, followed by embedding in paraffin and sectioning. Sections (5 μm) were deparaffinised in xylene followed by a graded series of alcohol washes. Sections were stained with Safranin-O/Fast Green (Sigma-Aldrich, Oakville, Ontario) according to the manufacturer's recommendations. Slides were evaluated by two independent readers in a blinded fashion. To determine the extent of cartilage deterioration, joint sections were stained with Safranin-O/Fast Green and histological scoring method issued by Osteoarthritis Research Society International (OARSI) was used for analysis as previously described (Glasson et al., 2010). To evaluate the degree of fibrosis in the synovium, 10 weeks post OA knee joint sections were stained with Masson's Trichrome stain. Stained sections were blindly scored for degree of fibrosis on a scale of 0-3 (grade 0 = no fibrosis, 1 = low degree of fibrosis, 2 = moderate fibrosis and 3 = severe fibrosis).

Immunohistochemistry (IHC)

IHC studies were performed using specific antibodies for target genes. For IHC analysis, Dakocytomation (Dako) labelled streptavidin biotin + System-horseradish peroxidase kit was used following manufacturer's recommended protocol as previously described(Vasheghani et al., 2013). 5 μm sections were deparaffinised in xylene followed by a graded series of alcohol washes. Endogenous peroxide was blocked for 5 minutes using 3% H2O2. Nonspecific IgG binding was blocked by incubating sections with BSA (0.1%) in PBS for 1 hour. Sections were then incubated with primary antibody in a humidified chamber and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes followed by streptavidin for 1 hour. The diaminobenzidine tetrahydrochloride chromogen substrate solution was then added until sufficient color developed.

Chondrocyte Primary Cell Culture

Primary chondrocytes were prepared from the articular cartilage of control and PPARγ KO mice (5 weeks post OA surgery) as well as age-matched control and PPARγ KO mice (no surgery) as previously described (Monemdjou et al., 2012). Articular cartilage was dissected, rinsed in phosphate buffered saline (PBS), and incubated at 37 °C for 15 minutes in trypsin-EDTA followed by digestion with 2 mg/ml collagenase P at 37 °C for 2 hours in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 5% CO2. The cell suspension was filtered through a 70-μm cell strainer (Falcon, Fort Worth, Texas), washed, counted and plated. At confluence, the cells were detached and plated for experiments. To retain the phenotype, only first-passage cultured chondrocytes were used throughout the study.

Transient Transfection

Transient transfection experiments were performed using the Transfectine TMLipid Reagent according to the manufactur's recommended protocol (Biorad Inc., Missisuga, Canada). Briefly, chondrocytes were seeded 24 hours prior to transfection at a density of $2x10^5$ cells/well in 6-well plates and transiently transfected with 1 μg of the PPARγ expression vector or pcDNA empty vector as a control in the presence of transfectin. The amount of transfected DNA was kept constant by using the corresponding empty vector. After 5 hours, medium was changed with

DMEM containing 1% FCS and samples were incubated at 37°C incubator containing 5% CO2 for 48 hours. PPARγ expression and pcDNA empty vectors were donated by Dr. R. Evans (The Salk Institute, San Diego, CA). Cells were then harvested for RNA and protein extractions as previously described (Monemdjou et al., 2012).

RNA Isolation & Real-time PCR

Total RNA was isolated from the chondrocytes using TRIzol (Invitrogen, Burlington, Ontario) and RNeasy (QIAGEN, Toronto, Ontario) according to the manufacturers' recommendations, reverse transcribed and amplified using the TaqMan Assays-on-Demand (Applied Biosystems, Streetsville, Ontario) in a reaction solution containing two unlabeled primers and a 6-carboxyfluorescein-labeled TaqMan MGB probe. Samples were combined with One-Step MasterMix (Eurogentec, San Diego, California). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems) according to the manufacturer's instructions. The expression values were standardized to values obtained with control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA primers using the ΔCt method. All experiments were performed in duplicate for each sample, and the primers were designed using Primer3 online software. Data were normalized to GAPDH mRNA levels and represent averages and standard error of the mean (SEM). Statistical significance of qPCR results was determined by two-way analysis of variance with the Bonferroni post-test using GraphPad Prism 3.00 for Windows.

Western Blotting

Cells were lysed in Tris-buffered saline containing 0.1% SDS, and the protein content of the lysates was determined using bicinchoninic acid protein assay reagent (Pierce Rockford) with BSA as the standard. Cell lysates were adjusted to identical equals of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10 mM Tris Buffered Saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with the respective antibodies in TBS-T. After washing the membranes with TBS-T, the membranes were incubated overnight with horseradish peroxidase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse IgG

(1:10,000 dilution in TBS-T containing 5% skim milk) at 4°C. Subsequently by further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad Chemidoc Apparatus.

Generation of Inducible Cartilage-specific PPARγ-mTOR Double KO Mice

Generation of inducible cartilage-specific PPARγ-mTOR double KO mice was performed by using the Cre-Lox methodology. Firstly, $mTOR^{fl/fl}$Col2-rt-TA-Cre mice were generated by mating mice containing a mTOR gene flanked by LoxP sites [C57BL/6-$mTOR^{fl/fl}$, Jackson Laboratory] with C57BL/6 Col2-rt-TA-Cre transgenic mice (Grover and Roughley, 2006) (obtained from Dr. Peter Roughley, McGill University, Montreal). Subsequently, $PPAR\gamma^{fl/fl}$Col2-rt-TA-Cre mice were crossed with $mTOR^{fl/fl}$Col2-rt-TA-Cre to create $PPAR\gamma^{fl/fl}$-$mTOR^{fl/fl}$Col2-rtTA-Cre animals. Next, 6 weeks old $PPAR\gamma^{fl/fl}$-$mTOR^{fl/fl}$Col2-rtTA-Cre mice were fed doxycycline (Sigma) dissolved at 10 µg/ml in PBS by oral gavage with the dose of 80µl/g body weight for 7 days. Routine genotyping of ear punch DNA followed by confirmation of loss of PPARγ and mTOR expression in the chondrocytes by RT-PCR and western blotting was performed (Grover and Roughley, 2006; Monemdjou et al., 2012). All animal procedures were approved by the Comité Institutionnel de protection des animaux (Institutional Animal Protection Committee) of the University of Montreal Hospital Research Centre CRCHUM. PPARγfl/fl-mTORfl/fl-Col2-rtTA-Cre treated with doxycycline are referred as PPARγ-mTOR Double KO Mice whereas PPARγfl/fl-mTORfl/flCol2-rtTA-Cre treated with PBS (control) are refereed as control mice. 10 weeks old mice were subjected to DMM OA surgery and mice were scarified at 10 weeks post-surgery for histo-morphometric evaluation.

Statistical analysis

Statistical analysis unless stated otherwise was performed using the two-tailed Student's t-test. $P<0.05$ was considered statically significant.

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FIGURE LEGENDS

Figure 1. PPARγ KO Mice Exhibit Accelerated OA phenotype: **(A)** Genotyping confirmed the presence of the Cre transgene in heterozygote ($PPAR\gamma^{fl/w}$) and homozygote ($PPAR\gamma^{fl/fl}$) mice and its absence in wild type mice. **(B and C)** qPCR and western blotting analysis of isolated chondrocytes confirmed absence of PPARγ expression in $PPAR\gamma^{fl/fl}$Cre mice treated with doxycycline compared to $PPAR\gamma^{fl/fl}$Cre mice treated with saline. (n=5, *: $p<0.05$). **(D)** Immunohistochemical staining for PPARγ confirmed the absence of PPARγ expression in the articular cartilage of $PPAR\gamma^{fl/fl}$Cre mice treated with doxycycline compared to $PPAR\gamma^{fl/fl}$Cre mice treated with saline (n = 4, Magnification: ×25). **(E)** Histological analysis using safranin O/fast green staining of 5 and 10 weeks post OA surgery knee joint sections demonstrate that PPARγ KO mice exhibit accelerated OA phenotype associated with greater cartilage degradation and loss of safranin O staining compared to control mice (Magnification: ×6.2 and ×25). **(F)** Osteoarthritis Research Society International (OARSI) scores are significantly higher in the medial tibial plateau and medial femoral chondyle of PPARγ KO mice compared to control mice (n=6, *: $p<0.05$). **(G and H)** Masson's trichrome staining of 10 weeks post OA surgery sections showed significantly enhanced synovial fibrosis in PPARγ KO mice compared to control mice (n=5, *: $p<0.05$**,** Magnification: ×10). (Images shown in D, E and G are representative specimen of at least 5 independent sections per genotype per time point).

Figure 2. (A) Dysregulated expression of mTOR and autophagy genes in PPARγ KO mouse subjected to OA surgery: (A) TUNEL assay demonstrated a significant increase in the percentage (%) of apoptotic cells in PPARγ KO mice cartilage (medial tibial plateau) compared to control cartilage at 10 weeks post OA surgery (n=4, *: $p<0.05$). Magnification: ×25. **(B and C)** qPCR and western blotting analysis on isolated chondrocytes (5 weeks post OA surgery) showed significant increase in mTOR mRNA and protein expression in PPARγ KO mice chondrocytes compared to control chondrocytes (n=4, *: $p<0.05$). **(D and E),** qPCR analysis on isolated chondrocytes (5 weeks post OA surgery) showed significant decrease in the expression of autophagy genes (ULK1, ATG5, LC3B and BNIP3), a significant decrease in the expression of ECM anabolic products (collagen type II and aggrecan) and increased expression of catabolic factors (MMP-13 and ADAMTS-5) and inflammatory enzymes (COX-2 and iNOS) in PPARγ KO mice chondrocytes compared to control mice chondrocytes (n=4, *: $p<0.05$).

Figure 3. PPARγ expression vector rescues the aberrant expression of catabolic, anabolic and inflammatory markers, and the dys-regulated expression of mTOR/autophagy genes in PPARγ KO-OA chondrocytes: PPARγ KO chondrocytes (isolated at 5 weeks post OA surgery) were cultured and transfected with PPARγ expression vector or empty vector. Rescue of PPARγ in PPARγ KO chondrocytes by PPARγ expression vector resulted in: **(A and B)** decreased mRNA and protein expression of mTOR, **(C)** increased mRNA expression of autophagy genes including ULK1, ATG5, LC3B and ATG5, **(D)** increase in the expression of ECM anabolic products (collagen type II and aggrecan) and decrease in the expression of catabolic factors (MMP-13 and ADAMTS-5) and inflammatory enzymes (COX-2 and iNOS). (n=4, *: $p<0.05$).

Figure 4. Inducible cartilage-specific PPARγ-mTOR double KO mouse are protected from DMM-induced OA: **(A)** Genotyping confirms the presence of the Cre transgene in heterozygote ($PPAR\gamma^{fl/w}$-$mTOR^{fl/w}$) and homozygote ($PPAR\gamma^{fl/fl}$-$mTOR^{fl/fl}$) mice and its absence in wild type mice. **(B and C)** qPCR and western blotting analysis of isolated chondrocytes confirmed absence of PPARγ and mTOR expression in $PPAR\gamma^{fl/fl}$-$mTOR^{fl/fl}$Cre mice treated with doxycycline compared to $PPAR\gamma^{fl/fl}$-$mTOR^{fl/fl}$Cre mice treated with saline. (n=5, *: $p<0.05$). **(D)** Histological analysis using safranin O/fast green staining of 10 weeks post OA surgery knee joint sections demonstrate that in comparison to control mice, all PPARγ-mTOR double KO mice exhibit significant protection from DMM-induced OA associated with reduced cartilage degradation, proteoglycan loss and reduced loss of articular chondrocyte cellularity (n=5, Magnification: ×6.2). **(E)** A significant reduction in the OARSI scale was observed at both medial tibial plateau and medial femoral chondyle in PPARγ-mTOR double KO mice compared to control mice at 10 weeks post OA surgery. (n=5, *: $p<0.05$). **(F)** A significant increase in the mRNA expression of autophagy genes including ULK1 and LC3B was observed in the chondrocytes isolated from PPARγ-mTOR double KO mice compared to control mice at 5 weeks post OA surgery (n=5, *: $p<0.05$). (Images shown in C and D are representative specimen of at least 5 independent observations per genotype).

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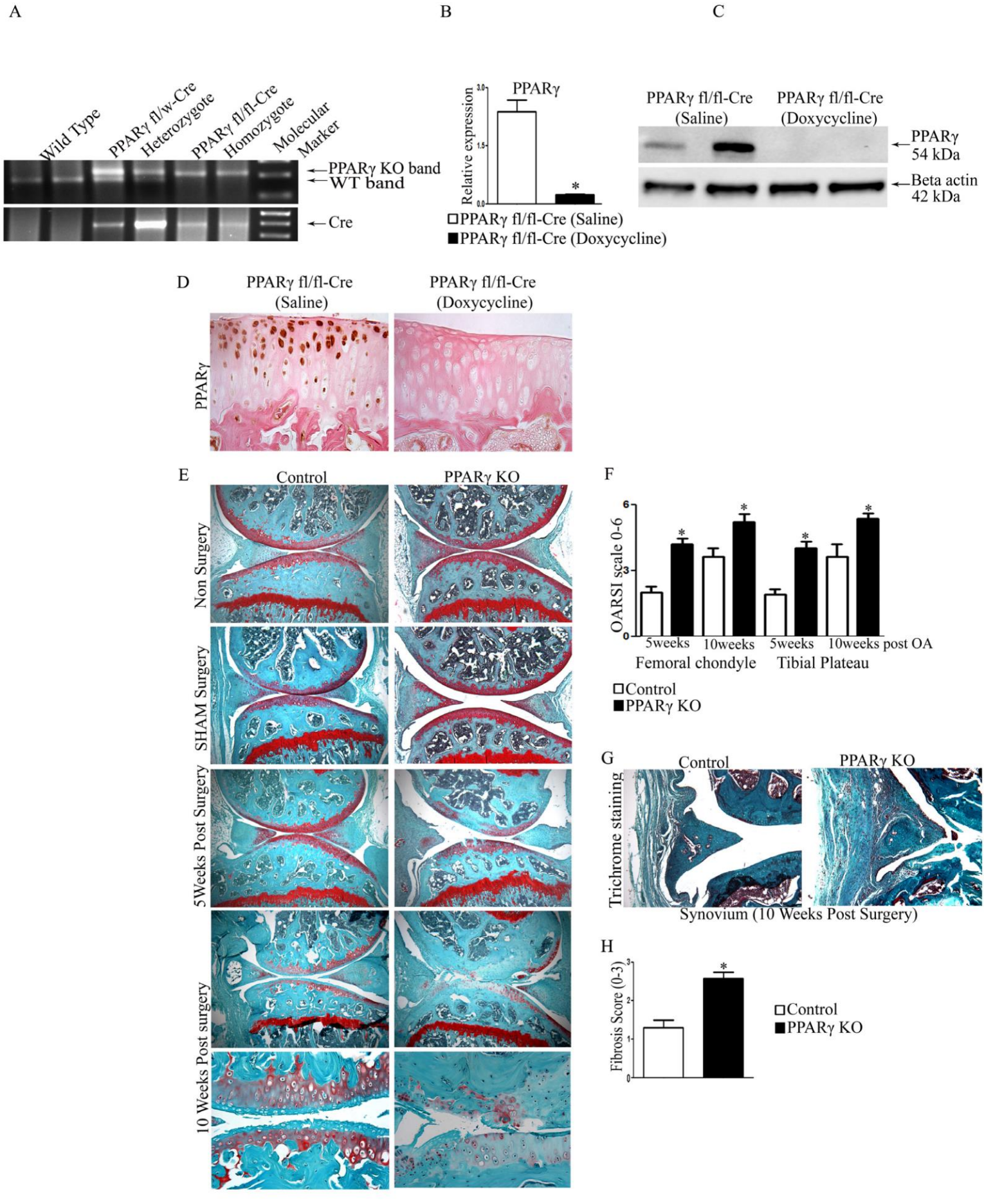
Figure 1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PPARγ fl/fl-Cre Homozygote
Molecular Marker
PPARγ KO band
WT band
Cre
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Relative expression
3.0
1.5
0.0
*
PPARγ fl/fl-Cre (Saline)
PPARγ fl/fl-Cre (Doxycycline)
C
PPARγ fl/fl-Cre (Saline)
PPARγ fl/fl-Cre (Doxycycline)
PPARγ 54 kDa
Beta actin 42 kDa
D
PPARγ fl/fl-Cre (Saline)
PPARγ fl/fl-Cre (Doxycycline)
PPARγ
E
Control
PPARγ KO
Non Surgery
SHAM Surgery
5Weeks Post Surgery
10 Weeks Post surgery
F
OARSI scale 0-6
6
3
0
*
5weeks
10weeks
5weeks
10weeks post OA
Femoral chondyle
Tibial Plateau
Control
PPARγ KO
G
Control
PPARγ KO
Trichrome staining
Synovium (10 Weeks Post Surgery)
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Fibrosis Score (0-3)
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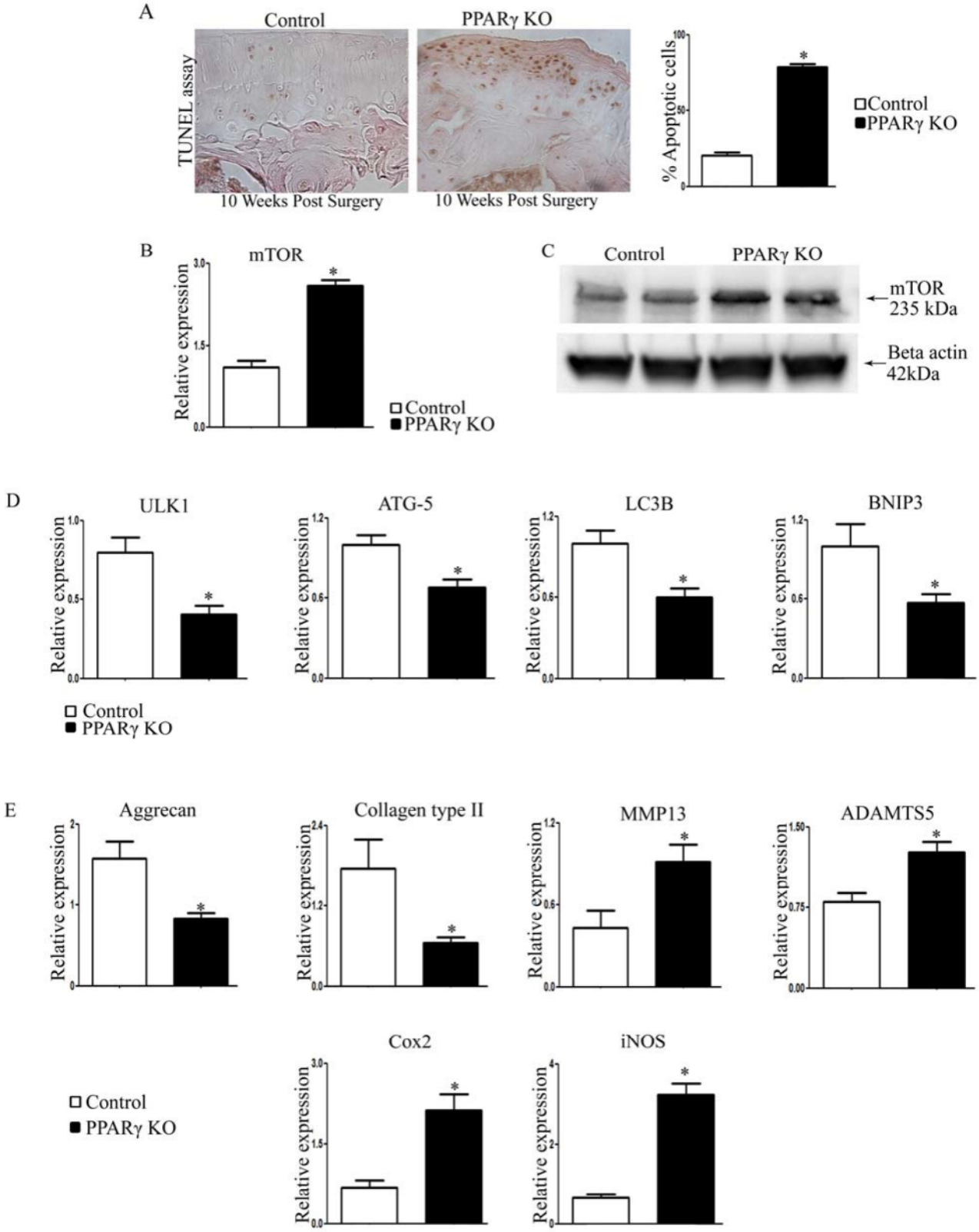
2
1
0
*
Control
PPARγ KO

Figure 2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Control
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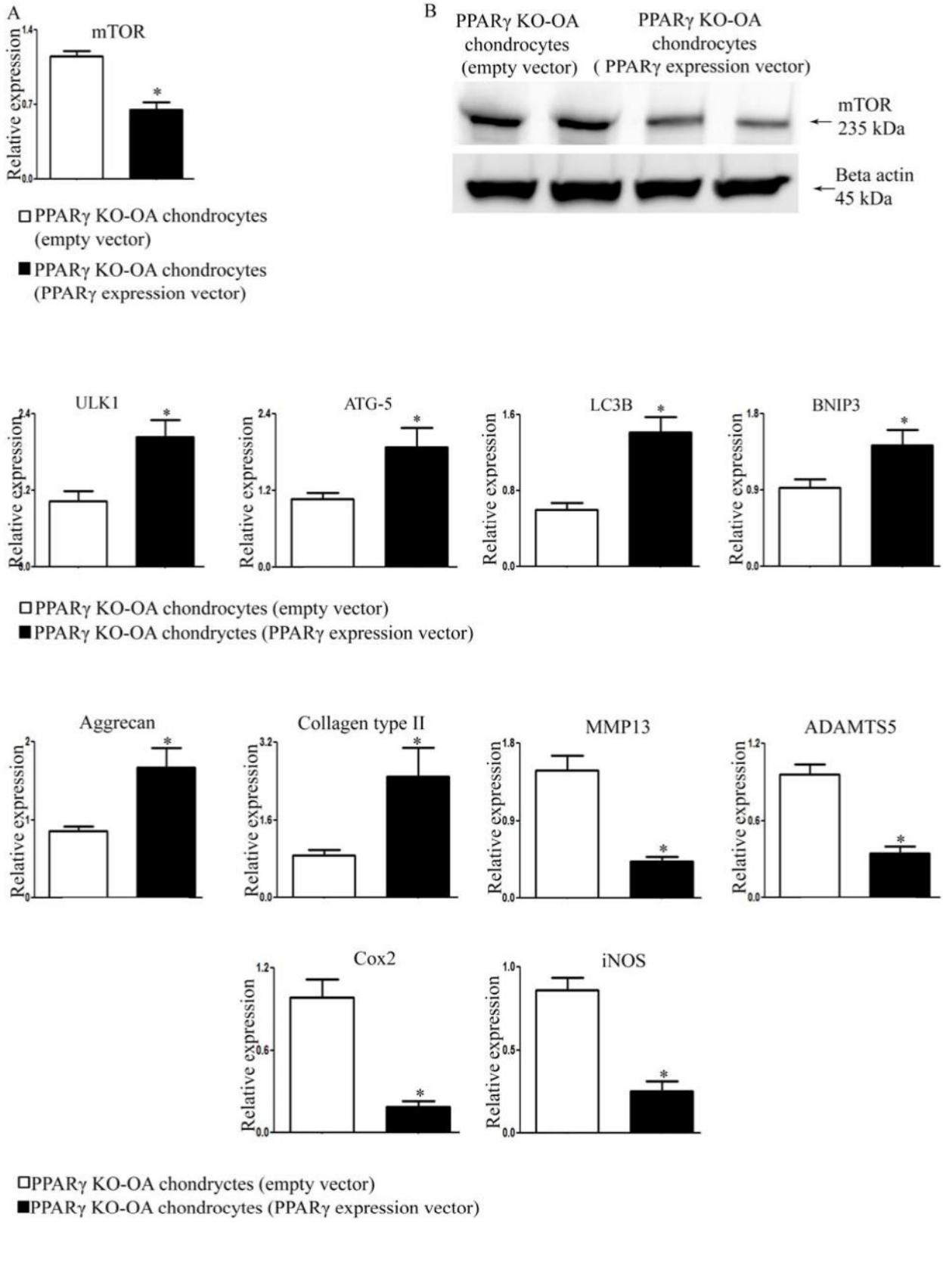
TUNEL assay
10 Weeks Post Surgery
10 Weeks Post Surgery
% Apoptotic cells
Control
PPARγ KO
B
mTOR
Relative expression
Control
PPARγ KO
C
Control
PPARγ KO
mTOR
235 kDa
Beta actin
42kDa
D
ULK1
ATG-5
LC3B
BNIP3
Relative expression
Control
PPARγ KO
E
Aggrecan
Collagen type II
MMP13
ADAMTS5
Cox2
iNOS
Relative expression
Control
PPARγ KO

Figure 3


A
mTOR
Relative expression
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0.7
0.0
*
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■ PPARγ KO-OA chondrocytes (PPARγ expression vector)
B
PPARγ KO-OA chondrocytes (empty vector)
PPARγ KO-OA chondrocytes (PPARγ expression vector)
mTOR 235 kDa
Beta actin 45 kDa
C
ULK1
ATG-5
LC3B
BNIP3
Relative expression
□ PPARγ KO-OA chondrocytes (empty vector)
■ PPARγ KO-OA chondryctes (PPARγ expression vector)
D
Aggrecan
Collagen type II
MMP13
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Relative expression
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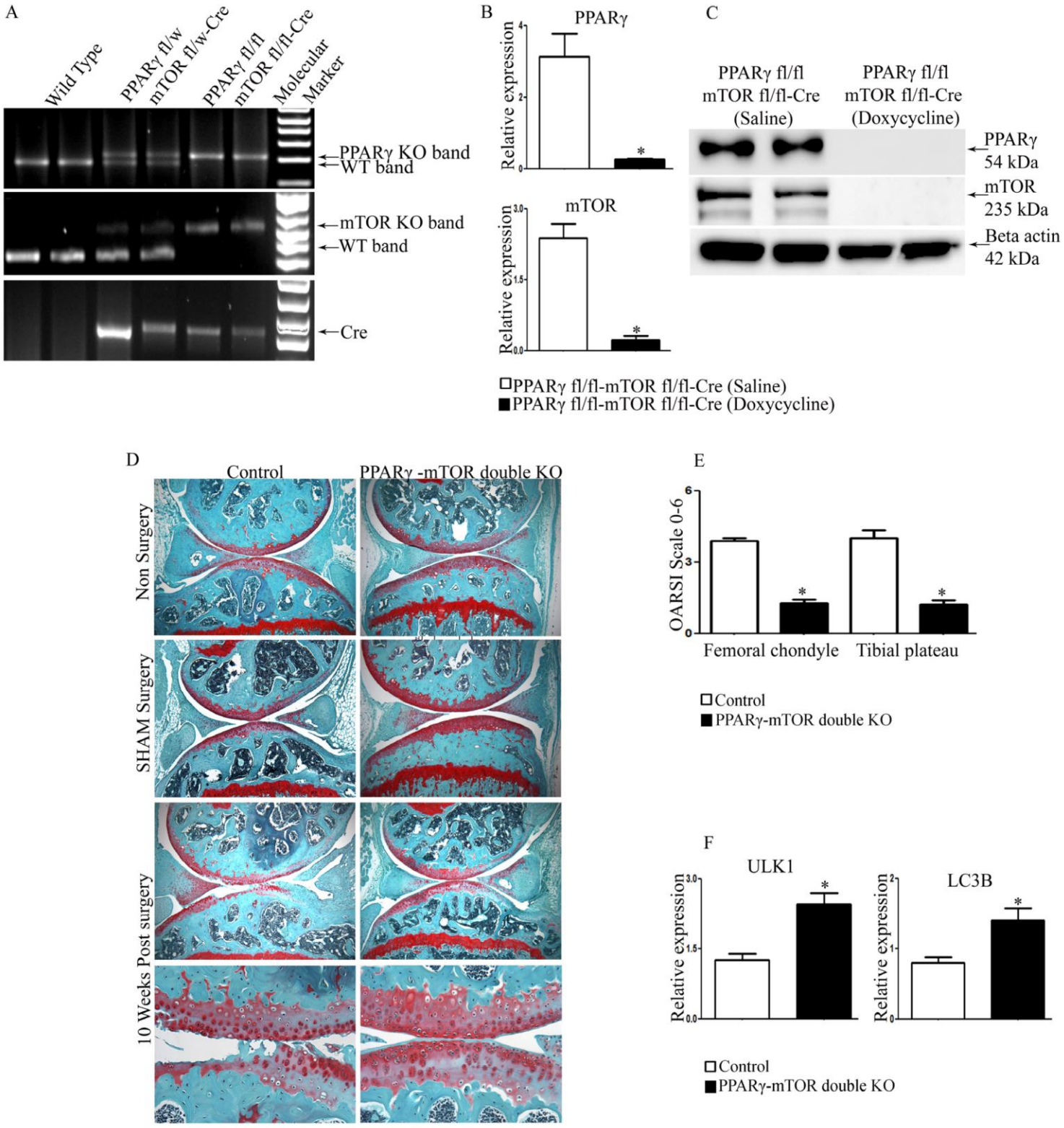
■PPARγ KO-OA chondrocytes (PPARγ expression vector)

Figure 4


A
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PPARγ fl/fl mTOR fl/fl-Cre
Molecular Marker
PPARγ KO band
WT band
mTOR KO band
WT band
Cre
B
PPARγ
Relative expression
mTOR
Relative expression
PPARγ fl/fl-mTOR fl/fl-Cre (Saline)
PPARγ fl/fl-mTOR fl/fl-Cre (Doxycycline)
C
PPARγ fl/fl mTOR fl/fl-Cre (Saline)
PPARγ fl/fl mTOR fl/fl-Cre (Doxycycline)
PPARγ 54 kDa
mTOR 235 kDa
Beta actin 42 kDa
D
Control
PPARγ -mTOR double KO
Non Surgery
SHAM Surgery
10 Weeks Post surgery
E
OARSI Scale 0-6
Femoral chondyle
Tibial plateau
Control
PPARγ-mTOR double KO
F
ULK1
LC3B
Relative expression
Control
PPARγ-mTOR double KO

ARTICLE 3

Association of cartilage-specific deletion of peroxisome proliferator-activated receptor γ with abnormal endochondral ossification and impaired cartilage growth and development in a murine mode

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Association of cartilage-specific deletion of peroxisome proliferator-activated receptor γ with abnormal endochondral ossification and impaired cartilage growth and development in a murine model

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Abstract

Objective—Long bones develop through the strictly regulated process of endochondral ossification within the growth plate, resulting in the replacement of cartilage by bone. Defects in this process result in skeletal abnormalities and can predispose to disease such as osteoarthritis (OA). Studies suggest that activation of the transcription factor peroxisome proliferator activated receptor gamma (PPARγ) is a therapeutic target for OA. In order to devise PPARγ-related therapies in OA and related diseases, it is critical to identify its role in cartilage biology. Therefore, we determined the *in vivo* role of PPARγ in endochondral ossification and cartilage development using cartilage-specific PPARγ knockout (KO) mice.

Methods—Cartilage-specific PPARγ KO mice were generated using LoxP/Cre system. Histomorphometric and immunohistochemical analysis was performed to account for ossification patterns, chondrocyte proliferation, differentiation, hypertrophy, skeletal organization, bone density and calcium deposition. Real-Time PCR and western blotting was performed to determine the expression of key markers involved in endochondral ossification.

Results—PPARγ KO mice exhibited reduced body length, weight, length of long bones, skeletal growth, cellularity, bone density, calcium deposition and trabecular bone thickness, abnormal growth plate organization, loss of columnar organization, shorter hypertrophic zones, and delayed primary and secondary ossification. Immunohistochemistry for Sox9, BrdU, p57, collagen X and PECAM revealed reduction in chondrocyte differentiation and proliferation, and hypertrophy and vascularisation in growth plates of mutant mice. Isolated chondrocytes and cartilage explants from mutant mice showed aberrant expression of ECM markers including aggrecan, collagen II and MMP-13.

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Conflict of Interest: None

Conclusion—PPARγ is required for normal endochondral ossification and cartilage development *in vivo*.

INTRODUCTION

Long bones are formed and lengthened through a process termed endochondral ossification whereby a cartilage anlagen grows through chondrocyte proliferation and hypertrophy [1] and, subsequently, cartilage is replaced by bone [2]. In this process, cartilage provides an intermediate template on which bone is laid down [3]. During endochondral bone growth, chondrocytes are organized into three zones within the epiphyses of the cartilage, namely, resting, proliferating, and hypertrophic zones. Resting and proliferating chondrocytes express high levels of aggrecan and collagen type II, the two main components of the cartilage extracellular matrix (ECM), while hypertrophic chondrocytes express collagen type X. Prior to entering the hypertrophic zone, cells exit the cell cycle and begin to differentiate to hypertrophic chondrocytes [4]. Subsequently, they mineralize their surrounding ECM in the cartilage centre and undergo apoptosis [5, 6]. Blood vessels then invade the hypertrophic cartilage region, bringing in osteoblasts and osteoclasts [6]. Osteoclasts degrade mineralized cartilage while osteoblasts replace it with bone tissue [1, 2, 7]. This mineralization process is termed primary ossification [8]. Secondary ossification centres are formed in the epiphyses post-natally [2].

The normal lengthening of long bones depends on the rate of production of hypertrophic chondrocytes from proliferating chondrocytes, the volume increase in hypertrophic chondrocytes, and the number of proliferative cycles a chondrocyte undergoes [4, 9–12]. Disturbances in the fine balance controlling endochondral bone growth result in growth- and development-related abnormalities such as dwarfism and skeletal deformities. Several other signaling molecules, including Indian hedgehog, fibroblast growth factors (FGFs), Akt, and Wnt/β-catenin have been shown to play roles in chondrogenesis and cartilage growth and development [13–16] (Reviewed in [12]). However, the exact mechanisms through which chondrocyte function and behaviour is controlled during chondrogenesis and cartilage growth and development are largely unknown. A better understanding of chondrogenesis and cartilage growth and development will help to advance our knowledge of the pathophysiology of diseases such as OA.

The transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) belongs to the family of ligand-activated nuclear receptors and plays a key role in lipid and glucose homeostasis [17, 18]. It regulates gene expression by binding as a heterodimer to retinoid X receptor. This heterodimer complex acts as a transcriptional regulator upon binding to sequence-specific PPAR response elements in the promoter region of target genes [17, 18]. Recent studies suggest that PPARγ is involved in the maintenance of bone homeostasis by contributing to osteoclastogenic and osteoblastogenic pathways. Mice lacking PPARγ in osteoclasts develop osteopetrosis, which results from impaired osteoclast differentiation [19]. In addition, *in vitro* studies suggest that PPARγ may play a role in chondrocyte biology [20]. However, the specific *in vivo* role of PPARγ in chondrogenesis and cartilage growth and development is still largely unknown. Therefore, this study examined, for the first time, the specific *in vivo* contribution of PPARγ to chondrogenesis and cartilage growth and development using cartilage-specific PPARγ knockout (KO) mice, as global PPARγ KO mice exhibit embryonic lethality due to placental defects [21].

MATERIALS AND METHODS

Materials

C57BL/6-PPAR$\gamma^{fl/fl}$ mice were obtained from Jackson Laboratory (Bar Harbor, Maine). C57BL/6 Col2-Cre transgenic mice were obtained from Shriners Hospital for Children, Montreal, QC, Canada [22]. The following antibodies were used in this study: platelet/endothelial cell adhesion molecule 1 (PECAM-1) #SC-1506, Sox9 #SC-20095, p57 #SC-8298, PPARγ #SC-7273, goat-anti-rabbit #SC-2004, goat-anti-mouse #SC-2005, rabbit-anti-goat #SC-2768 (Santa Cruz Biotechnology, Santa Cruz, California); collagen type X #C-7974, 5-bromo-2'-deoxyuridine (BrdU) #B-8434, Cre #C-7988, MMP-13 #M-4052 (Sigma-Aldrich, Oakville, Ontario); p38 #9212 and phospho-p38 #9216 (Cell Signaling, Danvers, Massachusetts); DMEM and trypsin/EDTA (Wisent, St-Bruno, Quebec).

Generation of cartilage-specific PPARγ KO mice

Genetically modified mice harbouring a cartilage-specific deletion of PPARγ were generated using the Cre Lox methodology in which mice carrying Cre recombinase under the control of the collagen type II promoter were used to induce specific recombination in chondrocytes as previously established [22]. Briefly, mice containing a PPARγ gene flanked by LoxP sites (C57BL/6-PPAR$\gamma^{fl/fl}$, Jackson Laboratory) were mated with C57BL/6 Col2-Cre transgenic mice [22–24] to generate mice bearing Col2-Cre and a floxed allele in their germline (genotype: PPAR$\gamma^{fl/+}$, Cre). These mice were backcrossed to homozygote floxed mice in the following cross: PPAR$\gamma^{fl/+}$, Cre X PPAR$\gamma^{fl/fl}$ to generate mice with both alleles inactivated in chondrocytes (genotype: PPAR$\gamma^{fl/fl}$, Cre). PPAR$\gamma^{fl/fl}$, Cre mice are referred to as homozygote PPARγ KO mice, PPAR$\gamma^{fl/+}$ Cre mice are referred to as heterozygote PPARγ KO mice, and PPAR$\gamma^{fl/fl}$ mice without Cre transgene are referred to as control mice.

All procedures involving animals were approved by the *Comité institutionnel de protection des animaux of the* CRCHUM, and the Animal Use Subcommittee of the Canadian Council on Animal Care at the University of Western Ontario. All animal studies including housing and breeding were performed as approved by the aforementioned committees. All mice were kept in a 12 hour light/dark cycle. Food and water were available *ad libitum*.

Primary culture of chondrocytes

Primary chondrocytes were prepared from long bones of embryonic day (E) 16.5 control, heterozygote and homozygote PPARγ KO mice as previously described [25]. Cartilage was dissected from long bones, rinsed in phosphate buffered saline (PBS), and incubated at 37 °C for 15 minutes in trypsin-EDTA followed by digestion with 2 mg/ml collagenase P at 37 °C for 2 hours in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 5% CO_2. The cell suspension was filtered through a 70-μm cell strainer (Falcon, Fort Worth, Texas), washed, counted and plated. At confluence, the cells were detached and plated for experiments. To retain the phenotype, only first-passage cultured chondrocytes were used throughout the study.

Western blotting

Cells were lysed in Tris-Buffered Saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined using bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Rockford, Illinois) with bovine serum albumin (BSA) as the standard. Cell lysates were adjusted to equal equivalents of protein and then were applied to SDS-polyacrylamide gels (10%) for electrophoresis. Next, the

proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10mM TBS containing 0.1% Tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with the respective primary antibody (anti-PPARγ; 1:1000 dilution) in TBS-T. After washing the membranes with TBS-T, the membranes were incubated overnight with the appropriate secondary antibody in TBS-T containing 5% skim milk at 4 °C. After further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad (Mississauga, Ontario) Chemidoc Apparatus.

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from chondrocytes or cartilage explants using TRIzol (Invitrogen, Burlington, Ontario) and RNeasy (QIAGEN, Toronto, Ontario) according to the manufacturers' recommendations, reverse transcribed and amplified using the TaqMan Assays-on-Demand (Applied Biosystems, Streetsville, Ontario) in a reaction solution containing two unlabeled primers and a 6-carboxyfluorescein-labeled TaqMan MGB probe [26, 27]. Samples were combined with One-Step MasterMix (Eurogentec, San Diego, California). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems) according to the manufacturer's instructions. The expression values were standardized to values obtained with control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA primers using the ΔCt method. All primers and probe sets were obtained from Applied Biosystems and data were normalized to GAPDH mRNA levels and represent averages and standard error of the mean (SEM) from direct comparison of KO and control littermates. Statistical significance of real time polymerase chain reaction (qPCR) results was determined by two-way analysis of variance with the Bonferroni post-test using GraphPad Prism 3.00 for Windows.

Skeletal staining

Newborn mice were skinned, eviscerated, and dehydrated in 95% ethanol and acetone overnight. Skeletons were stained with 0.015% alcian blue, 0.05% alizarin red, and 5% acetic acid in 70% ethanol for several days. Skeletons were then cleared in 1% KOH, passed through a decreasing KOH series and stored in glycerol/ethanol (1:1) [23, 28].

Histological and immunohistochemistry studies

Freshly dissected mouse long bones were fixed with 10% neutral buffered formalin and decalcified with 0.1 M EDTA at room temperature before paraffin embedding and sectioning at the Centre for Bone and Periodontal Research at McGill University (Montreal, QC, Canada). Sections (5 μm) were deparaffinized in xylene followed by a graded series of alcohol washes. Sections were stained with Safranin-O/Fast Green (Sigma-Aldrich, Oakville, Ontario) according to the manufacturer's recommendations. For immunohistochemistry (IHC) analysis, the Dakocytomation (Dako, Burlington, Ontario) labeled streptavidin biotin + System-horseradish peroxidase kit was used following the manufacturer's recommended protocol. Briefly, endogenous peroxide was blocked for 5 minutes using 3% H_2O_2. Nonspecific immunoglobulin G binding was blocked by incubating sections with BSA (0.1%) in PBS for 1 hour. Sections were then incubated with the primary antibody in a humidified chamber and left overnight at 4 °C. Next, sections were incubated with biotinylated link for 30 minutes followed by streptavidin for 1 hour. The diaminobenzidine tetrahydrochloride chromogen substrate solution was then added until sufficient color developed.

BrdU labeling

For BrdU labeling, pregnant female mice were injected one day before being sacrificed i.p. with BrdU at a dose of 0.01 ml/g. BrdU was detected in paraffin sections using an anti-BrdU antibody through IHC as detailed above.

Bone mineralization

Freshly dissected mouse long bones were fixed with 10% neutral buffered formalin before plastic embedding, sectioning, and staining at the Centre for Bone and Periodontal Research at McGill University (Montreal, QC, Canada). Sections (5 μm) were stained with von Kossa to determine calcium deposition in the bone, Goldner to determine bone density, and Safranin-O/Fast Green to determine trabecular bone thickness. Bone density and trabecular bone thickness were quantified using Bioquant Osteo II software as previously described [29].

Cartilage explant studies

Cartilage explant studies were conducted using femoral head cartilage from 3-week-old control and homozygote PPARγ KO mice. Cartilage was extracted, rinsed in PBS and RNA was isolated and subjected to qPCR using specific primers as described above.

Statistical analysis

Statistical analysis was evaluated by the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of cartilage-specific PPARγ KO mice

Due to the fact that global PPARγ KO mice die as a result of embryonic lethality, the LoxP/Cre system was used to generate cartilage-specific PPARγ conditional KO mice. Mice containing a PPARγ gene flanked by LoxP sites ($PPAR\gamma^{fl/fl}$) were mated with mice carrying Cre recombinase under the control of the collagen type II promoter to induce specific recombination in chondrocytes [22]. Generation of conditional KO mice was first determined by tail DNA genotyping, which confirmed the presence of the Cre transgene in heterozygote ($PPAR\gamma^{fl/w}$ Cre) and homozygote ($PPAR\gamma^{f/f}$ Cre) PPARγ KO mice and its absence in WT (wild-type) (control) mice (Figure 1A). Loss of PPARγ expression in chondrocytes isolated from homozygote and heterozygote PPARγ KO versus control mice was confirmed by Western blotting (Figure 1B) and qPCR (Figure 1C). Additionally, IHC studies performed on femurs of post-natal day (P) 0 control mice demonstrated strong expression of PPARγ in resting and hypertrophic chondrocytes of the growth plate. However, PPARγ positive chondrocytes were undetectable in the resting and hypertrophic zones of the growth plate of homozygote PPARγ KO mice, thus confirming that recombination occurred with high efficiency (Figure 1D).

Cartilage-specific deletion of PPARγ results in reduced growth

We first determined the effect of cartilage-specific ablation of PPARγ on body length, body weight, and skeletal growth of mice. Analyses of newborn litters demonstrated growth retardation in homozygote PPARγ KO mice compared to control mice (Figure 2A). Whole-mount skeletal staining with alcian blue and alizarin red confirmed that newborn homozygote PPARγ KO mice show reduced skeletal growth compared to control mice and skeletal staining in some regions of the limbs in mutant mice was weaker compared to control mice (Figure 2B). Measurements of growth over 42 days post-birth demonstrated that homozygote PPARγ KO mice had significantly reduced body length (Figure 2C) and

weight (Figure 2D) compared to control mice. Measurements of individual bones demonstrated that homozygote PPARγ KO mice had significantly reduced length of tibiae and femurs at time of birth (Figure 2E). No significant differences in the viability of mice were observed. A consistent pattern of growth and weight retardation was observed in both genders.

PPARγ-deficient mice show delayed ossification and disorganization of growth plates

We further examined the effect of PPARγ deficiency on the organization of growth plates and ossification patterns. Histological analysis by Safranin-O/Fast Green staining demonstrated that femurs of E16.5 homozygote PPARγ KO mice exhibit reduced length and delayed primary ossification compared to control mice (Figure 3A). In addition, femurs of P14 homozygote PPARγ KO mice exhibited delayed secondary ossification compared to control mice (Figure 3B).

Blinded histological analyses were also performed to examine growth plate organization on a cellular level at time of birth. Safranin-O/Fast Green staining of P0 mouse femurs of homozygote PPARγ KO mice demonstrated growth plate defects, which is associated with hypocellularity in all three zones, loss of columnar organization in the proliferating zone, altered chondrocyte shape in the hypertrophic zone, and a shortened hypertrophic zone compared to control mice (Figure 3C,D).

Cartilage-specific deletion of PPARγ results in reduced chondrocyte proliferation, differentiation, hypertrophy and vascular invasion

Subsequently, we determined if PPARγ deficiency altered chondrocyte differentiation, proliferation, hypertrophy and vascular invasion. IHC using antibodies against Sox9 (Figure 4A), a marker of early chondrocyte differentiation, BrdU (Figure 4B), a marker of proliferation, and p57 (Figure 4C), a cell cycle inhibitor required for normal hypertrophic differentiation, revealed that femurs of E16.5 homozygote PPARγ KO mice exhibit reduced chondrocyte differentiation and proliferation as demonstrated by a reduced percentage of positive cells compared to control mice. IHC using collagen type X, a marker of chondrocyte hypertrophy, demonstrated that the femurs of E16.5 homozygote PPARγ KO mice exhibit reduced chondrocyte hypertrophy compared to control mice (Figure 4D). IHC for PECAM, a cell surface marker for endothelial cells and a marker of new blood vessel formation, further demonstrated reduced vascular invasion in the femurs of E16.5 homozygote PPARγ KO mice compared to control mice (Figure 4E).

Cartilage-specific deletion of PPARγ causes reduced bone density

We further investigated whether the loss of PPARγ resulted in abnormal calcium deposition and bone density. Two bone mineralization staining methods, von Kossa (Figure 5A) and Goldner (Figure 5B), in combination with measurements to quantify bone density (Figure 5C) using Bioquant Osteo II software, demonstrated that P0 homozygote PPARγ KO mouse femurs exhibit reduced calcium deposition and bone density, respectively, compared to control mice. Measurements using Bioquant Osteo II software to quantify trabecular bone thickness in P40 and P70 mice further demonstrated that homozygote PPARγ KO mice display significantly reduced trabecular bone thickness compared to control mice (Figure 5D).

PPARγ-deficiency results in reduced expression of aggrecan and collagen type II, and increased expression of MMP-13

We next isolated chondrocytes from E16.5 control and homozygote PPARγ KO mice (Figure 6A), and femur head cartilage explants from 3-week-old control and homozygote

PPARγ KO mice (Figure 6B) and examined the expression of the ECM proteins, aggrecan and collagen type II, and the catabolic factor MMP-13. Our analyses indicated that homozygote PPARγ KO mouse chondrocytes and cartilage explants exhibit significantly reduced expression of aggrecan and collagen type II, and significantly increased expression of MMP-13 compared to control mouse chondrocytes and cartilage explants.

DISCUSSION

Our results, for the first time, demonstrate an important *in vivo* role of PPARγ in endochondral ossification and cartilage growth and development through the use of cartilage-specific PPARγ KO mice. We show that genetic ablation of PPARγ in cartilage *in vivo* results in marked alterations in the process of endochondral ossification, and cartilage and long bone development, with specific alterations at the tissue, cellular, and molecular levels. PPARγ deficiency caused reduced growth and skeletal size, shorter length of long bones, delayed primary and secondary ossification, disorganization of growth plates accompanied by hypocellularity, reduced chondrocyte proliferation, differentiation and hypertrophy, delayed vascular invasion, decreased bone density, calcium deposition and trabecular bone thickness, and aberrant expression of key markers involved in skeletogenesis (Sox9, p57, and collagen type X) as well as ECM synthesis (aggrecan and collagen type II) and degradation (MMP-13) products. Collectively, our results demonstrate that PPARγ plays a pivotal role in coordinating diverse aspects of skeletal morphogenesis.

As demonstrated by skeletal staining and measurements, cartilage-specific deletion of PPARγ resulted in abnormal endochondral bone growth, including reduced body size in terms of height and weight as well as reduced skeletal size and length of long bones. Histological analyses further revealed that PPARγ deficiency caused delayed primary and secondary ossification and disorganization of the growth plates. All three zones of the cartilage epiphyses of mutant mice showed signs of highly disorganized growth plates, including hypocellularity and reduced chondrocyte proliferation, differentiation and hypertrophy. Since the normal lengthening of long bones depends on the rate of production of hypertrophic chondrocytes from proliferating chondrocytes, the increased volume of hypertrophic chondrocytes, and the number of proliferative cycles a chondrocyte undergoes [9–12], improper coordination of these variables results in abnormal bone length by hindering mineralization, apoptosis, vascular invasion, and thus, overall endochondral bone development.

Some of the key essential factors required for normal ossification, ECM production, and vascularization processes during early development of cartilage and bone include Sox9, p57, aggrecan, collagen type II, and collagen type X. During chondrogenesis, chondrocytes begin expressing Sox9, a chondrogenic transcription factor that is essential for proliferation and differentiation of chondrocytes. Within the growth plate, Sox9 is expressed in resting and proliferating chondrocytes and regulates the expression of genes encoding aggrecan and collagen type II, the two main components of the cartilage ECM [30–32]. p57 is a cell cycle inhibitor essential for cell cycle exit and onset of hypertrophic differentiation. Collagen type X is a cartilage ECM component unique to the hypertrophic zone and serves as a marker of terminally differentiated hypertrophic chondrocytes [33]. In the final step of endochondral bone development, chondrocytes undergo hypertrophy and produce a calcified, cartilaginous ECM, and angiogenic factors, which initiate and propagate vascular invasion. As a result, avascular cartilage is replaced by densely vascularized bone.

Delayed ossification and growth plate abnormalities in PPARγ mutant mice can be explained by the abnormal expression of Sox9, p57, and collagen type X. Our results show that the growth plates of homozygote PPARγ KO mice exhibit significantly reduced

expression of Sox9 and p57, thus affecting the normal sequence of chondrocyte proliferation, differentiation, and hypertrophy. In addition, the growth plates of homozygote PPARγ KO mice exhibit significantly reduced expression of PECAM, indicating delayed vascularization. Growth plates of homozygote PPARγ KO mice showed reduced expression of collagen type X. Collagen type X facilitates endochondral ossification by regulating matrix mineralization and compartmentalizing matrix components [34]. Mice with altered collagen type X function exhibit skeletal defects including compressed growth plates with reduced proliferative and hypertrophic zones, and trabecular bone thickness [35–37]. Therefore, reduced length of long bones, bone density and trabecular bone thickness may in part be dependent on decreased collagen type X expression upon genetic deletion of PPARγ.

We have previously observed a close relationship between PPARγ and p38 signalling. We have shown that PPARγ expression is suppressed by p38 activity. We examined show that PPARγ deficient chondrocytes (E16.5) show increased phosphorylation of p-38 and reduction in the mRNA expression of Indian Hedgehog (Ihh). Previous study using transgenic mice that specifically expresses a constitutively active mutant of MKK6 in chondrocytes that specifically activates p38 results in mice exhibiting a dwarf phenotype associated with reduction in chondrocyte proliferation, inhibition of hypertrophic chondrocyte differentiation, and a delay in primary and secondary ossification (Zhang et al., 2006 PNAS), a similar phenotype we observe with PPARγ-deficient mice. Ihh is a key regulator of normal endochondral bone and skeletal development (St-Jacques et al., 1999, Genes & Dev). We and others have shown that p38 negatively regulates the expression of Ihh both in vitro (Stanton and Beier, 2007, Exp. Cell Res) and in vivo (Zhang et al., 2006 PNAS). Here we show that PPARγ deficient chondrocytes exhibit decreased expression of Ihh, suggesting that inactivation of PPARγ and subsequent increase in the phosphorylation of p38 may account for repression of Ihh and ultimately contributing towards abnormal endochondral ossification observed in PPARγ-deficient mice.

It should be mentioned that previous studies have shown that PPARγ activation suppresses collagen type X expression and other markers of hypertrophic chondrocyte differentiation [20, 38]. At first glance, this appears to be contradictory to the delay in hypertrophic differentiation upon inactivation of PPARγ that we observed here. However, several possibilities could explain these apparent discrepancies. First, it is possible that there is an optimal level of PPARγ activity to promote hypertrophy and that both overactivation and inactivation suppress this process. Second, it is possible that reduced hypertrophy in these two settings is due to different mechanisms; PPARγ activation might directly suppress hypertrophy, while the smaller hypertrophic zone in cartilage-specific PPARγ KO mice could be secondary to reduced chondrocyte proliferation (e.g. less cells entering the hypertrophic zone). Further studies will be required to decipher the mechanisms involved.

In closing, our results, for the first time, demonstrate that PPARγ is a critical regulator of endochondral cartilage health and physiology in early growth and development. Collectively, our results demonstrate that PPARγ plays a pivotal role in coordinating diverse aspects of skeletal morphogenesis. A better understanding of the role of PPARγ in cartilage biology will enable us to devise appropriate PPARγ related therapeutic strategies against diseases such as OA and related disorders.

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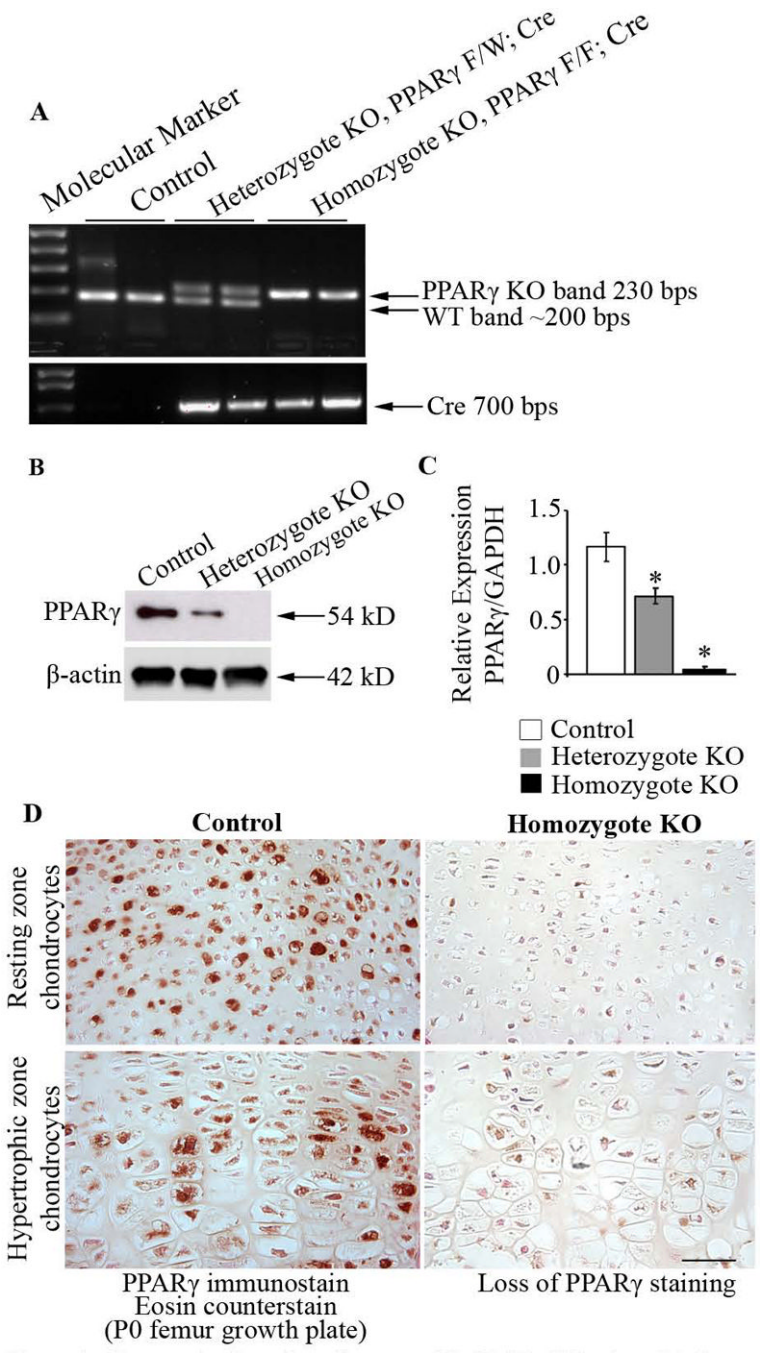

Figure 1.

Characterization of cartilage-specific PPARγ KO mice. (A) Genotyping confirmed presence of Cre transgene in P0 heterozygote and homozygote KO mice and its absence in control mice. PPARγ KO band was detected at 230 bps, WT band was detected at ~200 bps, and Cre band was detected at 700 bps. (B) Western blotting performed on isolated chondrocytes demonstrated reduced expression and complete absence of PPARγ in heterozygote and homozygote PPARγ KO mice, respectively, compared to control mice. (C) RT-PCR on isolated chondrocytes confirmed reduction of PPARγ mRNA levels by >96% in homozygote PPARγ KO mice compared to control mice. Representative data from n=6 independent isolated chondrocytes per group. Bar graph shows mean +/− SEM of each

group. *, P<0.05 (D) IHC studies confirmed the absence of PPARγ expression in resting and hypertrophic chondrocytes of P0 homozygote PPARγ KO mice compared to control mice. n=4 per group (bar, 100 μm). Figures show one representative experiment of at least four independent experiments.

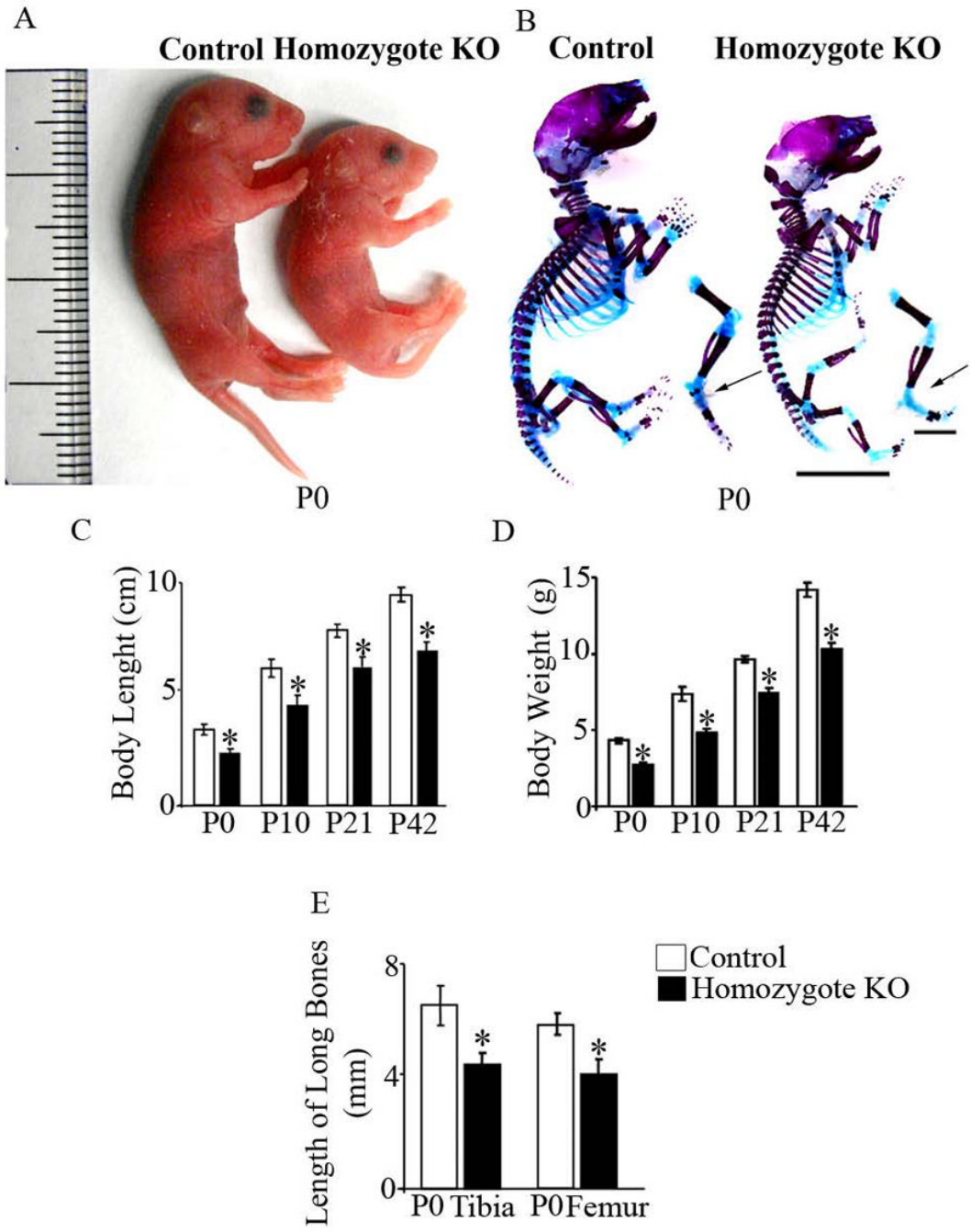

Figure 2.

Cartilage-specific PPARγ KO mice exhibit reduced growth. (A) Newborn homozygote PPARγ KO mice exhibited growth retardation compared to control mice. (B) Skeletal staining demonstrated reduced skeletal size (bar, 10 mm) and length of long bones (bar, 1 mm) in homozygote PPARγ KO mice compared to control mice. Arrows indicate loss of skeletal staining in homozygote PPARγ KO mice. (C) Body length and (D) weight measurements of P0, P10, P21, and P42 mice demonstrated that homozygote PPARγ KO mice exhibit delayed growth compared to control mice. (E) Measurements of P0 mice demonstrated that homozygote PPARγ KO mice have reduced length of tibiae and femurs

compared to control mice. n=6 per group. Figures show one representative experiment of six independent experiments. Bar graph shows mean +/− SEM of each group. *, P<0.05

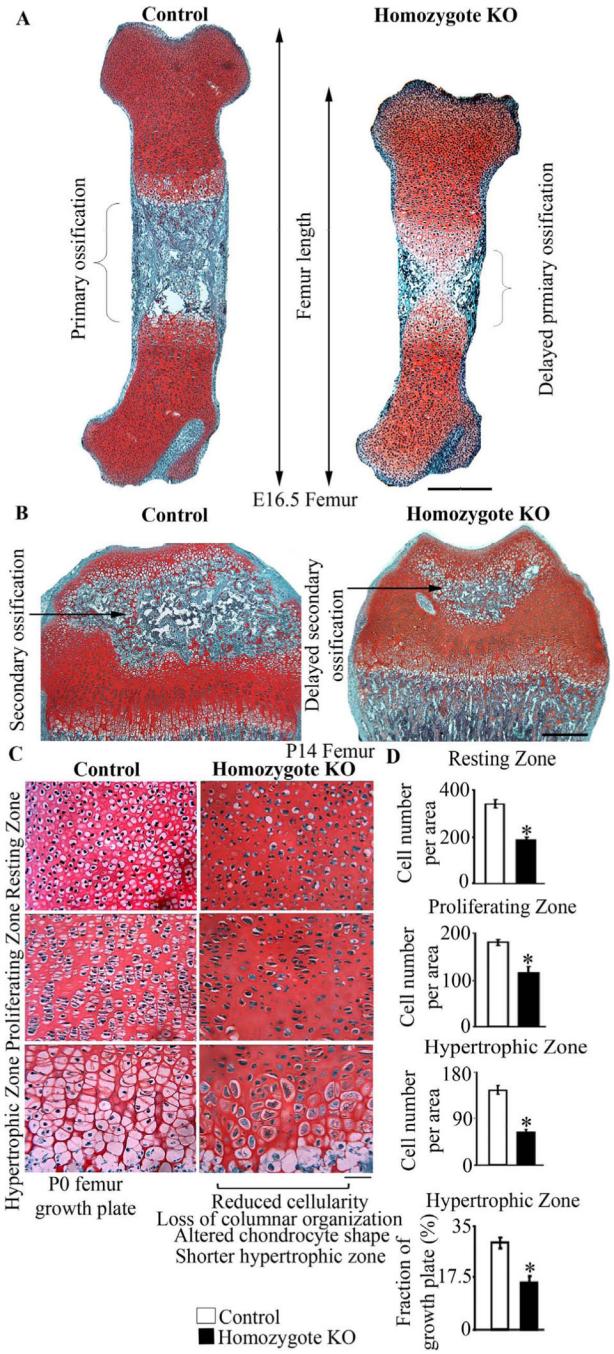

Figure 3.

Cartilage-specific PPARγ KO mice exhibit delayed ossification and disorganization of growth plates. (A) Safranin-O/Fast Green staining demonstrated that E16.5 homozygote PPARγ KO mice exhibit delayed primary ossification compared to control mice. n=6 per group (bar, 500 μm). (B) Safranin-O/Fast Green staining showed delayed secondary ossification in the femurs of P14 homozygote PPARγ KO mice compared to control mice. n=4 per group (bar, 200 μm). (C) Homozygote PPARγ KO growth plates showed disorganization with reduced cellularity in all the three zones loss of columnar organization in the proliferating zone and a shorter hypertrophic zone with altered chondrocyte shape compared to control mice. n=6 per group (bar, 100 μm). (D) Bar graphs show reduced

cellularity in the resting, proliferating, and hypertrophic zones, and shortened hypertrophic zone in homozygote PPARγ KO mice compared to control mice. Figures show one representative experiment of at least four independent experiments. Bar graphs show mean +/− SEM of each group. *, P<0.05

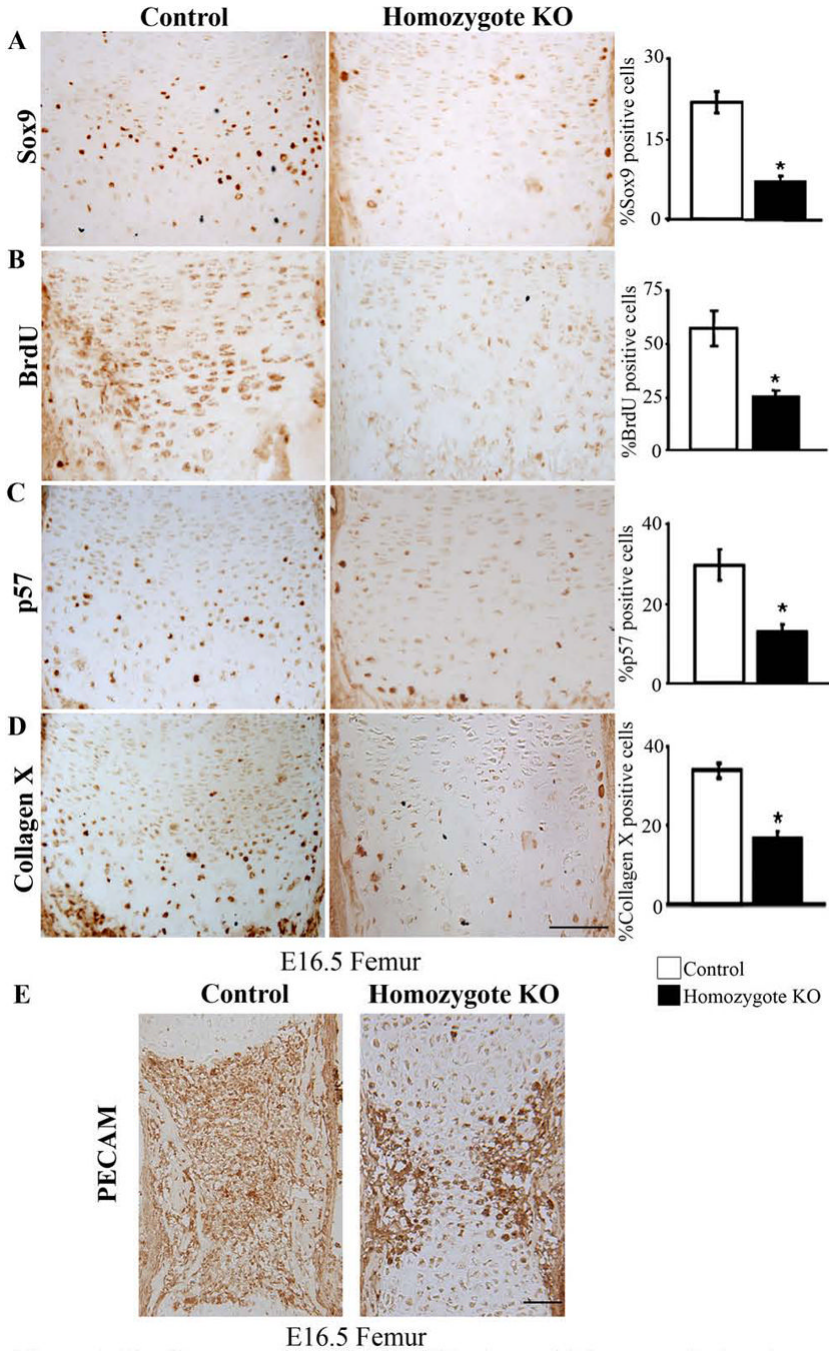

Figure 4.

Cartilage-specific PPARγ KO mice exhibit reduced chondrocyte proliferation, differentiation, hypertrophy and vascular invasion. Staining for (A) Sox9, (B) BrdU, (C) p57, and (D) collagen type X demonstrated reduced chondrocyte proliferation, differentiation and hypertrophy in E16.5 homozygote PPARγ KO mouse femurs compared to control mice. Bar graphs corresponding to IHC analysis show reduced percentage of positive cells for Sox9, BrdU, p57, and collagen type X in homozygote PPARγ KO mice compared to control mice (bar, 200μm). (E) IHC for PECAM confirmed reduced vascularity in the femurs of E16.5 homozygote PPARγ KO mice compared to control mice (bar,

200μm). n=4 per group. Figures show one representative experiment of four independent experiments. Bar graphs show mean +/− SEM of each group. * P<0.05

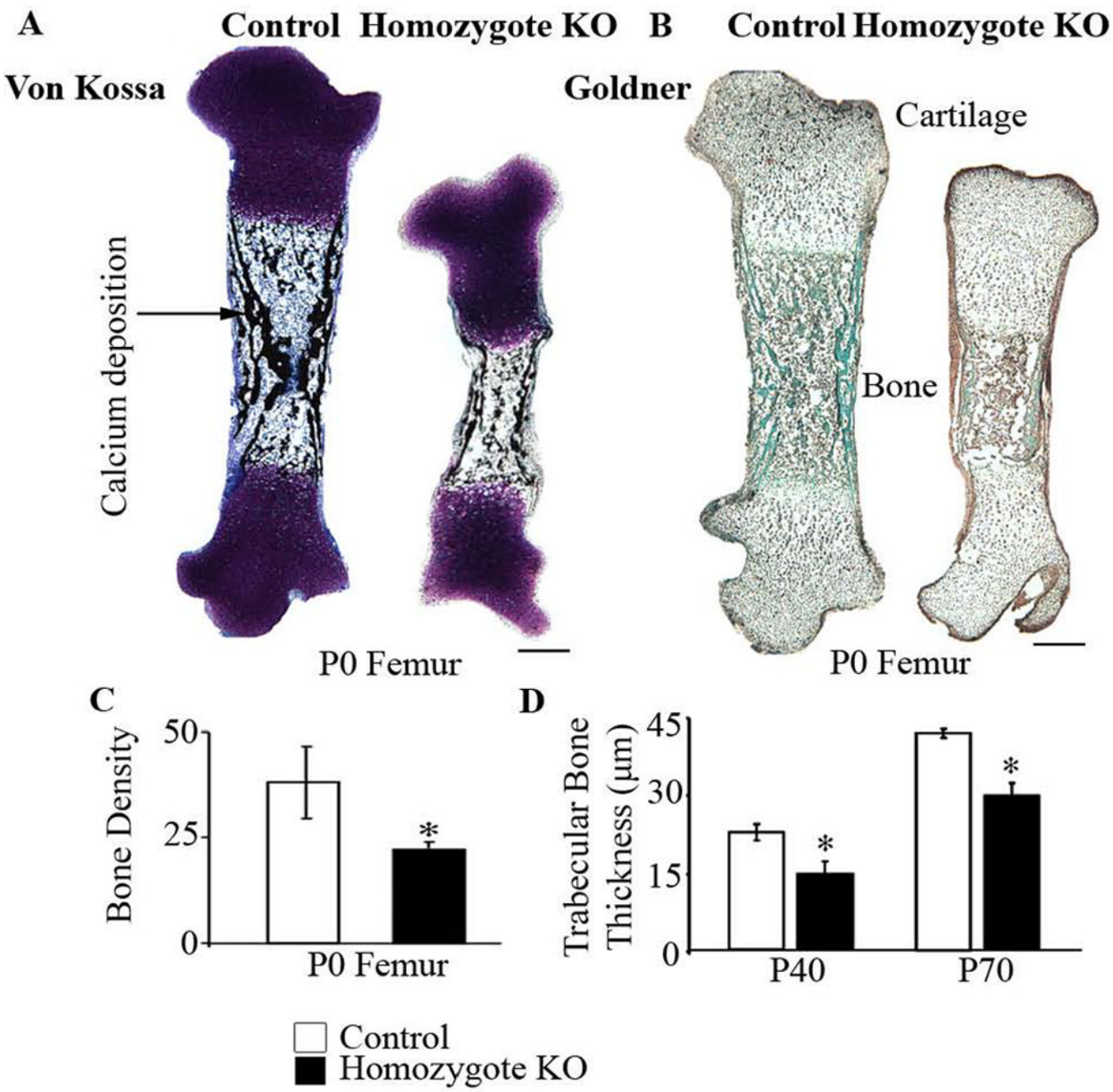

Figure 5.

Cartilage-specific PPARγ KO mice exhibit decreased calcium deposition, bone density and trabecular bone thickness. (A) Von Kossa staining of P0 mouse femurs showed that homozygote PPARγ KO mice exhibit reduced calcium deposition compared to control mice (bar, 500 μm). (B) Goldner staining and (C) bone density quantification using Bioquant Osteo II software demonstrated that the femurs of PO homozygote PPARγ KO mice show decreased bone density compared to control mice (bar, 500 μm). (D) Quantification of trabecular bone thickness using Bioquant Osteo II software demonstrated that P40 and P70 homozygote PPARγ KO mice exhibit decreased trabecular bone thickness compared to

control mice. n=4 per group. Figures show one representative experiment of four independent experiments. Bar graphs show mean +/− SEM of each group. *, P<0.05

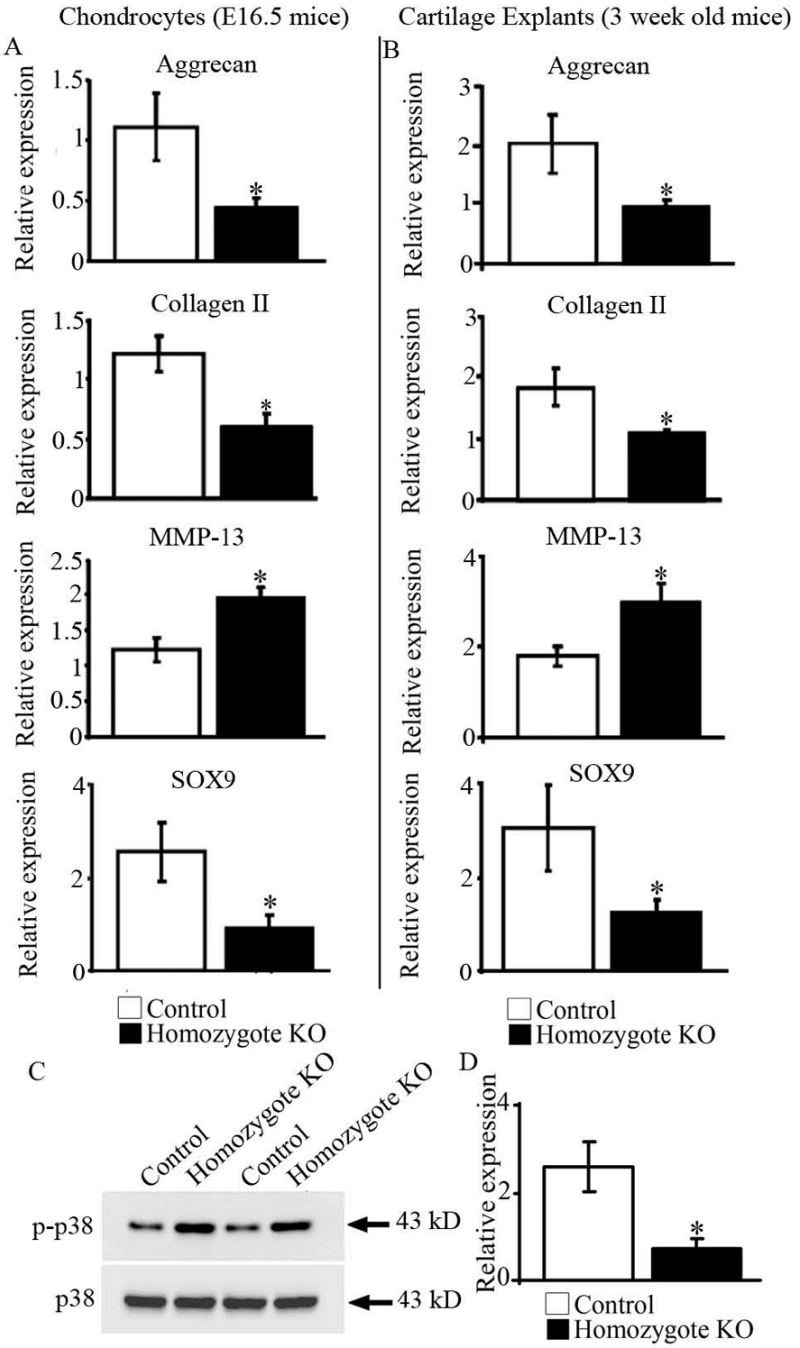

Figure 6.

Genetic ablation of PPARγ results in aberrant expression of endochondral ossification markers, ECM genes and p38 phosphorylation. (A) mRNA expression using RNA isolated from E16.5 mouse chondrocytes indicated decreased expression of aggrecan, collagen type II and SOX9, and increased expression of MMP-13 in homozygote PPARγ KO mice compared to control mice. (B) mRNA expression using RNA isolated from femur head cartilage explants of 3 weeks old mice also indicated decreased expression of aggrecan, collagen type II and SOX9, and increased expression of MMP-13 in homozygote PPARγ KO mice compared to control mice. (C) Increased phosphorylation of p38 was observed in E16.5 mouse chondrocytes isolated from homozygote PPARγ KO mice compared to control

mice. Representative blot from n=4 separate blots is shown. (D) Reduced mRNA expression of Ihh was observed in E16.5 mouse chondrocytes isolated from homozygote PPARγ KO mice compared to control mice. Representative data from n=6 independent isolated chondrocytes and cartilage explants experiments per group. Bar graphs throughout figure show mean +/− SEM of each group. *, $P<0.05$

ARTICLE 4

Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from Osteoarthritis

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EXTENDED REPORT

Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis

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ABSTRACT

Objectives Mammalian target of rapamycin (mTOR) (a serine/threonine protein kinase) is a major repressor of autophagy, a cell survival mechanism. The specific in vivo mechanism of mTOR signalling in OA pathophysiology is not fully characterised. We determined the expression of mTOR and known autophagy genes in human OA cartilage as well as mouse and dog models of experimental OA. We created cartilage-specific mTOR knockout (KO) mice to determine the specific role of mTOR in OA pathophysiology and autophagy signalling in vivo.

Methods Inducible cartilage-specific mTOR KO mice were generated and subjected to mouse model of OA. Human OA chondrocytes were treated with rapamycin and transfected with Unc-51–like kinase 1 (ULK1) siRNA to determine mTOR signalling.

Results mTOR is overexpressed in human OA cartilage as well as mouse and dog experimental OA. Upregulation of mTOR expression co-relates with increased chondrocyte apoptosis and reduced expression of key autophagy genes during OA. Subsequently, we show for the first time that cartilage-specific ablation of mTOR results in increased autophagy signalling and a significant protection from destabilisation of medial meniscus (DMM)-induced OA associated with a significant reduction in the articular cartilage degradation, apoptosis and synovial fibrosis. Furthermore, we show that regulation of ULK1/adenosine monophosphate-activated protein kinase (AMPK) signalling pathway by mTOR may in part be responsible for regulating autophagy signalling and the balance between catabolic and anabolic factors in the articular cartilage.

Conclusions This study provides a direct evidence of the role of mTOR and its downstream modulation of autophagy in articular cartilage homeostasis.

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis whose exact pathophysiology is still unknown. The major features of OA are cartilage degradation, synovial inflammation and subchondral bone remodelling. Loss of chondrocyte cellularity within the articular cartilage is one of the prominent events that contribute to its degradation. However, it is still uncertain as to what mechanisms control the fate of chondrocytes within the articular cartilage during normal versus OA conditions.

Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is a key regulator of cell growth, metabolism, survival and lifespan of organisms.[1] mTOR associates with raptor and rictor to form the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR is known to regulate protein synthesis by phosphorylation and inactivation of translational repressor 4E-binding protein (4E-BP1), and through the phosphorylation and activation of S6 kinase (S6K1).[2] It has been previously shown that TOR deficiency in the nematode *Caenorhabditis elegans* is able to double its lifespan.[3] In mice, treatment with mTORC1 inhibitor (rapamycin) extends lifespan of both male and female mice,[4] suggesting mTOR as a key mediator of lifespan regulation. One of the key functions of mTOR is the suppression of autophagy, a cell survival mechanism.[5] Autophagy is an essential homeostatic process by which cells break down their own components and is essential for survival, differentiation, development and homeostasis.[5] Hypoxia, reactive oxygen species and deprivation of nutrients and energy are among the key inducers of autophagy process[6] and when mTOR is inhibited.[7] It has been shown that mTOR prevents ULK1 activation by phosphorylating ULK1 and disrupting the interaction between ULK1 and AMPK.[8]

Recent studies by Caramés *et al*[9] [10] and Sasaki *et al*[11] have shown dysregulation in the expression of key autophagy genes in OA pathogenesis. It has been shown that the expression of ULK1 (the most upstream autophagy inducer), microtubule-associated protein 1 light chain 3 (LC3B; autophagy structural and functional factor) and Beclin1 (autophagy regulator) is reduced during OA.[9] Treatment with rapamycin (mTORC1 Inhibitor) has also been shown to reduce the severity of experimental OA in mice.[10]

The specific in vivo mechanism of mTOR signalling in OA pathophysiology is not fully characterised. This study was designed to first determine the expression of mTOR and autophagy genes in human OA cartilage as well as in mouse and dog models of experimental OA. To elucidate the specific in vivo role of mTOR in OA pathophysiology and regulation of autophagy during OA, we created cartilage-specific mTOR knockout (KO) mice using LoxP Cre technology and subjected these mice to DMM model of OA surgery to determine the effects of cartilage-specific ablation of mTOR on autophagy signalling and kinetics of OA in vivo. This study provides direct evidence on the role of mTOR and its downstream modulation of autophagy in articular cartilage homeostasis.

METHODS

Human specimens

Normal human cartilage was obtained from both femoral condyles and tibial plateaus, at autopsy within 24 h of death, from individuals with no history of arthritic diseases (n=15, 60.4 ±15.4 years (mean±SD)). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically as previously described.[12] Human OA cartilage was obtained from patients undergoing knee replacement (n=32, 65.2±17.6 years). In all patients, OA was diagnosed on the basis of criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA.[13] At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs or selective COX-2 inhibitors. None had received intra-articular steroid injections within 3 months prior to surgery. The Institutional Ethics Committee Board approved the use of the human articular tissues.

Generation of inducible cartilage-specific mTOR knockout mice

Inducible cartilage-specific mTOR KO mice were generated by mating mice containing a mTOR gene flanked by LoxP sites (C57BL/6- $mTOR^{fl/fl}$, Jackson Laboratory) with C57BL/6 Col2-rt-TA-Cre transgenic mice[14] (obtained from Dr. Peter Roughley, McGill University, Montreal). Five weeks old $mTOR^{fl/fl}$ Cre mice were fed doxycycline (Sigma-Aldrich Inc., Oakville, ON) dissolved at 10 μg/μL in phosphate buffer saline (PBS), pH 7.4 by oral gavage with the dose of 80 μg/g body weight for 7 days. $mTOR^{fl/fl}$ Cre mice without doxycycline (only PBS) treatment were used as control mice. Routine genotyping of tail DNA followed by confirmation of cartilage-specific loss of mTOR expression upon treatment with doxycycline was confirmed by western blotting and immunohistochemistry as described before.[14] All animal procedure protocols were approved by the Comité Institutionnel de protection des animaux of CRCHUM.

Chondrocyte culture studies

Human OA chondrocytes were released from cartilage by sequential enzymatic digestion, as previously described.[15] Cells were seeded at 3×10^5/well in 6-well culture plates in DMEM supplemented with 10% FBS and cultivated at 37°C for 48 h. Cells were washed and incubated for an additional 24 h in DMEM containing 0.5% FBS before treatment with rapamycin (100 nM) for 24 h. Cells were then harvested for RNA or protein isolation.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated by Trizol, then the RNeasy Mini kit (QIAGEN) was used including on-column DNase digestion to eliminate DNA as mentioned above (RNase-Free DNase Set, QIAGEN). RNA quantification was then performed using the QuantiTect Reverse Transcription PCR Kit (QIAGEN) on the Rotor Gene 3000 real-time PCR system (Corbett Research, Mortlake, Australia) according to the manufacturer's protocol and the fold increase in PCR products by $2^{-\Delta\Delta Ct}$ method and calculated using the housekeeping gene GAPDH.[16] All experiments were performed in triplicate for each sample, and the primers were designed using Primer3 online software.

Immunohistochemistry and TUNEL staining

The immunohistochemistry analysis was performed as previously described.[16] The percentage of chondrocytes staining positive for each specific antigen was determined as previously reported.[17] To detect chondrocyte apoptosis, TUNEL staining and Poly (ADP-ribose) polymerase (PARP)p85 immunostaining was performed in cartilage specimens obtained from normal and OA human cartilage as well as control and mTOR KO mice. TUNEL assay was performed using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer's recommendations (Millipore, Ontario, Canada).

Western blotting

Cells were lysed in Tris buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined using a bicinchoninic acid protein assay reagent (Thermo Fisher Scientific), with bovine serum albumin (BSA) as the standard. Cell lysates were adjusted to equal equivalents of protein and then were applied to SDS–polyacrylamide gels for electrophoresis as described before.[16]

Murine model of OA

OA was surgically induced in 10-week-old control and mTOR KO male mice by DMM or sham surgery (control) in the right knee, as previously described.[18 19] Histological and biochemical analysis were performed at 5 and 10 weeks post-OA surgery.

At 5 weeks post-OA surgery, right knee joint cartilage from femoral condyles and tibial plateaus was removed and primary chondrocytes were prepared from the mice as previously described.[16]

Assessment of progression and severity of osteoarthritis

Mouse knee joints sections (5 μm) at 5 and 10 weeks post-OA surgery were stained with Safranin-O/Fast Green (Sigma-Aldrich, Oakville, Ontario) according to the manufacturer's recommendations. Slides were evaluated by two independent readers in a blinded fashion. To determine the extent of cartilage deterioration, joint sections were stained with Safranin-O/Fast Green and histological scoring method issued by Osteoarthritis Research Society International (OARSI) was used for analysis as previously described.[20] To determine degree of synovial fibrosis, 5-week post-OA knee joint sections were stained with Masson's Trichrome stain. Stained sections were blindly scored for degree of fibrosis on a scale of 0–3 (0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, severe fibrosis).

Dog model of OA

Adult mixed breed Mongrel dogs (LAKA, St-Basile-le-Grand, Quebec, Canada) weighing 25±3 kg underwent surgical sectioning of the anterior cruciate ligament of the right knee as previously described,[21] and at 8 weeks postsurgery, cartilage from femoral condyles and tibial plateaus was removed and processed for RNA extraction as described above. Cartilage from femoral condyles and tibial plateaus of the left (contralateral) knee was used as control cartilage. All experimental procedures were approved by the Institutional Ethics Committee Board.

Gene silencing

Briefly, human OA chondrocytes were plated at 3×10^5/well in 6-well culture plates in DMEM supplemented with 10% FBS and pretreated with rapamycin (or DMSO as control) for 6 h. Cells were then transfected with ULK1 siRNA (20 nM, QIAGEN) or non-targeting (random) CTRL-ALLstars siRNAs (QIAGEN) using Lipofectamine RNAiMax Reagent (Invitrogen) for 48 h and protein and RNA were harvested for analysis.

Statistical analysis

The data are expressed as mean±SEM. The significance of differences in the levels of expression between the control (normal) and OA groups was determined using a two-tailed Mann–Whitney U test. For significance of differences between rapamycin-treated OA chondrocytes and DMSO-treated (control) chondrocytes, as well as siCTRL and siULK-treated OA chondrocytes, two-tailed t test was used. For autophagy PCR array analysis, two-tailed T test was used. $p<0.05$ was considered statistically significant.

RESULTS

Increased expression of mTOR in human OA as well as mouse and dog models of experimental OA

We first determined the expression of mTOR in human OA cartilage compared with normal human cartilage. Western blotting and immunohistochemical analysis showed enhanced protein expression of mTOR in human OA cartilage compared with normal human cartilage (figure 1A, B). Further, we also observed a significant upregulation ($p<0.05$) in the mRNA expression of mTOR in human OA cartilage compared with normal human cartilage (figure 1C). Similarly, mTOR expression was also significantly upregulated ($p<0.05$) in both dog OA cartilage and mouse OA chondrocytes compared with control dog cartilage and control sham surgery chondrocytes, respectively (figure 1D, E).

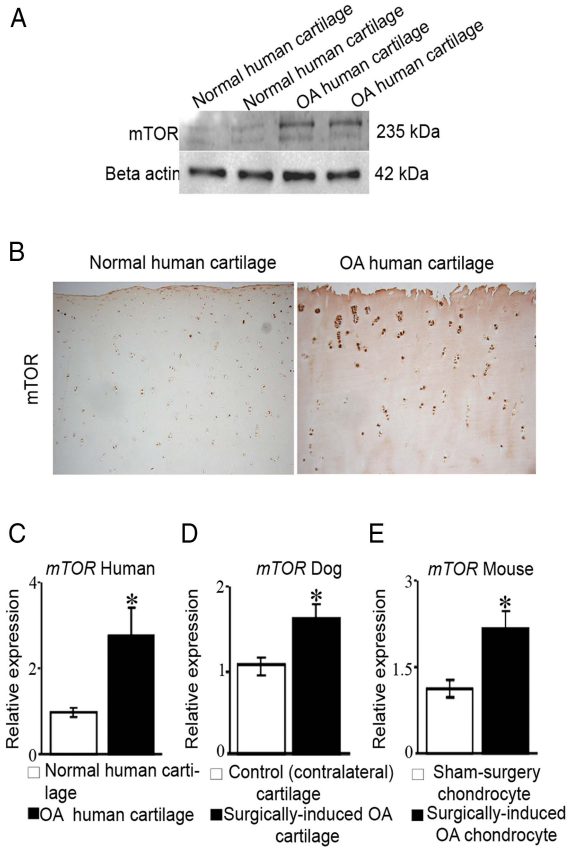

Figure 1 Enhanced expression of mTOR during OA. (A and B) Protein expression of mTOR was enhanced in human OA cartilage (n=4) compared to normal human cartilage (n=4) as determined by Western blotting and immunohistochemistry. Original magnification = ×25. (C) A significant increase (*$p<0.05$) in the mRNA expression of mTOR was observed in human OA cartilage (n=5) compared to normal human cartilage (n=5). (D) In dog OA cartilage (n=5), a significant increase (*$p<0.05$) in the mRNA expression of mTOR was observed compared to control contralateral cartilage (n=5). (E) A significant increase (*$p<0.05$) in the mRNA expression of mTOR was also observed in mouse OA chondrocytes (n=8) compared to sham-surgery control chondrocytes (n=8).

Aberrant expression of autophagy genes in human OA cartilage compared with normal human cartilage

Since mTOR is a master negative regulator of autophagy and we observed increased expression of mTOR in human OA cartilage compared with normal human cartilage, we further determined the expression of known autophagy genes in human OA cartilage compared with normal human cartilage. The expression patterns of 84 key autophagy genes and 5 housekeeping genes in human OA cartilage compared with normal human cartilage were profiled using a human autophagy PCR array.

The results show that in OA cartilage 20 autophagy-related genes were significantly downregulated and 5 autophagy-related genes were significantly upregulated compared with normal human cartilage ($p<0.05$) (see online supplementary table 1). Also, no significant change in the expression of 53 autophagy-related genes was observed in OA cartilage compared with normal human cartilage. 6 autophagy-related genes were undetected in both normal and OA cartilage samples. Results obtained from the PCR arrays showed that the key autophagy-related genes such as ATG3, ATG5, ATG12, ULK1, LC3B, Beclin-1 and GABA(A)receptor-associated protein like 1 (GABARAPL1) involved in initiating autophagy, autophagic vacuole formation and phagophore extention were downregulated in OA cartilage compared with normal human cartilage (see online supplementary table 1). Also, coregulators of autophagy and apoptosis including BNIP3, cyclin-dependent kinase inhibitor 1b (CDKN1B), TNF receptor superfamily member 6 (FAS) were downregulated in OA cartilage. Chaperone-mediated autophagy-related genes, such as HSP90AA1 and HSPA8, were also significantly downregulated in OA cartilage. Array data also revealed that critical regulators of cell death/apoptosis mechanisms including APP, CTSB, BCL2 and BCL2-associated agonist of cell death (BAD) were upregulated in OA cartilage compared with normal cartilage.

Increase in cell death and reduction in the production/expression of key autophagy markers in human OA cartilage versus normal cartilage

We performed TUNEL assay to determine the degree of cell death in human OA cartilage compared with normal human cartilage. Results clearly show greater percentage of TUNEL positive cells in OA cartilage compared with normal human cartilage (figure 2A). Immunostaining using PARPp85 antibody further confirmed increased number of apoptotic cells in human OA cartilage compared with normal human cartilage.

Since autophagy PCR array data showed that several autophagy genes involved in the induction of autophagy process were downregulated in human OA cartilage compared with normal human cartilage, we further analysed the expression of four crucial autophagy markers including ULK1 (most upstream autophagy inducer), LC3B (an autophagy structural and functional factor), ATG5 (an autophagy regulator) and BNIP3 (an interactor of LC3 in autophagy) by immunohistochemistry and qPCR analysis. Immunohistochemistry showed that all four autophagy genes (ULK1, LC3B, ATG5 and BNIP3) were constitutively expressed throughout the normal human cartilage. However, a significant ($p<0.01$) reduction in the number of positive cells for ULK1, LC3B, ATG5 and BNIP3 was observed

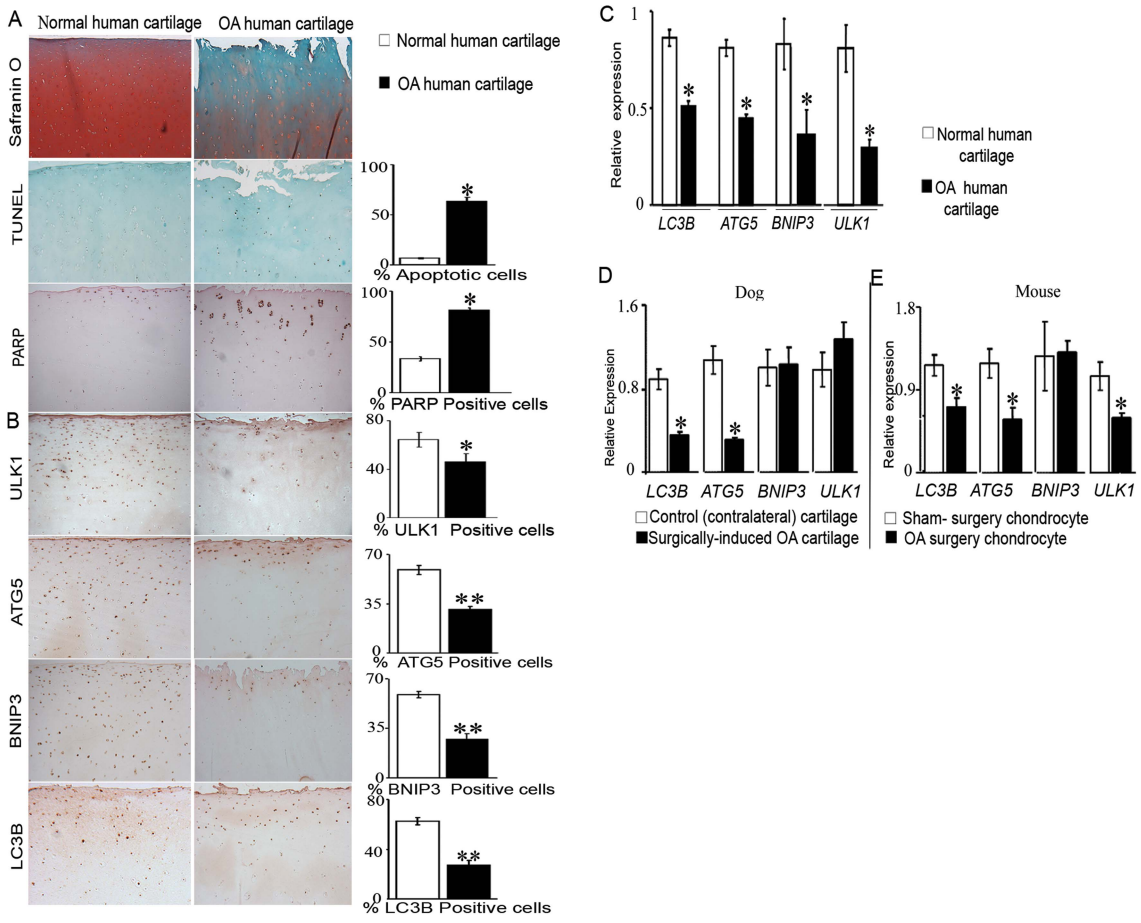

Figure 2 Increased chondrocyte apoptosis and decreased expression of autophagy genes during OA. (A) TUNEL assay and immunohistochemical analysis for PPAP p85 demonstrated a significant increase in the percentage (%) of apoptotic cells in human OA cartilage compared to normal human cartilage (n=6, *p<0.05). (B) Immunohistochemical analysis showed decreased % of positive cells for LC3B, ATG5, BNIP3 and ULK1 in human OA cartilage (n=5) compared to normal human cartilage (n=5, *p<0.05). Original magnification = ×25. (C) Significant down-regulation in the mRNA expression of autophagy genes including LC3B, ATG5, BNIP3, and ULK1 in human OA cartilage (n=8) compared to normal human cartilage (n=8; *p<0.05). (D) A significant reduction (n=5; *p<0.05) in the mRNA expression of LC3B and ATG5 (with no significant change in the expression of BNIP3 and ULK1) was observed in the dog OA cartilage (n=5) compared to control contralateral cartilage (n=5). (E) A significant (n=8; *p<0.05) reduction in the mRNA expression levels of LC3B, ATG5 and ULK1 (with no significant change in the expression of BNIP3) was observed in mouse OA chondrocytes (n=8) compared to sham-surgery chondrocytes (n=8).

in the human OA cartilage compared with normal human cartilage (figure 2B). qPCR analysis also showed a significant reduction in the mRNA expression of ULK1, LC3B, ATG5 and BNIP3 ($p<0.05$) in OA cartilage compared with normal human cartilage (figure 2C).

Reduction in the expression of autophagy markers in dog and mouse experimental OA

We further assessed the expression of LC3B, ATG5, BNIP3 and ULK1 in dog and mouse models of experimental OA. In the cartilage obtained from the knees of dogs subjected to the instability model of experimental OA (n=5) compared with cartilage obtained from the non-surgery contralateral knee joints (control, n=5), we observed a significant downregulation ($p<0.05$) in the mRNA expression levels of LC3B and ATG5 with no significant differences in the expression of BNIP3 and ULK1 in dog OA cartilage compared with control cartilage (figure 2D). In mouse model of experimental OA, we isolated chondrocytes from the knee joints of mouse subjected to DMM OA surgery (n=8) and control sham surgery (n=8). As observed in human OA cartilage, the mRNA expression of LC3B, ATG5 and ULK1 (but not BNIP3) was significantly ($p<0.05$) reduced in mouse OA chondrocytes compared with sham surgery chondrocytes (figure 2E). These results indicate the complexity and differential regulation of certain autophagy genes across species.

Cartilage-specific deletion of mTOR results in increased expression of autophagy markers

Since we observed dysregulation in the expression of mTOR and autophagy genes in OA conditions, we then created inducible cartilage-specific mTOR KO mice to elucidate the in vivo role of mTOR signalling in OA pathophysiology. Routine genotyping of tail DNA followed by confirmation of cartilage-specific loss of mTOR expression upon treatment with doxycycline was confirmed by western blotting and immunohistochemistry (figures 3A, B and 4A). Inducible mTOR KO mouse develop normally exhibit no developmental defects with no phenotypic changes in the articular cartilage till 10 weeks postbirth. First, we examined the effect of cartilage-specific ablation of mTOR on mTOR signalling pathway. Loss of mTOR in the cartilage resulted in decreased phosphorylation of ribosomal protein S6 Kinase (rpS6 K), a downstream target of mTORC1[2] (figure 3B). Furthermore, mTOR genetic deletion resulted in increased

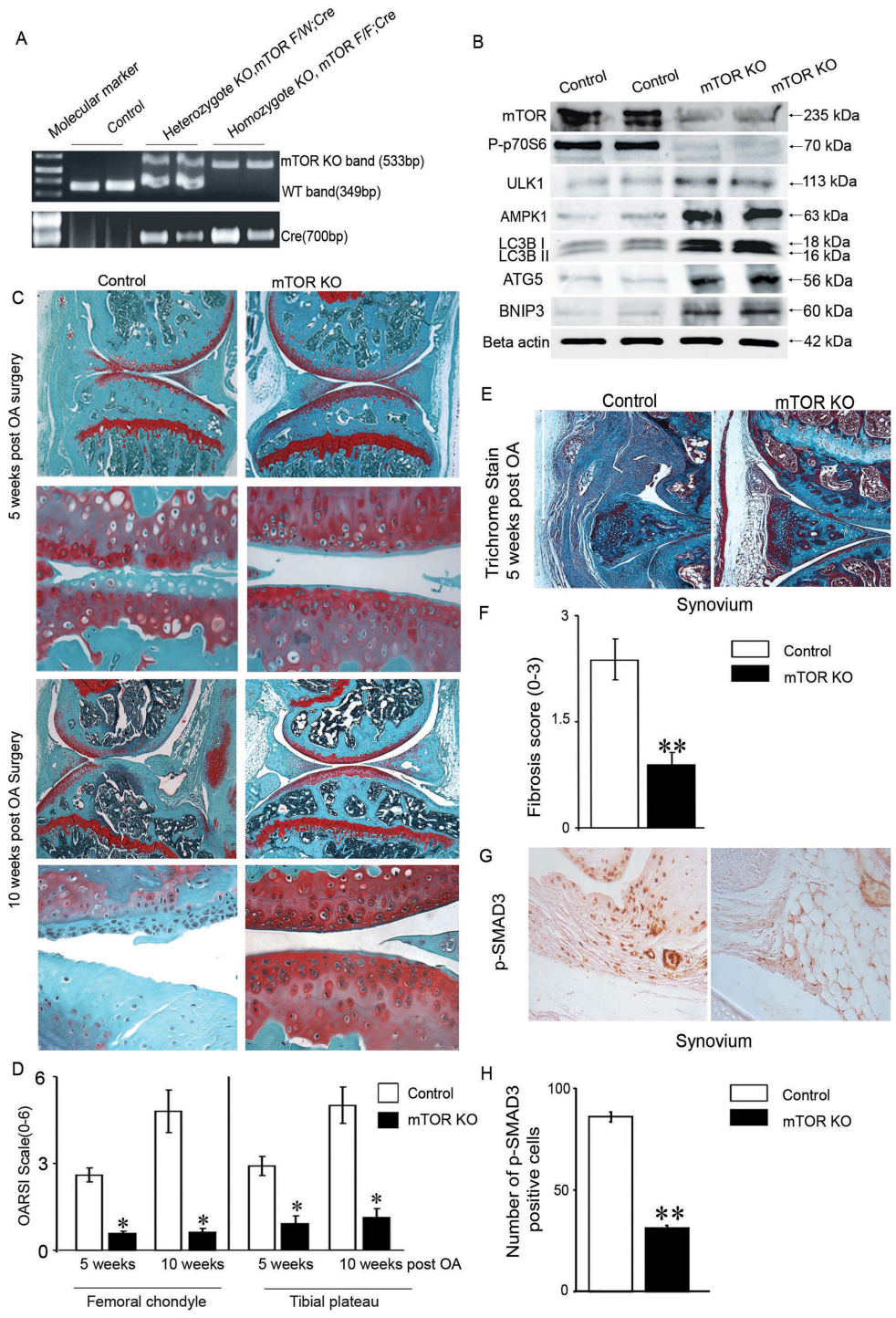

Figure 3 Inducible cartilage-specific mTOR KO mice exhibit protection from DMM-induced OA. (A) Characterization of inducible cartilage-specific mTOR KO mouse: Genotyping confirms the presence of the Cre transgene in mTOR heterozygote ($mTOR^{fl/w}$) and homozygote ($mTOR^{fl/fl}$) mice. The mTOR KO band is detected at 533 bp, the wild-type (WT) band at 349 bp, and the Cre band at 700 bp. (B) mTOR deficient cartilage exhibits increased expression of autophagy genes: Western blotting results showed abolishment of mTOR protein expression, decreased phosphorylation of P70S6 kinase and increased expression of ULK1, AMPK1, ATG5, BNIP3 and total LC3 expression as well as the conversion from LC3BI to LC3BII, in articular chondrocytes isolated from inducible cartilage-specific mTOR KO mice compared to control mice chondrocytes. (C) mTOR KO mice are protected from DMM-induced OA: Inducible cartilage-specific mTOR KO mice and control mice were subjected to DMM surgery and kinetics of cartilage degradation were assessed at 5 and 10 weeks post OA surgery. Safranin O-fast green stained sections showed that all mTOR KO mice exhibited significant protection from OA compared to control mice at 5 and 10 weeks post OA associated with reduced cartilage degradation/fibrillation, proteoglycan loss and reduced loss of articular chondrocyte cellularity. Original magnification = ×25. (D) A significant reduction in the OARSI scale was observed at both medial tibial plateau and medial femoral chondyle in mTOR KO mice compared to control mice (n=5/genotype/time point). (E) mTOR KO mice are protected from synovial fibrosis during OA: Trichrome staining showed decreased synovial fibrosis in mTOR KO mice (n=5) compared with control mice (n=5) 5 weeks post OA surgery. Original magnification = ×25. (F) A significant reduction in the fibrosis score in mTOR KO mice synovium compared to control mice synovium (n=5, **p<0.01). (G and H) Immunohistochemical analysis showing a significant decrease in number of p-Smad3 positive cells in the synovium of mTOR KO mice (n=4) compared to control mice (n=4). Original magnification = ×25.

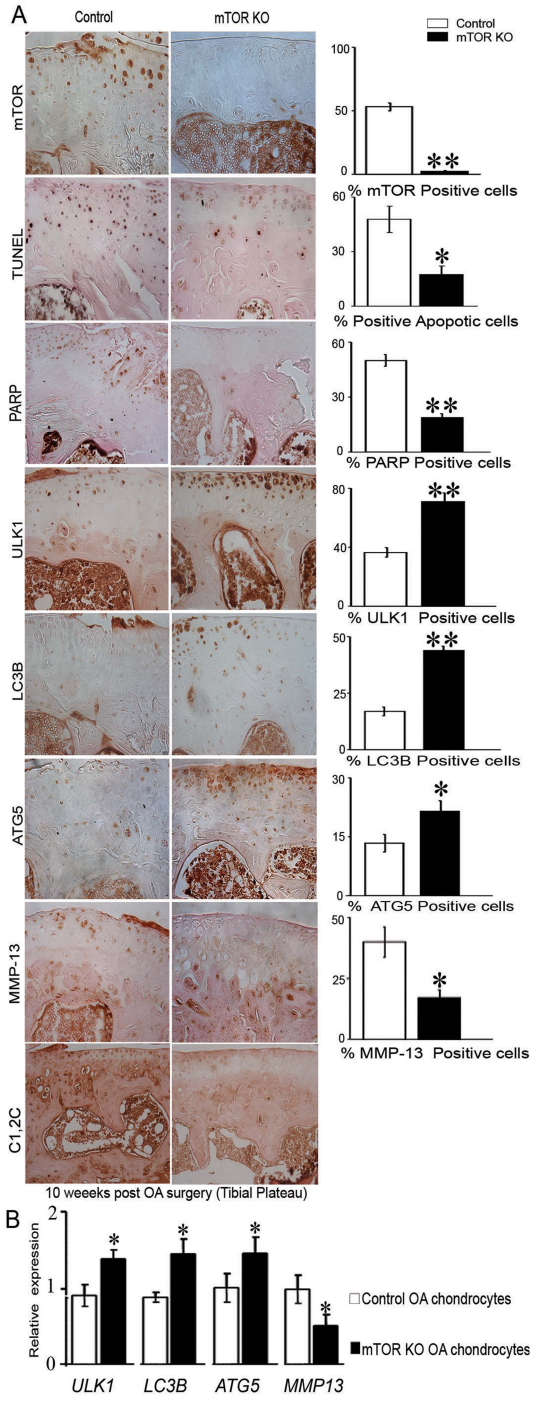

Figure 4 mTOR KO mice exhibit decreased articular chondrocyte apoptosis, increased expression of autophagy markers and decreased expression of MMP-13 during OA. TUNEL assay and immunohistochemical analysis for PPAP p85 showed a decreased number of apoptotic cells (chondrocytes) in the tibial plateau of mTOR KO mice articular cartilage compared to control mice articular cartilage 10 weeks post OA surgery. Immunohistochemical analysis showed significantly increased expression of autophagy genes including ULK1 (n=5, **p<0.01), LC3B (n=5, **p<0.01) and ATG5 (n=5, *p<0.05), and decreased expression of OA catabolic factor MMP-13 (n=5, *p<0.05) and decreased staining for the type II collagen breakdown product C1-2C (n=5) in the tibial plateau of mTOR KO mice (n=5) compared to control mice (n=5) 10 weeks post OA surgery. Original magnification = ×25. (B) A significant increase in the mRNA expression levels of ULK1, LC3B and ATG5 (n×5; *p<0.05) and significant decrease in the expression of MMP-13 (n=5; *p<0.05) was observed in mTOR KO OA chondrocytes compared to control mice OA chondrocytes.

expression of ULK1, AMPK1, ATG5, BNIP3 and increase in total LC3 expression as well as the conversion from LC3BI to LC3BII. These results show an increased autophagy signalling upon genetic deletion of mTOR in the articular cartilage.

Inducible cartilage-specific mTOR KO mice exhibit protection from DMM-induced OA

mTOR KO mouse were then subjected to DMM model of OA and kinetics of OA progression and severity were assessed at 5 and 10 weeks post-OA surgery. Compared with control mice, mTOR KO mice exhibited significant protection from DMM-induced OA manifestations including significant protection from cartilage degradation, significant reduction in proteoglycan loss and loss of articular chondrocyte cellularity (figure 3C). Two blinded observers further confirmed a significant reduction in the OARSI scale at both medial tibial plateau and medial femoral chondyle in mTOR KO mice compared with control mice (figure 3D).

Interestingly, protection from DMM-induced OA in mTOR KO mouse was not limited to the articular cartilage as significant reduction in the synovial fibrosis was also observed in mTOR KO mouse compared with control mouse (figure 3E–F). Since transforming growth factor-beta (TGF-β) is a major pro-fibrotic factor and its signalling through Smads is implicated in mediating fibrosis, we investigated the mechanism related to decreased synovial fibrosis observed in mTOR KO mice by performing immunostaining for pSmad3 in the synovium of mTOR KO mice compared with control mice. In 5 weeks post-OA mice synovium, a significant decrease in the number of pSmad3 positive cells was observed in the synovium of mTOR KO mice compared with control mice (figure 3G, H), suggesting that loss of mTOR is associated with decreased TGF-β/Smad3 signalling resulting in decreased synovial fibrosis.

Inducible cartilage-specific mTOR KO mice exhibit reduced apoptosis, enhanced production of autophagy markers and reduced expression of catabolic factor MMP13 during OA

TUNEL assay and PARPp85 on 10 weeks post-OA mouse cartilage showed a reduction in the apoptotic cells in mTOR KO mouse cartilage compared with control mouse cartilage (figure 4). The protection of articular chondrocytes from apoptosis in mTOR KO cartilage co-related with the increased expression of autophagy genes including ULK1, LC3B and ATG5 and decreased expression of major OA-catabolic factor matrix metalloproteinase-13 (MMP-13) and MMP-induced type II collagen breakdown product C1,2C (figure 4A) as assessed by immunohistochemistry. qPCR analysis further confirmed the increased expression of ULK1, LC3B and ATG5 and decreased expression of MMP-13 in chondrocytes isolated from mTOR KO mice at 5 weeks post-OA surgery compared with control mice chondrocytes (figure 4B). These results show that upon genetic deletion of mTOR, increase in autophagy signalling and the reduction in chondrocyte apoptosis may in part contribute to decreased catabolic activity observed in mTOR KO mouse, resulting in protection from OA.

mTOR modulates the expression of autophagy factors and the expression of catabolic and anabolic factors implicated in OA

We treated human OA chondrocytes with rapamycin (mTORC1 inhibitor)[22] and determined the effect of inhibition of mTOR signalling on the expression of autophagy-related genes as well as on the expression of key catabolic and anabolic factors implicated in OA pathophysiology. Rapamycin treatment significantly

reduced rpS6K phosphorylation in OA chondrocytes compared with control (DMSO-treated) chondrocytes confirming the inhibition of mTOR signalling pathway (figure 5A). Further, rapamycin treatment resulted in a significant increase in total LC3 expression and the conversion from LC3BI to LC3BII (figure 5A) as well as increase in the mRNA expression of LC3, ULK1 and AMPK1 in rapamycin-treated OA chondrocytes compared with DMSO-treated chondrocytes (figure 5C). Interestingly, treatment

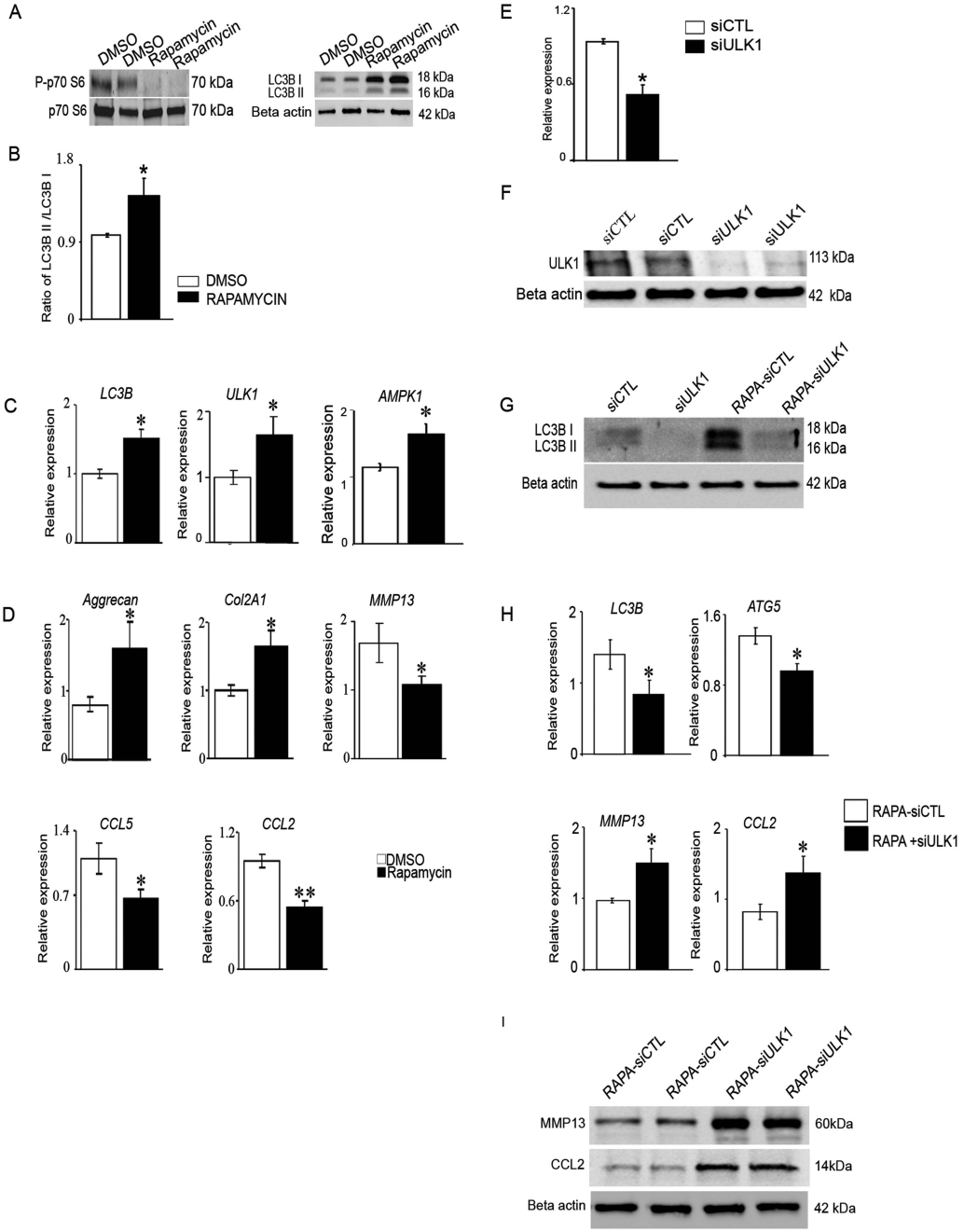

Figure 5 mTOR controls autophagy by modulating ULK1 expression in the articular cartilage. (A) Human OA chondrocytes weretreated with rapamycin for 24 hr. Results showed decreased phosphorylation of P70S6 Kinase in rapamycin-treated OA chondrocytes (n=4) compared to DMSO-treated (control) chondrocytes (n=4). Treatment of OA chondrocytes with rapamycin resulted in an increase in total LC3 expression. (B) The conversion from LC3BI to LC3BII compared to DMSO-treated (control) OA chondrocytes. (C) mRNA expression of LC3, ULK1 and AMPK1 was significantly increased (n=6; *p<0.05) in rapamycin-treated human OA chondrocytes (n=6) compared to control (DMSO-treated) chondrocytes (n=6). (D) A significant increase in the mRNA expression of aggrecan and Type II collagen (p<0.05), and a significant decrease in the mRNA expression of MMP-13 (n=6; *p<0.05), CCL5/RANTES (n=6; *p<0.05) and CCL2/MCP-1 (n=6; **p<0.01) was observed in rapamycin-treated OA chondrocytes (n=6) compared to DMSO-treated OA chondrocytes (n=6). (E-F) Silencing efficiency of ULK1 siRNAs in human OA chondrocytes (n=6) was confirmed by qPCR and western blotting analysis. A significant decrease in the expression of ULK1 was observed at both mRNA (E) and protein levels (F) in ULK1 siRNA-treated cells compared to control siRNA-treated cells.(G-H) Silencing of ULK1 in rapamycin-treated human OA chondrocytes resulted in a significant decrease in the protein expression of LC3B determined by Western blots (total LC3 expression and the conversion from LC3BI to LC3BII), significant decrease in the mRNA expression of LC3B and ATG5 and increase in the expression of OA catabolic factors including MMP-13 and CCL2 at both mRNA (H) and protein levels (I) (n=8; *p<0.05).

of OA chondrocytes with rapamycin significantly increased the mRNA expression of aggrecan and type II collagen (two major components of extracellular matrix) and decreased the expression of MMP-13 and chemokines including CCL5/RANTES and CCL2/MCP-1 compared with DMSO-treated chondrocytes (figure 5D). Similarly, we isolated mouse OA chondrocytes from control and mTOR KO mouse at 5 weeks post-OA surgery and treated these chondrocytes with rapamycin in vitro. As expected, in control mice OA chondrocytes, rapamycin treatment resulted in a significant rescue/increase in the expression of ULK1, LC3B, ATG5 and type II collagen and a significant decrease in the expression of MMP-13, thus mimicking the effects observed in human OA chondrocytes (see online supplementary figure 1). In mTOR-deficient chondrocytes, rapamycin treatment exhibited no significant differences in the expression of autophagy markers as well as the expression of catabolic and anabolic factors, thus confirming that in the absence of mTOR (mTOR KO mice chondrocytes) rapamycin exhibited no further rescue effect.

Since IL-1β is a major pro-inflammatory cytokine implicated in OA pathophysiology,[23] we treated human OA chondrocytes in the presence/absence of IL-β to determine whether IL-1β can alter the expression of mTOR. Indeed, our results show that IL-1β-treatment results in a significant increase in the expression of mTOR associated with a significant increase in the expression of MMP-13, CCL2 and CCL5 and a significant decrease in the expression of type II collagen (see online supplementary figure 2). These results suggest that IL-1β may play a critical role in increasing the expression of mTOR during OA.

Silencing of ULK1 can rescue the protective effects of rapamycin

Previously, it has been reported that mTOR regulates ULK1 in combination with AMPK.[8] Since ULK1 is the most upstream autophagy inducer and we observed that genetic deletion of mTOR in mouse (in vivo) and rapamycin treatment in human OA chondrocytes (in vitro) resulted in the upregulation of ULK1 and AMPK1, we hypothesised that the ability of mTOR to modulate ULK1 signalling may in part be responsible for controlling autophagy as well as the balance between catabolic and anabolic processes in the articular cartilage. To test this, we pretreated OA chondrocytes with rapamycin and transfected these cells in the presence/absence of ULK1 siRNA to determine if silencing of ULK1 can rescue the protective effects of rapamycin. Indeed, silencing of ULK1 (figure 5E, F) in rapamycin-treated OA chondrocytes resulted in a significant decrease in the expression of LC3B (total LC3 expression and the conversion from LC3BI to LC3BII) (figure 5G–H) as well as mRNA expression of LC3 and ATG5 (figure 5H). Furthermore, silencing of ULK1 in rapamycin-treated OA chondrocytes resulted in a significant increase in the expression of OA catabolic factors (MMP-13 and CCL2) (figure 5H, I). These results suggest that in the articular cartilage, ability of mTOR to suppress autophagy and create imbalance between catabolic and anabolic processes may in part be regulated by shutting down ULK1 pathway.

DISCUSSION

Adult articular cartilage comprises only one type of cells 'chondrocytes' that are essential for maintaining the integrity of extracellular matrix as well as imparting/maintaining adequate homeostasis and balance between catabolic and anabolic activities in the cartilage milieu.[24] One of the critical events during OA is the loss of chondrocyte cellularity within the articular cartilage that can disrupt the balance between catabolic and anabolic processes, resulting in destruction of the cartilage. However, the mechanisms that control the fate of the chondrocytes in the articular cartilage during normal versus OA conditions remain to be elucidated.

Recent studies by Caramés *et al*[9 10] and Sasaki *et al*[11] have suggested a dysregulation in the process of autophagy during OA. Specifically, it has been shown that the expression of some of the key autophagy genes is downregulated in human OA and ageing-related and mouse experimental OA cartilage.[9] Subsequently, rapamycin has been shown to induce autophagy and protect mice from experimentally induced OA.[10] These results suggest that compromised autophagy may contribute to decreased chondroprotection and development of OA. However, it still remains to be determined what controls autophagy in the articular cartilage.

In the present study, we provide comprehensive in vivo and in vitro evidence that one of the key central factors that control autophagy signalling and articular cartilage homeostasis is mTOR. We first show that mTOR is overexpressed in human OA cartilage (compared with normal human cartilage), as well as mouse and dog models of experimental OA. This upregulation in the expression of mTOR during OA correlates with enhanced chondrocyte apoptosis and decreased expression of key genes involved in the induction of autophagy including ULK1 (most upstream autophagy inducer),[25] LC3B (an autophagy structural and functional factor), ATG5 (an autophagy regulator)[26] and BNIP3 (an interactor of LC3 in autophagy).[27]

We then created inducible cartilage-specific mTOR KO mouse (mTOR global KO mouse are not viable)[28] to specifically elucidate the in vivo role of mTOR/autophagy signalling in OA pathophysiology. Our results show that the genetic loss of mTOR in the articular cartilage results in increased autophagy signalling, reduced expression of OA catabolic factor MMP-13, decreased chondrocyte apoptosis and significant protection from DMM-induced OA.

To extrapolate our in vivo mouse findings to human OA and dissect the mTOR signalling pathway operative during OA pathogenesis, we treated human OA chondrocytes with rapamycin to inhibit mTOR signalling. Rapamycin-treated OA chondrocytes exhibited suppression in the phosphorylation of rpS6K (confirming the inhibition of mTOR signalling) associated with a significant increase in the expression of AMPK1 and autophagy genes including LC3 (total LC3 expression and the conversion from LC3BI to LC3BII) and ULK1. Interestingly, rapamycin treatment elevated the expression of aggrecan and type II collagen, two major components of cartilage ECM, and decreased the expression of OA catabolic factors including MMP-13 and chemokines (CCL5/RANTES and CCL2/MCP-1), suggesting the potential of mTOR inhibition in correcting the imbalance between catabolic and anabolic factors during OA.

ULK1 is the most upstream autophagy inducer and a key initiator of autophagy process.[29] It is believed that the interaction between ULK1/AMPK and mTOR signalling pathway are critical components in the regulation of autophagy.[30] In mammalian cells it has been shown that activation of mTORC1 results in phosphorylation of ULK-1 resulting in disruption of interaction between AMPK and ULK-1, thus inhibiting AMPK-mediated ULK1 activation and hence loss of autophagy.[8 31] Similarly, upon starvation mTORC1 receives stress signals that promote ULK1 complex formation, mTORC1 kinase activity is inhibited and autophagosome formation occurs.[29 32] Our in vivo and in vitro studies using mTOR KO mouse as well as rapamycin treatment (mTORC1 Inhibition) in human OA chondrocytes respectively show upregulation in the expression of ULK1 and AMPK1 upon genetic deletion of mTOR as well as pharmacological inhibition of mTOR signalling (rapamycin) in OA chondrocytes. To

confirm our hypothesis that mTOR controls autophagy in part by modulating ULK1 expression in the articular cartilage, we pretreated OA chondrocytes with rapamycin and transfected these cells in the presence/absence of ULK1 siRNA to determine if silencing of ULK1 can rescue the protective effects of rapamycin. Our results clearly show that silencing of ULK1 in rapamycin-treated OA chondrocytes resulted in a significant decrease in the expression of other autophagy regulators including LC3B and ATG5. Furthermore, silencing of ULK1 in rapamycin-treated OA chondrocytes resulted in a significant increase in the expression of OA catabolic factors (MMP-13 and CCL2). Collectively, these results suggest that in the articular cartilage, ability of mTOR to modulate ULK1/AMPK autophagy pathway may in part be responsible for the imbalance between catabolic and anabolic processes and decreased chondroprotection and ultimately cartilage destruction observed during OA. The involvement of other signalling pathways through which mTOR mediates its actions in the articular cartilage cannot be ruled out and needs further investigation.

Targeting ECM degradative products such as MMPs as well as inflammatory cytokines have yielded disappointing results. Thus far, all MMP inhibitors tested in clinical trials have been unsuccessful due to specificity issues as well as being associated with adverse effects.[23] OA is currently managed by using treatments such as acetaminophen, opioids and non-steroidal anti-inflammatory drugs (NSAIDs) including selective cyclooxygenase-2 (COX-2) inhibitors, all of which only provide symptomatic relief. Thus, targeting cellular homeostasis mediators such as mTOR and its downstream signalling by autophagy pathway may be a promising therapeutic strategy to achieve chondroprotection and correct the imbalance between catabolic and anabolic processes during OA and related disorders.

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Notes