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# The Role of ULK1 in the Pathophysiology of Osteoarthritis

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# The Role of ULK1 in the Pathophysiology of Osteoarthritis

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# RÉSUMÉ

L'arthrose est la maladie musculo-squelettique la plus commune dans le monde. Elle est l'une des principales causes de douleur et d'incapacité chez les adultes, et elle représente un fardeau considérable sur le système de soins de santé. L'arthrose est une maladie de l'articulation entière, impliquant non seulement le cartilage articulaire, mais aussi la synoviale, les ligaments et l'os sous-chondral. L'arthrose est caractérisée par la dégénérescence progressive du cartilage articulaire, la formation d'ostéophytes, le remodelage de l'os sous-chondral, la détérioration des tendons et des ligaments et l'inflammation de la membrane synoviale. Les traitements actuels aident seulement à soulager les symptômes précoces de la maladie, c'est pour cette raison que l'arthrose est caractérisée par une progression presque inévitable vers la phase terminale de la maladie.

La pathogénie exacte de l'arthrose est encore inconnue, mais on sait que l'événement clé est la dégradation du cartilage articulaire. Le cartilage articulaire est composé uniquement des chondrocytes; les cellules responsables de la synthèse de la matrice extracellulaire et du maintien de l'homéostasie du cartilage articulaire. Les chondrocytes maintiennent la matrice du cartilage en remplaçant les macromolécules dégradées et en répondant aux lésions du cartilage et aux dégénérescences focales en augmentant l'activité de synthèse locale. Les chondrocytes ont un taux faible de renouvellement, c'est pour cette raison qu'ils utilisent des mécanismes endogènes tels que l'autophagie (un processus de survie cellulaire et d'adaptation) pour enlever les organelles et les macromolécules endommagés et pour maintenir l'homéostasie du cartilage articulaire.

L'autophagie est une voie de dégradation lysosomale qui est essentielle pour la survie, la différenciation, le développement et l'homéostasie. Elle régule la maturation et favorise la survie des chondrocytes matures sous le stress et des conditions hypoxiques. Des études effectuées par nous et d'autres ont montré qu'un dérèglement de l'autophagie est associé à une diminution de la chondroprotection, à l'augmentation de la mort cellulaire et à la dégénérescence du cartilage articulaire. Carames et al ont montré que l'autophagie est constitutivement exprimée dans le cartilage articulaire humain normal. Toutefois, l'expression des inducteurs principaux de l'autophagie est réduite dans le vieux cartilage.

Nos études précédentes ont également identifié des principaux gènes de l'autophagie qui sont exprimés à des niveaux plus faibles dans le cartilage humain atteint de l'arthrose. Les mêmes résultats ont été montrés dans le cartilage articulaire provenant des modèles de l'arthrose expérimentaux chez la souris et le chien. Plus précisément, nous avons remarqué que l'expression d'Unc-51 like kinase-1 (ULK1) est faible dans cartilage humain atteint de l'arthrose et des modèles expérimentaux de l'arthrose. ULK1 est la sérine / thréonine protéine kinase et elle est l'inducteur principal de l'autophagie. La perte de l'expression de ULK1 se traduit par un niveau d'autophagie faible. Etant donné qu'une signalisation adéquate de l'autophagie est nécessaire pour maintenir la chondroprotection ainsi que l'homéostasie du cartilage articulaire, nous avons proposé l'hypothèse suivante : une expression adéquate de ULK1 est requise pour l'induction de l'autophagie dans le cartilage articulaire et une perte de cette expression se traduira par une diminution de la chondroprotection, et une augmentation de la mort des chondrocytes ce qui conduit à la dégénérescence du cartilage articulaire. Le rôle exact de ULK1 dans la pathogénie de l'arthrose est inconnue, j'ai alors créé pour la première fois, des souris KO ULK1

spécifiquement dans le cartilage en utilisant la technologie Cre-Lox et j'ai ensuite soumis ces souris à la déstabilisation du ménisque médial (DMM), un modèle de l'arthrose de la souris pour élucider le rôle spécifique in *vivo* de ULK1 dans pathogenèse de l'arthrose. Mes résultats montrent que ULK1 est essentielle pour le maintien de l'homéostasie du cartilage articulaire. Plus précisément, je montre que la perte de ULK1 dans le cartilage articulaire a causé un phénotype de l'arthrose accéléré, associé à la dégénérescence accélérée du cartilage, l'augmentation de la mort cellulaire des chondrocytes, et l'augmentation de l'expression des facteurs cataboliques. En utilisant des chondrocytes provenant des patients atteints de l'arthrose et qui ont été transfectées avec le plasmide d'expression ULK1, je montre qu'ULK1 est capable de réduire l'expression de la protéine mTOR (principal régulateur négatif de l'autophagie) et de diminuer l'expression des facteurs cataboliques comme MMP-13 et ADAMTS-5 et COX-2. Mes résultats jusqu'à présent indiquent que ULK1 est une cible thérapeutique potentielle pour maintenir l'homéostasie du cartilage articulaire.

Mot clés : Arthrose, ULK1, cartilage articulaire, autophagie, mort cellulaire, chondrocytes

## SUMMARY

Osteoarthritis (OA) is the most common musculoskeletal disease worldwide. It is one of the leading causes of pain and disability among adults, and represents a considerable burden on the healthcare system. OA is a disease of the entire joint, involving not only the articular cartilage but also the synovium, ligaments and subchondral bone. It is characterized by the progressive degeneration of the articular cartilage, osteophyte formation, remodelling of the subchondral bone, deterioration of tendons and ligaments and various degrees of inflammation of the synovium. While current therapies and management strategies can help alleviate symptoms early in the disease process, OA is characterized by almost inevitable progression towards end-stage disease.

The exact pathogenesis of OA is largely unknown but the key event in OA is the degradation of the articular cartilage. The articular cartilage is only composed of chondrocytes; cells responsible for the synthesis of the extracellular matrix (ECM) and maintenance of articular cartilage homeostasis. Chondrocytes maintain the articular cartilage matrix by replacing degraded macromolecules and respond to focal cartilage injury or degeneration by increasing local synthesis activity. Since chondrocytes exhibit low levels of turnover, they rely on endogenous mechanisms such as autophagy (a cell survival and adaptation process) to remove damaged organelles and macromolecules in order to maintain articular cartilage homeostasis.

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development and homeostasis. It regulates maturation and promotes survival of terminally differentiated chondrocytes under stress and hypoxic conditions. Studies by us and others have shown that compromised autophagy is associated with decreased chondroprotection, increased cell death and articular cartilage degeneration. Carames et al showed that autophagy is

constitutively expressed in normal human articular cartilage. However, expression of key autophagy inducers is reduced in ageing cartilage. Our previous studies have also identified a panel of key autophagy genes that are expressed in low levels in human OA cartilage as well as in the articular cartilage from mouse and dog models of experimental OA. Specifically, we identified that expression of unc-51 like kinase-1 (ULK1) is suppressed in human OA cartilage and experimental OA models. ULK1 is a serine/threonine protein kinase and is the most upstream autophagy inducer. Loss of ULK1 results in disruption of autophagy induction. Since adequate autophagy signaling is required for maintaining chondroprotection as well as articular cartilage homeostasis, we hypothesized that ULK1 is required for autophagy induction in the articular cartilage and loss of it will result in decreased chondroprotection and enhanced chondrocyte death leading to the degeneration of articular cartilage. Since the exact role of ULK1 in pathogenesis of OA is unknown, I created for the first time, an inducible cartilage-specific ULK1 knockout (KO) mice using Cre-Lox technology and subjected these mice to the destabilization of the medial meniscus (DMM) mouse OA model to specifically elucidate the specific in *vivo* role of ULK1 in OA pathogenesis. My results show that ULK1 is essential for maintaining articular cartilage homeostasis. Specifically I show that loss of ULK1 in the articular cartilage results in an accelerated OA phenotype; which is associated with accelerated cartilage degeneration, enhanced chondrocyte cell death, increased expression of catabolic MMP-13. Using human OA chondrocytes transfected with ULK1 expression plasmid I show that ULK1 is able to reduce the expression of mTOR (major negative regulator of autophagy) and decrease the expression of OA catabolic factors including MMP-13, ADAMTS-5 and COX-2. My results so far suggest that ULK-1 is a potential therapeutic target to maintain articular cartilage homeostasis.

Key words: osteoarthritis, ULK1, articular cartilage, autophagy, cell death, chondrocytes.

## CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

**1**. I for the first time generated cartilage-specific ULK1 KO mice.

**2.** I showed that loss of ULK1 in the cartilage results in accelerated cartilage degeneration in mice subjected to the mouse model of experimental OA.

**3.** I showed that loss of ULK1 in the cartilage results in accelerated chondrocyte cell death and catabolic activity.

4. I showed that loss of ULK1 in the cartilage results in enhanced synovial inflammation.

5. I transfected human OA chondrocytes with the ULK1 plasmid and showed a panel of genes that are regulated by ULK1.

My studies thus far suggest that ULK1 is essential for maintaining articular cartilage homeostasis; loss of which results in accelerated OA.

I performed all animal experiments, including breeding, genotyping, characterization of ULK1 KO mice, tissue dissection, histopathology, expression analysis, PCR arrays, qPCR analysis, chondrocyte isolation, chondrocyte culture studies using the ULK1 plasmid. I performed all data analysis, writing my thesis and manuscript under the supervision of my supervisor Dr. Kapoor. I did not perform the DMM OA surgery as this specialized surgery in my laboratory was performed by Dr. Anirudh Sharma.

My thesis has resulted in 1 original manuscript that will be submitted for publication at the end of August 2015 (see Manuscript 1 attached).

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# LIST OF ABBREVIATIONS

4E-BP1: 4E-binding protein 1

ADAMTS-4: a disintegrin and metalloproteinase with thrombospondin motifs 4

ATG-13: autophagy-related protein 13

BMPs : bone morphogenic proteins

CRP: C-reactive protein

COX-2: cycloxygenase-2

DMOADs: Disease-modifying OA drugs or agents

ECM : extracellular matrix

FIP200: 200 kDa focal adhesion kinase family interacting protein

HA: hyaluronic acid

IL-1β: interleukin-1 beta

IL-1: interleukin-1

IL-6 : Interleukin-6

IFP : infrapatellar fat pad

Ihh: Indian hedgehog

KO: knockout

LC3-1: microtubule-associated protein 1A/1B-light chain 3

mTORC1: mammalian target of rapamycin complex 1

MTORC1: MTOR complex 1

MMP : matrix metalloproteinases

MMP-1 : collagenase-1

MMP-13: collagenase-3

MSCs: Mesenchymal stem cells

NK: natural killer

NOS: NO synthase

NSAIDs: non-steroidal anti-inflammatory drugs

NF-kB: nuclear factor kB

OA: osteoarthritis

PTHrP: parathyroidhormone-related peptide

PPARγ: peroxisome proliferator-activated receptor gamma

$PGE_2$: prostaglandin $E_2$

PI(3)P: phosphatidylinositol 3-phosphate

PARP: Poly-ADP Ribose polymerase

ROS: reactive oxygen species

TGF- β: transformation growth factor B

TNF-α: tumor necrosis factor α

TIMPs: tissue inhibitors of MMPs

ULK1: unc-51-like kinase 1

VEGFA: vascular endothelial growth factor A

WT: wild type

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# INTRODUCTION

# 1. INTRODUCTION

## 1.1 Epidemiology of Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis. It is a chronic disease that has a devastating effect on the lives of many [1]. Data from National Arthritis Data workgroup in the US showed that the prevalence of clinical hand, hip and knee joint OA increased in just over a decade from 21 million US adults aged 25 years or older in 1995 to 27 million adults[2]. OA is also a burden on the economy mainly because of its debilitating effect on the lives of patients and the expenses associated with the treatment [3]. Many risk factors act together to trigger the onset of OA, those factors can be classified into 3 categories: modifiable local risk factors, modifiable systemic risk factors and non-modifiable systemic risk factors [4]. Age, sex, genetics and ethnicity fall into the category of non-modifiable systemic risk factors. Age is a strong predictor, a high percentage of individuals over the age of 65 show radiographic or clinical evidence of OA [5, 6]. With age, joints and cells lose their ability to adapt to mechanical stress. Though incidence of OA has been shown to be higher in females, it's unclear why this happens but some studies showed that this incidence increases in women at the time of menopause, which led to the hypothesis that estrogen plays a role in OA [7]. Studies suggested that the loss of estrogen caused by menopause increases women's risk of having OA, others studies showed that hormone replacement therapy in knee OA has a protective effect [8, 9]. Obesity, a modifiable systemic risk factor, is one of the main causes leading to OA at peripheral weight bearing joints such as the knee and the hip [10]. Other local risk factors include joint injury, muscle strength and physical activity [11-13].

## 1.2 Joint structures affected during OA

OA patients experience pain in their joints, tenderness, stiffness, locking and reduced range of motion. OA is characterized by the degradation of the cartilage, remodeling of the subchondral bone and the inflammation of the synovium. It is not just a cartilage disease like it was initially perceived; the entire joint is affected. OA progresses in three stages: in stage I, breakdown of cartilage matrix by proteolytic enzymes is observed. In stage II, there is fibrillation and erosion of the cartilage surface accompanied by the release of breakdown products into the synovial fluid. Finally in stage III, synovial cells ingest a breakdown product through phagocytosis and produce proteases and proinflammatory cytokines leading to the inflammation of the synovium [14]. Other changes in the joint during OA include formation of osteopthytes, changes in the joint capsule, ligaments and periarticular muscles and meniscal tears and extrusion [15, 16]. Loss of articular cartilage is hallmark of OA and will be discussed in detail later in the introduction.

### Subchondral Bone

Subchondral bone is now regarded as an active player in pathogenesis of OA. Subchondral bone consists of the subchondral bone plate, the underlying trabecular bone and bone marrow space. The subchondral bone plate consists of cortical bone and is separated from the articular cartilage by the zone of the calcified cartilage [17]. Bone remodeling and modeling are two mechanisms that modify the subchondral bone and allow the repair of any damage caused during mechanical loading. The remodeling cycle is initiated by osteoclasts that mediate bone resorption, followed by bone formation mediated by osteoblasts. There is an equilibrium between those two processes in order to maintain the bone mass [18, 19]. During OA, these mechanisms are altered causing changes in the subchondral bone. These changes, including sclerotic changes and the

development of bone marrow lesions, can be seen using magnetic resonance imaging (MRI). Bone cysts formation and osteophytes-formation of new bone at the joint margins- follow [20, 21]. Bone cysts are frequently observed in advanced stages of OA in areas where severe destruction of the cartilage occurs, the synovial fluid enters the bone marrow leading to the formation of the cysts [17]. The formation of osteophytes is one of the main radiographic signs of OA, they can form in early stages of OA and can lead to pain as well as loss of function. The exact process with which osteophytes are formed is unknown. However, it is believed that mesenchymal stem cells (MSCs) present in the periosteum (the membrane covering the outer surface of all bones) or present in the synovial lining at the joint margin differentiate into chondrocytes which then undergo differentiation and hypertrophy followed by endochondral ossification and deposition of bone [22]. Transformation growth factor B (TGF- β) is expressed in osteophytes and is involved in the initial steps of their formation as well as development [23].

## Synovium

Synovium is another key joint structure that is actively involved in pathogenesis of OA. Inflammation of the **synovium** also known as synovitis is associated with pain and progression of OA. It is believed that inflammation occurs secondary to the onset of the degradation of the cartilage, which suggests that it is caused by cartilage breakdown[24].

In contrast, other studies showed that synovial changes are likely to occur primarily in OA. One study for example showed that most patients with early OA show thickening of the synovial membrane and this thickening was caused by the infiltration of mononuclear leukocytes, mostly T cells. This infiltration indicates synovial inflammation thus confirming that the thickening in early OA was due to synovitis and that synovial changes occur first.[25, 26]

In the first case, as a result of the breakdown, microcrystals, osteochondral fragments and products of the extracellular matrix (ECM) degradation are secreted in the joint cavity leading to the secretion of cytokines, chemokines and matrix metalloproteinases (MMP) that will further degrade the cartilage and deregulate its homeostasis [27]. Under normal conditions, the cells of the synovial membrane secrete lubricin and hyaluronic acid to protect the surface of the articular cartilage. Macrophages like cells as well as fibroblasts like synoviocytes are the major source of synovial fluid components [28, 29]. During OA, inflammation leads to the recruitment of T cells, mainly CD4+ and CD8+, B cells, monocytes and macrophages to the synovial membrane [27]. Fragments of the degraded cartilage activate the innate immune system by interacting with toll-like receptors expressed on the synovial cells. This interaction causes the activation of transcription factors mainly nuclear factor kB (NF-kB) which leads to the secretion of cytokines, chemokines, reactive oxygen species (ROS) and MMPs. Those secreted factors provoke tissue damage, recruitment and activation of further immune cells and angiogenesis [24]. It is believed that angiogenesis in OA does not initiate inflammation but it permits its persistence by allowing infiltration of inflammatory cells [30]. Pro-inflammatory cytokines in the synovial fluid include interleukin-1 beta (IL-1β), tumor necrosis factor α (TNF-α). IL-1β is able to induce cartilage destruction as it suppresses aggrecan and collagen synthesis in chondrocytes, and also can up-regulate the proteolytic enzymes ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs 4) and MMP-13 [31-34]. Like interleukin-1 (IL-1), TNF α can activate chondrocyte-mediated catabolic protease production [35]. IL-1 β and TNF α also induce the E2 [36-38].

## Menisci and Infrapatellar fat pad

The menisci are two crescent-shaped fibrocartilaginous structure in a joint cavity between the femur and the tibia in the medial and lateral side. Its function is to spread the load of the weight of the body, reduce friction during movement and absorb shocks that the joint undergoes. A healthy meniscus protects the articular cartilage and lubricates the joint. In OA, the meniscus appears to be torn, fragmented or even completely destroyed [39]. The degeneration begins on the surface of the meniscus and then spreads to the articular surface, progressing to total loss predominantly in the avascular zone [40]. The meniscus also loses type I collagen in all its zones gradually increasing from the surface to the deep zone; but the loss in type II collagen is severe in the surface zone as well as the middle and deep zones [41]. The meniscus loses its ability to withstand loading and force transmission with the development of the disease leading to further degenerative tears and meniscal extrusion [42, 43].

The **infrapatellar fat pad (IFP),** also known as Hoffa's fat pad, is a cylindrical piece of fat that is situated intracapsularly and extrasynovially underneath the patella bone (knee cap) within the knee. IFP is composed of adipose tissue and improves lubrication of the knee joint by enlarging the synovial area [44]. The IFP is thought to have an important role in knee function; studies have shown that it is conserved under extreme starvation conditions when the subcutaneous adipose tissue is eliminated [45]. Besides this role, very little is known about the function of the IFP in the knee joint but it is thought to play an important role in the development and progression of knee OA [46]. The IFP has been shown to be a site of inflammation; studies showed that IFP in OA patients contains significant levels of IL-6, TNFα, vascular endothelial growth factor and fibroblast growth factor [47]. The IFP is a source of adipokines such as

adiponectin and leptin who have been shown to be upregulated in late stage OA [48]. Leptin is known to stimulate IL1β production, to increase the effect of pro-inflammatory cytokines and induce the expression of MMPs in the OA cartilage [49-51]. Adiponectin induces MMP1 and IL6 production in synovial fibroblasts [52]. The adipose tissue in the IFP is composed mainly of adipocytes and is considered to be a site where a large number of immune cells are present. These immune cells include macrophages, mast cells, natural killer (NK) cells, NKT cells, T cells, and B cells and are involved in inflammation [53, 54]. The IFP also contains peptidergic C-fibers that are nociceptive nerve fibers staining positive for substance P, which is an important element in pain perception and causes vasodilation leading to diapedesis of immune cells and edema. This proves that IFP is an important source of pain in knee OA [55]. Infiltrated immune cells vary in functions: activated macrophages produce various growth factors, cytokines and enzymes that enhance osteophyte formation. Neutrophils play a role in cartilage breakdown and necrosis of adipose tissue by the production of cytokines such as IL1, IL8 and MMP8 [56, 57]. Eosinophils and basophils release histamine, which increases production of matrix degrading enzymes and pro- inflammatory mediators in synovial fibroblasts and cartilage [58]. Lymphocytes from OA joints express Th1 cytokines which can directly degrade cartilage or activate macrophages through cell-cell interactions to produce cartilage degrading mediators [59].

As we can see OA is a whole joint disease.

## Cartilage

### (a) Cartilage growth and development

The cartilage is a connective tissue composed of one type of cells: the chondrocytes formed by the process called chondrogenesis. Intramembranous ossification and endochondral ossification are the two processes by which the skeleton develops. While the first process only occurs in the flat bones of the skull, the second process takes place in the rest of the skeleton. In intramembranous ossification, the MSCs differentiate directly into osteoblasts, while in endochondral ossification the cartilage is formed when embryonic MSCs condense and differentiate into chondrocytes. The cartilaginous matrix template is then replaced by bone matrix [60, 61]. Chondrocytes secrete the components of cartilage ECM including type II collagen and the proteoglycan aggrecan [62]. Type II collagen provides tensile strength to the cartilaginous matrix, while aggrecan attracts water molecules and forms large aggregates in the cartilage and provides a cushioning role to the matrix. Aggrecan also plays a role in the immobilization and storage of growth factors thus acting as an organizer of the ECM and cartilage [63-65].

Endochondral ossification begins during fetal life and continues until growth stops in the early adulthood [61]. The first step of chondrogenesis is the condensation of MSCs that leads to the expression of the transcription factor Sox9, a key regulator of chondrogenesis. Cells undergoing chondrogenesis acquire a distinct spherical morphology [66]. Studies have shown that inactivation of Sox9 before mesenchymal condensations resulted in a complete absence of both cartilage and bone and that Sox9 deletion after mesenchymal condensations showed a severe

generalized chondrodysplasia as condensed mesenchymal cells and did not undergo differentiation into chondrocytes [67].

The morphology of chondrocytes in the growth plate changes progressively based on their role in the process of endochondral ossification. Chondrocytes are initially small and round in a resting phase, after which they will undergo proliferation that will allow the expansion of the cartilage. The proliferating cells are flat, stacked in columns and located in the center of the cartilage. They express the transcription factors Sox5, Sox6 and Sox9, and the structural proteins collagen, type II, α1 and aggrecan. Following this step, proliferating chondrocytes mature into pre-hypertrophic chondrocytes that express both parathyroid hormone 1 receptor and Indian hedgehog (Ihh). This is followed by maturation into early hypertrophic chondrocytes that express collagen, type X and α1. Hypertrophic chondrocytes will then deposit forming the primary center of ossification. Late hypertrophic chondrocytes secrete vascular endothelial growth factor A (VEGFA) and MMP-13 whose expression permits the invasion of the growth plate by osteoclasts and osteoblast precursors. Osteoclasts remove cartilage ECM and osteoblasts deposit bone allowing the bone to elongate longitudinally [61, 63, 68, 69]. Most hypertrophic chondrocytes undergo apoptosis before the ossification front [70].

Endochondral ossification is an important process in the creation and development of long bones and articular cartilage, thus proper regulation is necessary [61]. It is also essential in the natural healing of bone fractures [71]. Imbalance in the regulation could lead to skeletal deformities and dwarfism [72]. The proliferation of chondrocytes in the growth plate is controlled by three signaling molecules synthesized by the chondrocytes themselves: parathyroidhormone-related peptide (PTHrP), Ihh, and TGF- β. The role of TGF- β on the cell is mediated in part by the

transcription factor, Smad 3. TGF- β signaling through Smad-3 is not critical for development of a normal skeleton at birth, but it is important for normal postnatal growth and development.[72] Studies have showed that mice deficient in Smad-3 have a completely normal skeleton at birth, but when they reach 3 weeks, they begin to exhibit cartilage abnormalities, including premature hypertrophy of both growth plate and articular chondrocytes, and disorganization of the growth plate columns resulting in decreased longitudinal growth.[73] TGF- β signal transduction pathway and its components can also regulate PPARγ (peroxisome proliferator-activated receptor gamma) expression[74]. A study showed that PPARγ KO mice exhibited reductions in body length, body weight, length of the long bones, skeletal growth, cellularity, bone density, calcium deposition, abnormal organization of the growth plate. They also showed loss of columnar organization, shorter hypertrophic zones, and delayed primary and secondary ossification.[75]

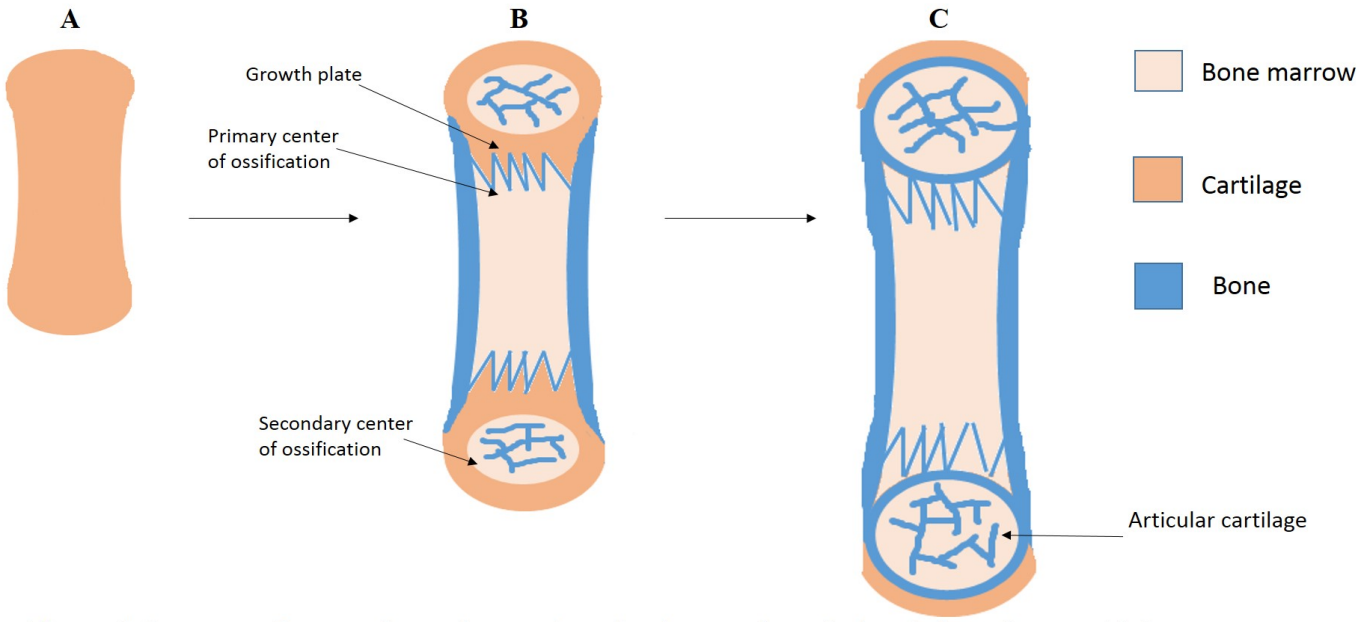


***Figure 1***| *Overview of bone and articular cartilage development by endochondral ossification. (A) In the first phase the cartilage model the future bone is formed. (B)The primary center of ossification is then followed by the formation of the secondary center of ossification leaving a cartilaginous growth plate between primary and secondary centers of ossification. (C) In the last phase, the growth plate cartilage is then completely replaced by bone, leaving articular cartilage only at the ends.*

**(b) Articular Cartilage**

The articular cartilage is a hyaline cartilage with a thickness of 2 to 4 mm; it is a highly specialized connective tissue of diarthrodial joints (movable joints characterized by the presence of cartilage that lines opposing bony surfaces). The articular cartilage lacks blood vessels, nerves and lymphatics [76]. Chondrocytes are the only cell type present in the articular cartilage; they differ in size, shape and metabolic activity in different zones of the cartilage [77, 78]. Chondrocytes are responsible for the synthesis of the ECM the main constituent of the articular cartilage. The ECM is composed mainly of water, collagen, proteoglycans and smaller amounts of non-collagenous proteins and glycoproteins. The role of these components is to maintain the mechanical properties of the cartilage by retaining water [79]. Therefore, the function of the

articular cartilage is to provide a smooth, lubricated surface for articulation and to facilitate the transmission of loads with a low frictional coefficient. Articular cartilage has a limited ability to repair itself and this ability decreases with age. Because it is subjected to a harsh biomechanical environment, the preservation of a healthy articular cartilage is important for keeping a healthy joint [76].

The articular cartilage is divided into four different zones: the superficial zone, the middle (or transitional) zone, the deep zone and the calcified zone. Each zone is then divided into 3 regions: the pericellular region, the territorial region and the interterritorial region [76]. The superficial zone is responsible for the tensile properties of the cartilage allowing it to resist compressive forces imposed by articulation [80]. This zone contains flattened chondrocytes that synthesize a matrix with high collagen concentration and low proteoglycan concentration. In the middle zone, chondrocytes are spherical and at low density and they are responsible for the secretion of thicker collagen fibrils and a high concentration of proteoglycans. The middle zone is the first line of resistance to compressive forces [76, 78]. The deep zone provides the most resistance to compressive forces; it consists of the largest diameter of collagen fibrils, highest concentration of proteoglycans between the zones and lowest water concentration. The collagen fibrils are arranged perpendicularly to the surface and the chondrocytes are stacked in columns also perpendicular to the joint surface [76, 78]. A tidemark separates the deep zone from the calcified zone. The calcified layer anchors the collagen fibrils from the deep zone to the subchondral bone therefore securing the connection between cartilage and bone. This zone is rare in cells and chondrocytes are hypertrophic [76, 81].

The relationship between the chondrocytes and the ECM consists of the chondrocytes secreting the matrix macromolecules and the matrix protecting the chondrocytes, their shape and

phenotype from mechanical damage during the use of the joint. Chondrocytes maintain the articular cartilage matrix by replacing degraded macromolecules and responding to focal cartilage injury or degeneration by increasing local synthesis activity. This is controlled by anabolic and catabolic cytokines. IL-1 plays a role in the degradation of the matrix macromolecules by inducing the secretion of matrix metalloproteinases. On the other hand, insulin-dependent growth factor 1 and TGF- β stimulate matrix synthesis and cell proliferation [82-84].

Each chondrocyte establishes a microenvironment and is responsible for the turnover of the ECM in its immediate vicinity. Tissue fluid represents between 65% to 80% of the total weight and the macromolecules account for the remaining weight [85]. Among the structural macromolecules of the ECM, collagen is the most abundant, contributing to about 60% of the dry weight of articular cartilage. Type II collagen is the principal molecular component constituting 90% to 95% of the collagen in the ECM. Collagen types- I, IV, V, VI, IX, X and XI all contribute to the mature matrix but in minor proportions. They help form and stabilize the collagen type II fibril network [76, 86]. All members of the collagen family contain a region of 3 polypeptide chains wound intro a triple helix whose function is to stabilize the matrix [87]. The second-largest group of macromolecules in the ECM are proteoglycans; they represent 10% to 15% of the wet weight. The largest and most abundant type of proteoglycan is aggrecan, it interacts with hyaluronan to form large proteoglycan aggregates via link proteins. This aggregation anchors aggrecans within the ECM, which provides the cartilage with its osmotic properties that are essential for its ability to resist compressive loads. The second type of proteoglycans is the small non-aggregating proteoglycans: decorin, biglycan and fibromodulin. Their role is to interact with collagen: decorin and fibromodulin play a role in in fibrillogenesis

and interfibril interactions by interacting with type II collagen fibrils. And biglycan is present in the immediate surroundings of chondrocytes where it may interact with type VI collagen [88-92].

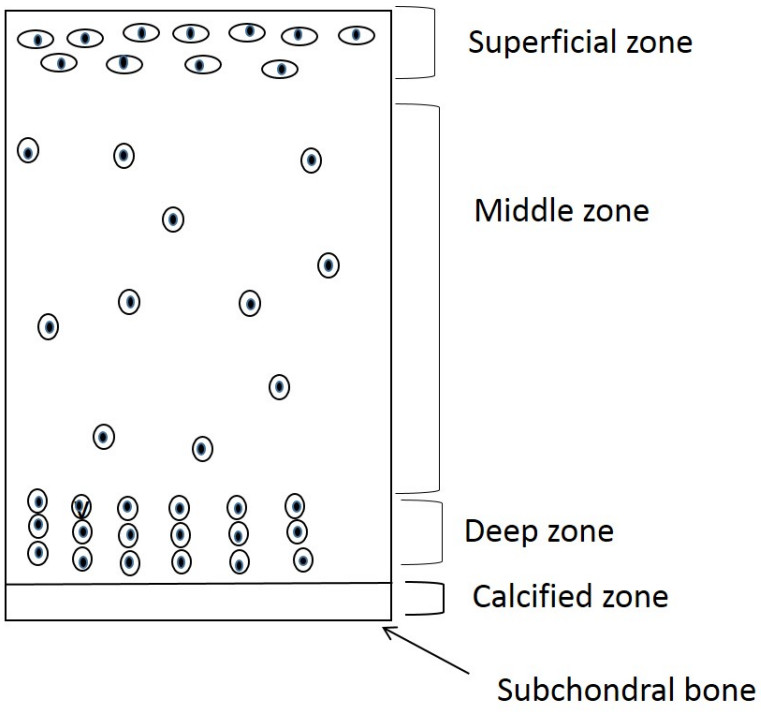


***Figure 2|*** *Schematic showing the cellular organization in the different zones of the articular cartilage.*

**(c) Articular Cartilage degeneration during Osteoarthritis**

OA is characterized by degeneration of the whole joint; however, articular cartilage degeneration can be regarded as the major structural change within the joint. In fact, the earliest OA microscopic change observed is the roughening and degeneration of the superficial zone of the articular cartilage: the dense collagenous matrix of the superficial zone is either disrupted or remodelled. Those changes in this zone alter its role of resisting compressive forces therefore contributing to further destruction of articular cartilage. The superficial zone also acts as a barrier

in isolating the cartilage from the immune system so disruption of this zone may allow the release of molecules that stimulate an immune or inflammatory response in the cartilage [93, 94]. As the disease progresses, the articular cartilage becomes roughened and irregular and fibrillation extends deeper into the zones of the cartilage until the cleft reaches the subchondral bone. Fragments of the cartilage get released into the joint space decreasing its thickness. Enzymatic degradation of the matrix leads to a decrease in the cartilage volume. With the progression of OA from the early to severe form, the patient is left with no cartilage and eburnated bone [95-97].

The progressive loss of articular cartilage in OA can be divided into three overlapping phases: cartilage matrix damage or alteration, chondrocyte response to tissue damage, progressive loss of tissue due to the decrease in the chondrocyte synthetic response [98-101]. In the first stage, there is damage or alteration in the matrix at the molecular level and the water content increases. There is a decrease in the proteoglycan aggregation and aggrecan concentration, and a decrease in the length of the glycosaminoglycan chains, while the concentration of type II collagen stays stable. Those changes lead to the increase in the permeability and decrease in the stiffness of the matrix causing an increase of the vulnerability of the cartilage to mechanical damage. There is a rapid loss of proteoglycans with the progression of OA. And although the collagen content is initially maintained, its organization is disrupted leading to a decrease in tensile stiffness and strength in the cartilage [98, 99, 102-104].

The second stage starts when the chondrocytes detect the tissue damage or the alterations in the osmolarity, in response they secrete catabolic and anabolic factors and proliferate. Anabolic factors including TGF- β, bone morphogenic proteins (BMPs) and insulin-like growth factor I stimulate synthesis of ECM macromolecules and proliferation of chondrocytes [78, 105]. In

response to mechanical or chemical stress chondrocytes secrete nitric oxide, which induces the production of the cytokine IL-1 β. This IL-1 β stimulates the expression of metalloproteases that degrade the matrix macromolecules [106]. The production of IL-1 β as well as proteases is further induced by the presence of damaged tissue. Degradation of the type IX and type XI collagens and other molecules may destabilize the type II collagen fibril meshwork. Although it doesn't harm the type II fibrils, it allows expansion of the aggrecan and increasing its water content. A repair response is triggered to counteract the catabolic effect of the proteases, which will stabilize or even restore cartilage tissue [78, 107].

The third stage is characterized by a progressive loss of articular cartilage after failure of stabilizing the loss in stage two and a decline in the anabolic response of chondrocytes as well as their proliferation. Mechanical damage, chondrocyte cell death and loss of the chondrocyte's ability to respond leads to a decline in the anabolic response [98, 99, 108]. The ability of chondrocytes to maintain a healthy cartilage decreases with age therefore increasing the risk joint degeneration and OA [109]. With aging, chondrocytes undergo stress that causes alterations in their content, composition and the structural organization of collagen and proteoglycan [110].

MMPs are responsible for the degradation of the articular cartilage in OA. Collagenases, in particular collagenase-1 (MMP-1) and collagenase-3 (MMP-13) are involved in type II collagen degradation [14, 111]. Proteoglycan degradation is mainly caused by stromelysin-1 (MMP-3) and aggrecanase-1 (ADAMTS-4) [112, 113]. Plasminogen activator/plasmin and cathepsin B act as activators of MMPs in the joint, and tissue inhibitors of MMPs (TIMPs) inhibit their activity. An imbalance between MMPs and TIMPs is observed in OA tissues [104]. MMPs act as the principal effectors in the degradation of the matrix; their actions are triggered by mechanical loading that affects the viability of chondrocytes and matrix breakdown [114]. The response by

the MMPs is further amplified by the expression of inflammatory cytokines. Proinflammatory cytokines such as TNF- α, IL-1 β and IL-6 have been shown to modulate chondrocyte metabolism to increase the synthesis of MMPs and inhibit the synthesis of TIMPs, collagen and proteoglycans. In other words, proinflammatory cytokines disrupt cartilage homeostasis and help in its degradation in OA. They mediate their activity through interactions with high affinity cell surface receptors [14].

IL-1 β is the main cytokine involved in destruction of the articular cartilage and TNF- α drives the inflammatory process. IL-1 β is synthesized as an inactive precursor and is activated by IL-1 β converting enzyme or ICE or caspace-1, a protease belonging to the cysteine-dependent protease, to reach its maturity and become active. ICE is produced in the synovial membrane and cartilage; its expression is increased in OA tissues compared to normal tissue [115, 116]. TNF- α acts as a trimer, it is also synthesized in an inactive form and is activated by TNF- α converting enzyme (TACE), whose levels have been shown to be elevated in OA [117, 118]. The effect of IL-1 β on MMP synthesis and the production of proteoglycans are amplified by IL-6, which increases the amount of inflammatory cells in synovial tissue [119-121].

As mentioned previously, OA is associated with inflammation. Different systemic markers can be measured to track the progression of the disease. For example, an increased concentration of systemic C-reactive protein (CRP) more specifically high sensitivity CPR (hsCRP) can be used as a predictor of rapid disease progression. Several studies have showed that CRP levels are highly elevated in patients with OA compared to normal controls. CRP levels are also associated with level of pain, clinical severity, and disability [122-124].

Neoepitope antibodies recognize the newly created N or C terminus of protein degradation products. The ones that recognize the C-terminal neoepitope of this cleavage

site have been developed as well as antibodies to denatured collagen. These antibodies have been used in many studies to demonstrate increased collagen cleavage and degradation in human and animal models of OA. Antibodies to the neoepitopes generated in aggrecan by the enzymatic activity of MMPs and aggrecanases were also developed. For example, NITEGE and DIPEN epitopes are generated in the cartilage because of the action of aggrecanases and MMPs on the aggrecan.[125, 126]

***Table 1|*** *The inflammatory and catabolic factors that play a role in articular cartilage degeneration.*

| Inflammatory factors | Catabolic factors |
|---|---|
| TNF- α | MMP-1 |
| IL-1 β | MMP-13 |
| IL-6 | MMP-3 |
| IL-8 | ADAMTS-4 |
| MCP-1 | ADAMTS-5 |
| NO | |
| PGE2 | |

Tumor necrosis factor α (TNF-α),interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), monocytes chemoattractant 1 (MCP-1), nitric oxide (NO), prostaglandin E2 (PGE2). Collagenase-1 (MMP-1), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), aggrecanase-1 (ADAMTS-4), and aggrecanase-2 (ADAMTS-5).

## 1.3 Current therapies to treat Osteoarthritis

Despite all the efforts and the clinical trials conducted to find a treatment for OA, an effective treatment that will prevent or stop the disease as well as its progression is yet to be discovered. Diagnosis of OA is based on radiological and clinical features; pain is the main symptom that leads patients with OA to seek medical help. They also experience stiffness and loss of

movement that decreases their quality of life. Current therapies for OA rely on symptom control using a combination of pharmacological and non-pharmacological approaches as well as surgical approaches in end stage cases [127, 128].

Non-pharmacological approaches include patient access to information and education about their disease, exercise programs both land and water based and weight loss especially in overweight patients. Those approaches help with the pain but also with the physical function making the life of patients a little bit easier [129].

Pharmacological treatments are also used to treat pain and stiffness but none of these treatments have shown to have any effect on the progression of the disease. These therapies include analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), and intra-articular injections of corticosteroids and of hyaluronate. Disease-modifying OA drugs or agents (DMOADs) are another class of drugs that target the changes occurring in OA, they have the most potential with their effect being the future of curing OA. Unlike RA, we currently have no approved DMOADs [129, 130].

The most commonly used analgesic and the first-line treatment agent for OA is acetaminophen. It is less effective than NSAIDs but has less adverse effects as well, making it the first line of treatment; patients who do not respond to acetaminophen will get NSAIDs [131, 132]. Side effects of acetaminophen include gastric ulcerations and bleeding, increased risk of loss of renal function with long-term consumption and hypertension when used in high doses [133, 134].

NSAIDs act as anti-inflammatory agents to reduce joint inflammation, the most commonly used type is cycloxygenase-2 (COX-2) inhibitors also called coxibs. NSAIDs have several side effects including prolonged bleeding time by interfering with platelet function, gastrointestinal ulceration and bleeding, renal toxicity and an increased risk of cardiovascular events [135].

Intra-articular injections of corticosteroid have an anti-inflammatory effect and are recommended for OA inflammatory flares. Injections of hyaluronic acid (HA) are also frequently used in OA patients. HA is a constitutive component of the ECM and of the synovial fluid; its injection should increase the viscoelasticity of synovial fluid that was decreased in OA and possibly prevent the degradation of the articular cartilage [135].

DMOADs should be able to slow the progression of OA, not only treat the pain. No DMOAD has been approved yet but several drugs are currently undergoing development. DMOADs fall into three categories: DMOADs targeting the cartilage by inhibiting catabolism (inhibitors of MMPs) and stimulating anabolism (growth factors). Second category includes DMOADs that target the synovial membrane (cytokines and IL-1 β inhibitors). Finally, thirds category includes DMOADs targeting the subchondral bone (inhibitors of bone resorption and stimulation of bone formation) [136]. Tissue engineering using MSCs has been emerging as an alternative method for treating OA. MSCs are multipotent progenitor cells found in various tissues, most commonly bone marrow and adipose tissue. MSCs are capable of differentiating into osteocytes, adipocytes, and chondrocytes [137] and their ability to halt or regenerate articular cartilage is being tested in various pre-clinical studies.

## 1.4 Articular Cartilage homeostasis

Since chondrocytes are the only cell types present in the articular cartilage, they are therefore critical for maintaining homeostasis as well as integrity of the ECM within the articular cartilage. Chondrocytes are postmitotic cells, they exhibit low rate of cell replication; because of that the articular cartilage has a decreased ability to maintain and repair itself. Chondrocytes rely on endogenous processes to remove damaged organelles and macromolecules that accumulate due

to the low turnover. [104, 105, 138]. In normal articular cartilage, the role of the ECM is to deal with mechanical stress without structural or cellular damage; it is responsible for protecting chondrocytes from the damaging biomechanical force. On the other hand, the role of the chondrocytes is to produce and maintain the ECM by sensing and responding to changes in the ECM and degrading or synthesizing appropriate types and amounts of macromolecules. With age, the capacity of chondrocytes to synthesize certain proteoglycans, and their proliferative capacity decrease. Their response to extracellular stimuli for example anabolic stimuli including growth factors is impaired. As a result, the ability of chondrocytes to maintain and restore articular cartilage decreases, and gene expression as well as cell differentiation are impaired. Understanding the exact chondrocyte cell death/survival mechanisms could lead prevention of cartilage degradation and to several promising OA therapeutic strategies [78, 139, 140].

**Cell senescence**

Mitotic cells lose their ability to divide in culture after 30-40 population doublings; this phenomenon is known as cell senescence. “Hayflick limit” or “replicative senescence” is when the proliferation of dividing cells declines after many cell doublings and eventually all cells lose their ability to divide[141, 142]. Cell senescence prevents cells with damaged DNA from replication. Replicative senescence is associated with changes in DNA structure and function including a shortening in the telomeres accompanied by telomeres dysfunction as well as gene mutations and damaged mitochondrial DNA [143, 144]. Progressive telomere shorting due to repeated cycles of cell division does not explain senescence in post-mitotic cells such as neurons, or quiescent cells such as chondrocytes. This lead to the discovery of other forms of cell senescence which are referred to as “extrinsic” or “stress-induced” senescence as opposed to the

intrinsic senescence resulting from replication. Stress induced senescence can be caused by different factors including: ultraviolet radiation, oxidative damage, activated oncogenes, and chronic inflammation. Oxidative damage can cause damage to DNA at the chromosomes' ends, which are sensitive to this kind of damage, leading to telomeres shortening which is similar to what happens in replicative senescence [139, 144, 145]. The expression and activity of regulatory proteins that control growth and proliferation are altered in senescent cells; this includes p53 and the cyclin-dependent kinase inhibitors $p21^{CIP1}$, and $p16^{INK4A}$. P53 activation is triggered by DNA damage or by telomere shortening and its role is to inhibit cell-cycle progression. The activation of p53 leads then to the increased expression of p21, which contributes to senescence. As p21 declines in senescent cells, p16 is increased leading to a long-term inhibition of cell-cycle progression through inhibition of retinoblastoma protein [145, 146]. Senescent secretory phenotype is another change that occurs in senescent cells resulting in increased production of cytokines, growth factors and metalloproteinase. This phenotype can lead to the development and progression of OA because of the inflammation caused by cellular senescence through the senescence associated secretory phenotype. This inflammation is caused by molecules like TNF-α, IL-6, MMPs, monocyte chemoattractant protein-1 (MCP-1), and IGF binding proteins (IGFBPs) that increase in multiple tissues with chronological aging [145, 147-149]. Chondrocyte senescence is induced by chronic stress. Studies have shown that telomere shortening noted in chondrocytes could be due to DNA damage from reactive oxygen species (ROS) [150, 151]. Aging can cause an increase in chondrocyte ROS levels. However, excessive mechanical loading and/or stimulation by cytokines could generate ROS and contribute to DNA damage and subsequent telomere shortening. Oxidative stress results when the amount of ROS exceeds the anti-oxidant capacity of the cell. Oxidative stress may play a major role in the link

between aging and the development of OA by leading to chondrocyte senescence and cartilage ageing [152-155]. In a study, bovine articular cartilage explants were exposed to pro-oxidant hydrogen peroxide. The results showed that oxidative stress induces expression of OA markers that are related to abnormal chondrocyte phenotype, like 3B3(-), nitrotyrosine and procollagen type IIA epitopes in the explants[156]. Another study showed that increased oxidative stress with aging makes chondrocytes more susceptible to oxidant mediated cell death through the dysregulation of the glutathione antioxidant system. This might highly contribute to the development of OA in older people[152].

**Cell death**

Apoptotic cell death has become a focus of interest and has been suggested to be a critical event in the degeneration of the articular cartilage in OA. The number of apoptotic cells in OA cartilage is higher than the one in normal cartilage [157, 158]. Apoptosis is programmed cell death and plays an important role in physiologic cell removal, especially during fetal development. Necrosis describes non-programmed cell death while apoptosis is used for programmed cell death. Apoptosis is also important for the removal of hypertrophic chondrocytes in the fetal growth plate cartilage, and represents a complex process involving an effector phase, a degradation phase, and a clearing phase [159]. The effector phase is characterized by an increase in intracellular free $Ca^{2+}$, activation of endonucleases, tissue transglutaminase, and cellular proteases (especially the caspases), as well as mitochondrial dysregulation. In addition, the activity of several genes, most importantly, c-myc and members of the bcl-2 family, as well as p53, is increased. These factors lead to a “point of no return” in

which the apoptotic destiny of the cells is fixed. In the degradation phase, extensive degradation of the nucleic acids and proteins occurs. Endonucleases and tissue transglutaminase are further activated, and the cytoskeleton is reorganized. The clearing phase is characterized by the clearance of the apoptotic cells by macrophages, this feature does not occur in adult articular cartilage because of its lack of macrophages. Chondrocyte-derived apoptotic bodies express functional properties that may contribute to the pathological cartilage degradation [160].

Chondrocyte apoptosis is a complex process, and it is important to understand the mechanisms that cause it. Lower zones of OA cartilage show progressive matrix calcification, which led to believe that the increase in phosphate and calcium ions could be a mechanism responsible for chondrocytes apoptosis.[161] *In vitro* culture was used to study the stimuli causing apoptosis in chondrocytes. Cultured chondrocytes undergo apoptosis in response to various stimuli, including serum deprivation, or in response to treatment with Fas ligand or anti-Fas/CD95 antibodies, NO-donor sodium nitroprusside, staurosporine/dihydrocytochalasin B, ceramide, and retinoic acid [161-164].

The role of NO synthase (NOS) has been carefully studied in the pathogenesis of OA, even before it was thought to have a role in the induction of chondrocyte apoptosis. OA cartilage is thought to produce a large amount of NO, which is why NO could be an initiator of chondrocyte apoptosis in the cartilage. NO generated from sodium nitroprusside induces expression of cyclooxygenase 2, possibly through the extracellular signal-regulated kinase 1/2 and p38 kinase pathways, which results in an increase in release of prostaglandin $E_2$ ($PGE_2$) in human OA chondrocytes. The exact role of $PGE_2$ however is still not clear. One study showed that the treatment of chondrocytes from bovine articular cartilage with $PGE_2$ resulted in the induction of DNA fragmentation. This DNA fragmentation was accompanied with a marked dose-dependent

increase in intracellular cAMP. However, a different study using human OA chondrocytes reported that $PGE_2$ sensitizes chondrocytes to NO-induced apoptosis, rather than inducing apoptosis directly [165, 166].

Another well-known apoptosis-inducing pathway is CD95/CD95 ligand–mediated signal transduction. Chondrocytes in OA chondrocytes express Fas receptor; therefore, activation of this pathway was suggested to mediate chondrocyte apoptosis in human OA. The proteasome inhibitors MG-132 and PS1 potentiated CD95 antibody-induced apoptosis, and this was associated with a reduced nuclear translocation of NF-kappaB. Proteasome inhibitors also caused the induction of DNA fragmentation by tumor necrosis factor alpha. These results suggest that CD95-dependent cell death is enhanced by NF-kappaB inhibition at and/or downstream of caspase 8 activation [163, 167].

Attachment of cells to one another or to the ECM is essential for growth, function and survival. This is known as anchorage dependence; cells that fail to do so will undergo apoptosis. During OA, the cartilage degradation is accompanied with loss of proteoglycan and perturbation of the collagen network; this will alter the environment and the anchorage of chondrocytes to the ECM leading to an increase in apoptosis [168]. Studies have showed that type II collagen, the major component of the ECM, is important for the survival of chondrocytes in articular cartilage. In mouse model lacking type II collagen chondrocyte apoptosis increased during embryogenesis, also *in vitro* cell culture showed that removal of collagen by collagenase resulted in chondrocyte apoptosis, but re-addition of collagen restored cell viability [169, 170].

It is still unclear whether apoptosis is primary or secondary to the destruction of the articular cartilage. Chondrocyte apoptosis combined with matrix loss forms a vicious cycle leading to accelerated development of OA.

## 1.5 Autophagy

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development, and homeostasis. In lower eukaryotes, it was thought to be just a response to starvation; however, in higher eukaryotes autophagy has various physiological functions. There are two types of protein degradation system: selective degradation carried out by ubiquitin-proteasome system and autophagy a non-selective degradation of cytoplasmic components that occurs in the lysosome [171, 172]. Autophagy occurs to in response to extracellular stress including nutrient starvation, energy deprivation, hypoxia, overcrowding and high temperature; and in response to intracellular stress including accumulation of damaged organelles and cytoplasmic components and reactive oxygen species [173].

The three types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy. In chaperone-mediated autophagy, substrate proteins are specifically recognized by chaperones and directly transported across the lysosomal membrane to the lumen. Microautophagy occurs by invagination of the lysosomal membrane into the lumen, the cytoplasmic material is directly engulfed into the lysosome. Macroautophagy, which we will focus on hereafter, is mediated by the autophagosome. Once autophagy is induced, a portion of the cytoplasm is enclosed by an autophagosome. Not only cytosolic proteins but also organelles such as mitochondria and endoplasmic reticulum are often sequestered into autophagosomes. The outer membrane of the autophagosome then fuses with the lysosome, allowing lysosomal enzymes to degrade the sequestered cytoplasmic materials [172, 174]. The physiological functions of autophagy include: maintenance of the amino acid pool, intracellular quality control, development and cell death, tumor suppression and anti-aging.

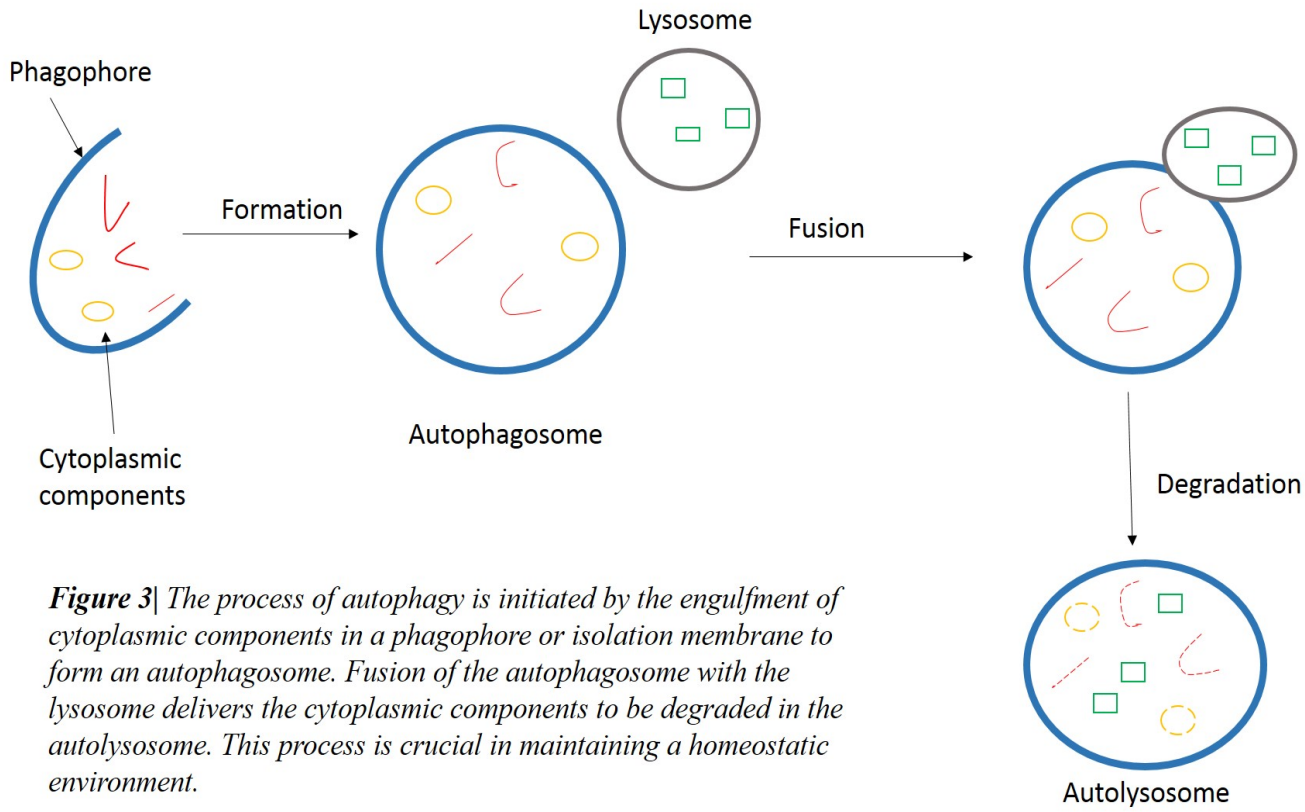


***Figure 3***| *The process of autophagy is initiated by the engulfment of cytoplasmic components in a phagophore or isolation membrane to form an autophagosome. Fusion of the autophagosome with the lysosome delivers the cytoplasmic components to be degraded in the autolysosome. This process is crucial in maintaining a homeostatic environment.*

Under starvation, autophagy generates amino acids inside the cell by degrading cytoplasmic proteins; by doing so the cell can adapt to starvation conditions. This role is carried by the proteasome under normal conditions. Amino acids produced by autophagy can also be used for energy production [175, 176]. Autophagy is not only active during starvation conditions; it is constitutively active at low levels under normal conditions. This basal level of autophagy plays a role in the turnover of cytoplasmic components preventing the accumulation of abnormal ubiquitinated proteins and organelles thus controlling intracellular quality [177].

Autophagy is also involved in the processes of differentiation and development as an adaptation to stress or in normal conditions. Both processes require cells to go through phenotypic changes that require the breakdown and the recycling obsolete cellular components. Silencing of some

autophagy genes will result in death during early development: for example, deletion of beclin-1 in mice results in early embryonic lethality. Autophagy is also thought to be a cell death-inducing process, specifically during development. It is referred to as "type 2 cell death" or "autophagic cell death." In cells lacking the mechanism of apoptosis, autophagy leads to cell death induced by apoptogenic stimuli [172, 173, 178].

There is a controversy around the role of autophagy in the development of a tumor. Many studies have shown that autophagy acts as a tumor suppressor, but it has also been shown that it contributes to the survival of tumor cells thus allowing the tumor to develop [179, 180]. Finally, autophagy functions as an anti-aging mechanism. It is upregulated during calorie restriction. The activation of autophagy leads to a reduction of reactive oxidative species, increased mitochondrial turnover and decreased cellular damage therefore extending the lifespan in animals. In aged cells, damaged proteins are accumulated due to the reduced activity of autophagy. The reduction of autophagy could lead to the development of age related diseases like OA, its regulation is important [173, 181].

**Autophagy Pathway**

Autophagy genes, the molecular components of the autophagy machinery, called Atg genes were first identified in yeast. Their homologues were then discovered in higher eukaryotes. Among these genes, the four major regulators are ATG1, ATG6, ATG8 (ULK1, BECN1, and MAP1LC3B in humans, respectively), and ATG5 [173]. The mammalian target of rapamycin complex 1 (mTORC1) is a key regulator of autophagy activation in humans. mTORC1 is activated when the level of nutrients is high. It will then stimulate protein synthesis through activation of ribosomal protein S6 kinase β-1 and the eukaryotic translation initiation factor 4E-

binding protein 1 (4E-BP1). When mTORC1 is activated, autophagy is inhibited. It inhibits autophagy by binding to and phosphorylating unc-51-like kinase 1 (ULK1) the most upstream inducer of autophagy and disrupting the interaction between ULK1 and AMPK [182].

When active, mTOR forms a complex with ULK1, 200 kDa FAK family kinase- interacting protein (FIP200) and autophagy-related protein (ATG) 13 inhibiting autophagy. When nutrients are rare, mTOR is inhibited and dissociates from the complex. Once ULK1 is activated, it activates further downstream autophagy genes and leads to the initiation of the formation of the autophagosome. The first step is the formation of the phagophore- an isolated double membrane structure. This process is driven by the production of phosphatidylinositol 3-phosphate (PI(3)P) enriched membrane domains by beclin-1 associated class III PI3K on vesicles,which acts as a nucleus for recruitment of further proteins involved in the autophagosome production. The cytoplasmic components that need to be autophagocytosed are also recruited during this step. The phagophore will then undergo growth and elongation and closure leading to the formation of the autophagosome. This process is driven by two ubiquitin-related conjugation systems, the conjugation of phosphatidylethanolamine with microtubule-associated protein 1A/1B-light chain 3 (LC3)-I (cytosolic form of LC3), resulting in the formation of the autophagy marker LC3-II (membrane bound form of LC3), and ATG12– ATG5 conjugation. The amount of LC3-II is correlated with the extent of autophagosome formation [183]. The following step involves the fusion of the enclosed by double-layered or multilayered membranes autophagosome with the lysosome forming the autolysosome. Finally, lysosomal enzymes degrade the components inside the autolysosome and the constituents are released and reutilized in the cell [183-185].

Longevity factors SIRT1, mTOR, FoxO3, NF-κB and p53 regulate autophagy. mTOR and NF-κB are repressors of the autophagy pathway under stress and inflammation signals.[186] SIRT1,

a stress resistance and longevity factor, and FoxO3, a major regulator of cellular metabolism, proliferation and stress resistance, enhance autophagy [187].

**Autophagy and OA**

Chondrocytes in the articular cartilage maintain the homeostasis of the ECM. Because they exhibit low levels of turnover, they rely on different mechanisms to remove damaged organelles and macromolecules in order to keep the cartilage healthy. One of the mechanisms used is autophagy, a mechanism of cell survival. The articular cartilage is highly susceptible to the accumulation of aging-related changes caused by trauma, mechanical and oxidant stress. Autophagy regulates maturation and promotes survival of terminally differentiated chondrocytes under stress and hypoxia conditions. But as mentioned before, autophagy decreases with age causing the accumulation of damaged organelles and increasing the risks of developing OA. The loss of autophagy with aging is caused by failure of the lysosomal hydrolases, resulting in an increase of toxic protein products and slow clearance of autophagosomes in the aging tissues. Autophagy could then have a protective role in OA, compromised autophagy may contribute to decreased chondroprotection and development of OA. Autophagy also plays a protective role during endochondral ossification; its inactivation leads to skeletal abnormalities. It is also essential for early embryonic development [188-190].

It has been shown that ULK1, Beclin1 and LC3-II are expressed in human normal chondrocytes but exhibit significant suppression in their expression in OA chondrocytes. Similarly, ULK1 as well as Beclin1 and LC3 are expressed in normal murine articular cartilage, suggesting presence of autophagy; LC3-II was also present indicating autophagosome formation. However, in OA human cartilage and chondrocytes, the expression of these autophagy markers was significantly

decreased. Similar observations were also made in mouse model of spontaneous OA as well as surgically induced OA [191-194]. Interesting, suppression of autophagy during OA in human OA cartilage as well as experimental models of OA was associated with significant increase in apoptosis and chondrocyte cell death [195, 196].

mTOR is an inhibitor of autophagy as mentioned before. Treatment with rapamycin (inhibitor of mTORC1) has been shown to reduce the severity of experimental OA in mice [193].

Many studies showed the effect of rapamycin. In one study, surgically induced OA was induced in mice and then they were administrated rapamycin intraperitoneally. The severity of cartilage degradation was significantly reduced in the rapamycin-treated group compared with the control group. Rapamycin treatment also maintained cartilage cellularity and decreased ADAMTS-5 and IL-1β expression in articular cartilage.[193] In another study, cartilage explants were treated rapamycin and tested for protective effects against mechanical injury. The results showed that rapamycin increased expression of autophagy regulators and prevented cell death in mechanically injured explants. Rapamycin also protected against cell death [196]. Another study, using chondrocytes showed that rapamycin treatment resulted in a significant increase in total LC3 expression and the conversion from LC3BI to LC3BII as well as increase in the mRNA expression of LC3, ULK1 and AMPK1 in rapamycin-treated OA chondrocytes compared to the control group. Also, treatment of OA chondrocytes with rapamycin significantly increased the mRNA expression of aggrecan and type II collagen, the two major components of extracellular matrix, and decreased the expression of MMP-13 and chemokines.[194]

It has also been shown that mTOR is upregulated in human OA cartilage compared to normal cartilage. Upregulation in mTOR in OA causes an increase in chondrocyte apoptosis as well as a decrease in the expression of essential genes for autophagy (including ULK1, LC3 and ATG5).

mTOR KO mice show upregulated autophagy and a protection from surgically induced OA [194]. Understanding the exact mechanism of autophagy and how it affects the development of OA could lead to a promising therapeutic strategy for OA.

## 1.6 Unc-51 Like Autophagy Activating Kinase 1 (ULK1)

ULK1 (unc-51 like kinase 1) is a serine/threonine protein kinase encoded by the ULK1 gene. It is the most upstream inducer of autophagy and it is important for the formation of the autophagosome. ULK1 also has other non-autophagic functions. Homologues of ULK1 in *C.elegans* (UNC-51) and Drosophila were shown to be essential in the development of neurons [197]. *C. elegans* with a mutation in Unc-51 were shown to be paralyzed because of a defect in the axonal elongation [198]. Similarly, in mice, ULK1 was shown to be critical in the development of neuronal populations [199].

Once autophagy is induced, ULK1 is recruited to the phagophore and binds to the regulatory proteins ATG13 and RB1CC1/FIP200. The phagophore elongates leading to the engulfment of the targeted cytoplasmic constituents and forming the autophagosome [200, 201]. ULK1 contains more than 30 phosphorylation sites, the change in the phosphorylation status of those sites in response to upstream signals, including nutrient and energy signals, leads to the activation or inhibition of autophagy [202]. ULK1 acts upstream of many autophagy genes: LC3 which is responsible for the formation and the expansion of autophagosome, it executes autophagy. ATG5, which is a regulator of autophagy and Beclin 1, forms a complex that allows the nucleation of the autophagic vesicles [195]. Studies have showed that ULK1 directly phosphorylates AMBRA1, a Beclin1-interacting protein and regulatory component of the PI3K

class III complex. During starvation conditions, once ULK1 is activated, it phosphorylates AMBRA1; this leads to the release of the PI3K complex and its translocation to the ER, where it initiates autophagosome formation [203].

Beclin-1 phosphorylation by ULK1 is required for full autophagic induction. When ULK1 is activated, it phosphorylates Beclin-1 on S14, thereby, enhancing the activity of the ATG14L-containing VPS34 complexes. VPS34 complexes are pro-autophagic and are responsible for generating PI3P at the phagophore: a critical event in the autophagosome formation [204].

MTOR binds to RPTOR and MLST8 forming the MTOR complex 1 (MTORC1). MTORC1 acts upstream of ULK1 and phosphorylates it through the interaction of raptor to ULK1, leading to the inhibition of autophagy. But during starvation conditions, ULK1 is not bound to the complex leading to the activation of autophagy [205, 206]. A study has shown that the relation between the ULK1 and mTOR is more complex. The study showed that ULK1 phosphorylates Raptor on multiple sites and inhibits mTORC1 activity when nutrients are limited [207].

While mTOR acts as an inhibitor of autophagy, AMPK acts as an activator. AMP activated protein kinase or AMPK acts as a sensor of energy and is important in maintaining energy homeostasis by regulating cellular metabolism. Under starvation conditions, AMPK activates ULK1 through phosphorylation of Ser 317 and Ser 777 leading to the activation of autophagy [208, 209]. When nutrients are sufficient, high mTOR activity prevents activation of ULK1 by phosphorylating it at Ser 757 and disrupting the interaction between ULK1 and AMPK; autophagy is then inhibited [210, 211].

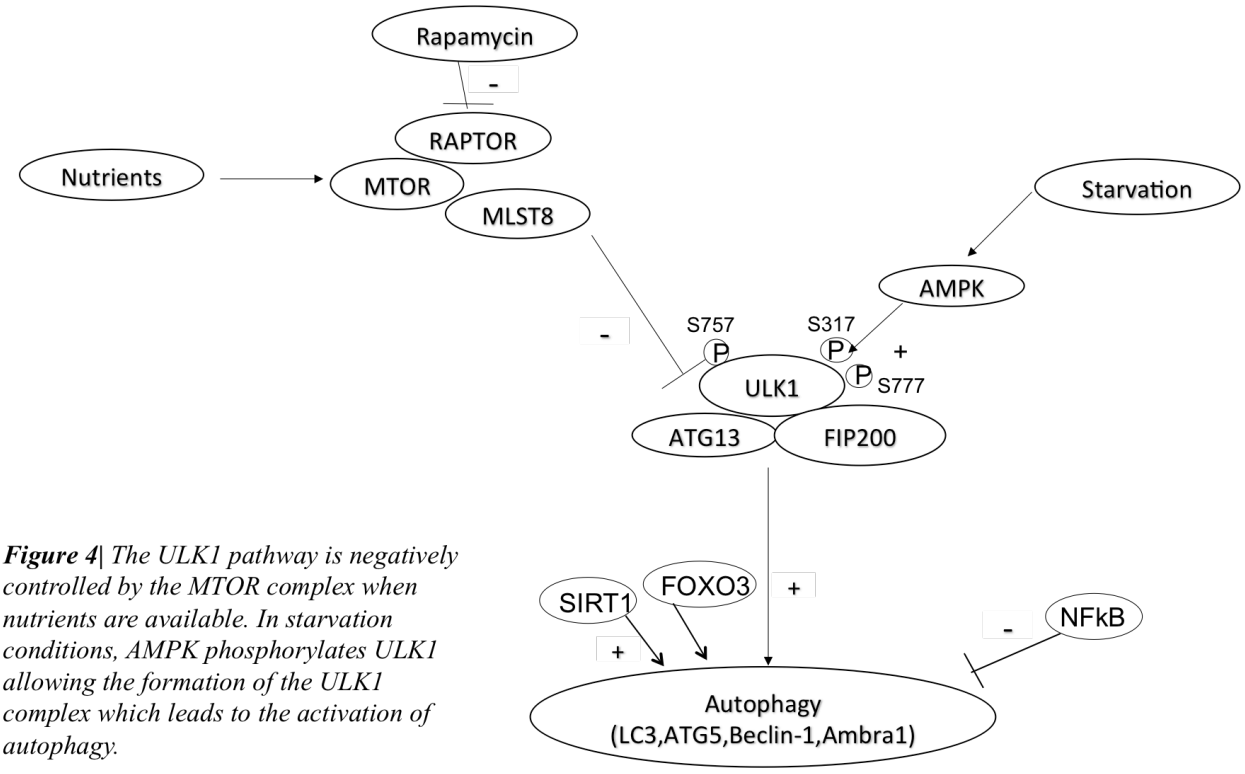


***Figure 4**| The ULK1 pathway is negatively controlled by the MTOR complex when nutrients are available. In starvation conditions, AMPK phosphorylates ULK1 allowing the formation of the ULK1 complex which leads to the activation of autophagy.*

**ULK1 in articular cartilage degeneration**

The exact role of ULK1 is articular cartilage degeneration is unknown. However, recent studies have shown that ULK1 expression is decreased in OA human cartilage compared to normal human cartilage. Similarly, ULK1 expression is significantly reduced in mouse experimental OA model. The genetic deletion of mTOR in mice resulted in an increase in the expression of ULK1 and an increase in autophagy, same results where observed in rapamycin (inhibitor of mTOR) treated OA chondrocytes. To further test that ULK1 is the main inducer of autophagy, OA chondrocytes were pretreated with rapamycin and transfected in the presence/absence of ULK1 siRNA to determine if silencing of ULK1 can rescue the protective effects of rapamycin. Indeed, silencing of ULK1 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) as

well as mRNA expression of LC3 and ATG5. Furthermore, silencing of ULK1 in rapamycin-treated OA chondrocytes resulted in a significant increase in the expression of OA catabolic factors [193, 194].

In the articular cartilage, ULK1 has the ability to activate autophagy and create a balance between catabolic and anabolic processes, the shutting down of this pathway could lead to an imbalance between the catabolic and anabolic factors, degradation in the articular cartilage and therefore OA. The study of the exact in *vivo* role of ULK1 and how its signaling can be modulated could lead to a potential therapy for OA.

## 2. PURPOSE OF THE STUDY

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 which mechanisms control the fate of chondrocytes within articular cartilage during normal versus OA conditions. Understanding the exact chondrocyte cell death/survival mechanisms could lead to several promising OA therapeutic strategies. Studies by my group and others have recently shown that the process of autophagy, a form of programmed cell survival, is impaired during OA and may contribute to decreased chondroprotection, resulting in the degradation of articular cartilage. Specifically, *in vitro* studies strongly suggest that one of the key central factors that controls autophagy induction and ultimately the fate of the chondrocytes within the articular cartilage is the ULK1. ULK1 is the most upstream autophagy inducer and inhibiting ULK1

results in loss of autophagy. Since the exact *in vivo* role of ULK1 in OA pathophysiology is unknown; my MSc project focuses on elucidating the specific *in vivo* role of ULK1 in (1) in OA pathogenesis using cartilage-specific ULK1 knockout (KO) mice in murine model of OA and subsequently (2) to identify ULK1 signalling pathway operative during OA.

## 3. HYPOTHESIS AND AIMS

***Hypothesis:*** *ULK1 is essential for maintaining adequate autophagy in the articular cartilage. Loss of ULK1 results in the suppression of autophagy leading to decreased chondroprotection, enhanced chondrocyte death and increased catabolism ultimately resulting in destruction of articular cartilage.*

To test this hypothesis, I generated inducible cartilage-specific ULK1 KO mouse using Cre-Lox technology in which Cre is under the control of collagen II promoter to achieve specific inactivation of ULK1 in chondrocytes. Cartilage-specific ULK1 KO mice, were subjected to destabilization OA model of medial meniscus (DMM) to determine:

The effect of cartilage specific ablation of ULK1 on:

***1. Articular cartilage degeneration.***

***2. Articular chondrocyte cellularity.***

***3. Synovial inflammation.***

***4. Expression of Apoptotic (cell death) and catabolic markers.***

***5. Determine the effect of ULK1 plasmid treatment in human OA chondrocytes on the expression of various genes involved in autophagy and cell death process.***

# 4. MANUSCRIPT 1

## Cartilage-specific ablation of Unc-51 like kinase 1 results in accelerated Osteoarthritis phenotype

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**Status: To be submitted by end of August 2015**

**Cartilage-specific ablation of Unc-51 like kinase 1 results in accelerated Osteoarthritis phenotype**

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**Key words**: osteoarthritis; articular cartilage; cartilage degradation; ULK1 KO

## Abstract

**Objectives:** Unc-51 like kinase-1 (ULK1) is a serine/threonine protein kinase and is the most upstream autophagy inducer. Recent studies suggest that ULK1 expression is suppressed in the articular cartilage during Osteoarthritis (OA). However, the exact role of ULK1 in pathogenesis of OA is not known. Therefore, we created cartilage-specific ULK1 knockout (KO) mice using Cre-lox technology and subjected them to mouse model of experimental OA to determine the specific *in vivo* role of ULK1 in OA pathophysiology.

**Methods:** Inducible cartilage-specific ULK1 KO mice were generated using Cre-Lox technology and subjected to the destabilization of the medial meniscus (DMM) mouse model of OA. At 10 weeks post OA, degree of cartilage degeneration, loss of cellularity and expression of cell death and catabolic markers were determined. Human OA chondrocytes were transfected with ULK1 expression plasmid to determine the expression of genes involved in cell death and survival mechanisms.

**Results:** We generated for the first time, cartilage-specific ULK1 KO mice and showed that cartilage-specific ablation of ULK1 results in an accelerated OA phenotype in mouse model of experimental OA. ULK1 KO mice, in comparison to control (wild type) mice exhibited accelerated cartilage degeneration, proteoglycan loss, chondrocyte cell death, synovial inflammation and expression of OA catabolic factors. Furthermore, using OA chondrocytes transfected with ULK1 expression plasmid shows that ULK1 modulates the expression of mTOR and OA catabolic factors such as ADAMTS-5.

**Conclusion**: This study provides the first *in vivo* evidence of the role of ULK1 in maintaining articular cartilage homeostasis.

## **Introduction**:

Osteoarthritis (OA) is the most common musculoskeletal disease worldwide. It is one of the leading causes of pain and disability among adults, and represents a considerable burden on the healthcare system [1, 3]. OA is a disease of the entire joint, involving not only the articular cartilage but also the synovium, ligaments and subchondral bone. It is characterized by the progressive degeneration of the articular cartilage, osteophyte formation, remodeling of the subchondral bone, deterioration of tendons and ligaments and various degrees of inflammation of the synovium. While current therapies and management strategies can help alleviate symptoms early in the disease process, OA is characterized by almost inevitable progression towards end-stage disease.

The exact pathogenesis of OA is largely unknown but the key event in OA is the degradation of the articular cartilage, which is only composed of chondrocytes; cells responsible for the synthesis of the extracellular matrix (ECM) and maintenance of articular cartilage homeostasis [79]. Chondrocytes maintain the articular cartilage matrix by replacing degraded macromolecules and responding to focal cartilage injury or degeneration by increasing local synthesis activity. Since chondrocytes exhibit low levels of turnover, they rely on endogenous mechanisms such as autophagy (a cell survival and adaptation process) to remove damaged organelles and macromolecules in order to maintain articular cartilage homeostasis [104, 105].

Autophagy is a lysosomal degradation pathway that is essential for survival, differentiation, development and homeostasis. It regulates maturation and promotes survival of terminally differentiated chondrocytes under stress and hypoxic conditions [171]. Studies by others and us have shown that compromised autophagy is associated with decreased chondroprotection,

increased cell death and articular cartilage degeneration. Carames et al showed that autophagy is constitutively expressed in normal human articular cartilage. However, expression of key autophagy inducers is reduced in ageing cartilage [195]. Our previous studies have also identified a panel of key autophagy genes that are expressed in low levels in human OA cartilage as well as in the articular cartilage from mouse and dog models of experimental OA. Specifically, we identified that expression of unc-51 like kinase-1 (ULK1) is suppressed in human OA cartilage and experimental OA models [212]. ULK1 is a serine/threonine protein kinase and is the most upstream autophagy inducer. Loss of ULK1 results in disruption of autophagy induction [212]. Since adequate autophagy signaling is required for maintaining chondroprotection as well as articular cartilage homeostasis, we hypothesized that ULK1 is required for autophagy induction in the articular cartilage and loss of it will result in decreased chondroprotection and enhanced chondrocyte death leading to the degeneration of articular cartilage. Since the exact role of ULK1 in the pathogenesis of OA is unknown, we created for the first time, an inducible cartilage-specific ULK1 knockout (KO) mice using Cre-Lox technology and subjected these mice to the destabilization of the medial meniscus mouse OA model to specifically elucidate the specific *in vivo* role of ULK1 in OA pathogenesis.

## Materials and Methods:

### Generation of inducible cartilage specific ULK1 knockout mice

Inducible cartilage specific ULK1 KO mice were generated by mating Col2-rt-TA-Cre mice [213] (obtained from Dr. Peter Roughley, McGill University, Montreal) with mice containing the ULK1 gene flanked by LoxP sites (C57BL6 ULK1fl/fl, Jackson Laboratory). When ULK1 f/f Cre mice reached 5 weeks of age, they were fed doxycycline by oral gavage (Sigma-Aldrich Inc., Oakville, ON) that was dissolved at 10 mg/mL in phosphate buffer saline (PBS), pH 7.4 with the dose of 80 mg/g body weight for 7 days. ULK1 f/f Cre mice fed with only PBS were used as controls. Mice were genotyped to make sure they all contained the floxed gene and the cre gene. The loss of ULK1 expression in the cartilage after treatment with doxycycline was confirmed by immunohistochemistry. All animal procedures protocols were approved by the Institutional Animal Care Committee at the Toronto Western Research Institute.

### Murine model of OA

10-week-old male control and ULK1 KO mice were subjected to destabilization of the medical meniscus surgery (DMM) to induce OA. The surgery was performed in the right knee as previously described before [214, 215].

Briefly, after anesthesia with a cocktail of ketamine and xylazine, the mouse knee was prepared. A small incision was created, allowing direct visualization of the meniscal tibial ligament that was then incised. Histology and immunohistochemistry were performed on knee joints extracted at 10 weeks post-surgery.

**Histology**

Knee joints dissected from the mice were fixed in 10% neutral buffered formalin for 48 hours and then decalcified for 1.5 hours in decalcifying solution (RDO, rapid decalcifier). The samples were then processed and embedded in paraffin then cut as previously shown [212]. Sections (5um) of mouse knee joints isolated at 10 weeks post OA-surgery were stained with either Safranin-O/Fast green or Harris's Hemotoxylin/Eosin staining.

**Assessment of progression and severity of OA**

Sections (5um) of mouse knee joints isolated at 10 weeks post OA-surgery were deparaffinized in xylene followed by graded series of alcohol washes. The sections were then stained using the Safranin-O/Fast green method according to the manufacturer's recommendations. The severity of the degradation of the cartilage was determined using the OARSI score, a scoring method issued by Osteoarthritis Research Society International. OARSI score was determined by two blinded observers as previously reported [216]. Chondrocyte cellularity was assessed by calculating the average of three equal squares drawn on the articular cartilage.

Synovial inflammation was assessed using the inflammation scoring. The inflammation score was calculated according to the number of inflammatory cells present in the synovium, and the thickness of the synovium using a scale from 0 to 3.

**Immunohistochemistry**

Immunohistochemistry studies were performed using specific antibodies for target genes. Immunohistochemistry was performed using the Dakocytomation (Dako)-labelled streptavidin biotin + system horseradish peroxidase kit following the manufacturer's recommended protocol as previously described [217]. After deparaffinization and blocking with 1% BSA, the slides are

incubated overnight in a humidified chamber at 4$^{o}$C with primary antibodies (1:50 dilution in 1% BSA for matrix metalloproteinases-13 (MMP-13) and PARP, 1:100 for ULK1). The sections were then incubated with biotinylated link followed by incubation in red streptadivin. The diaminobenzidine tetrahydrochloride chromogen substrate solution was used to develop the color and counterstained with Eosin as previously shown [212]. The quantification of the number of positive cells for each antigen was performed by the determination of the total number of chondrocytes and the total number that stained positive for the antigen. The final results were expressed as the percentage of positive cells for the antigen.

**Human specimens**

Human OA cartilage was obtained from patients undergoing total knee replacement surgery. In all patients, OA was diagnosed on the basis of criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA [218]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of non-steroidal anti-inflammatory drugs or selective cycloxygenase-2 (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.

**Chondrocyte culture studies**

Human chondrocytes were isolated from OA cartilage by enzymatic degradation as previously described [219]. Briefly, cells were seeded at 4 x $10^5$ cells/ well in a 6-well culture plate in

DMEM supplemented with 10% FBS and 1% antibiotics and kept in the incubator at 37$^{o}$C for 48 hours.

After 48 hours, the chondrocytes were transfected with the ULK1 plasmid (2ug) for 24 hours using transfectin lipid reagent (Biorad, 1:50 dilution in DMEM). Chondrocytes were transfected with an empty vector as a control (2ug). Cells were then harvested for RNA or protein isolation.

**RNA extraction and quantitative real time PCR (qPCR) and PCR array:**

Total RNA was isolated using Trizol and DNA was eliminated using DNA-free $^{TM}$ kit (DNA-free $^{TM}$ DNA removal kit, Life Technologies). RNA concentration was then measured using the Nanodrop machine (Nanodrop 1000, Thermo Scientific, then cDNA (using 1ug of RNA) was created using the reverse transcription kit (QUIAGEN). RNA quantification was then performed on the Light Cycler 480 system, Roche. The fold increase in the PCR products was calculated using the $2^{-\Delta\Delta Ct}$ method and GAPDH was used as the housekeeping gene [75]. All experiments were performed in duplicates for each sample. All the primers used for QPCR were listed in the supplementary table 1. The human autophagy PCR array was used to study the expression of genes involved in the autophagy machinery and of the genes involved in the regulation of autophagy after transfection of OA chondrocytes with ULK1 plasmid.

**Statistical analysis:**

The data are expressed as mean ± SEM. The significance of differences in the levels of expression between the control and OA groups was determined using the two-tailed t test with $p< 0.05$ considered statistically significant.

## Results:

### Expression of ULK1 in early development and adult mouse cartilage

The expression of ULK1 in mouse cartilage during early development (post natal Day 0) and adult articular cartilage (P70) was determined by immunohistochemistry. Results showed ULK1 is highly expressed in the hypertropic zone of the P0 growth plate during early phases of cartilage development. Results further showed that articular cartilage chondrocytes express ULK1 in all the zones of the articular cartilage (Figure 1).

### Inducible cartilage-specific ULK1 KO mice exhibit accelerated OA phenotype

Cartilage specific ULK1 KO mice were generated to determine the specific role of ULK1 in the pathophysiology of OA. All male mice used contained the ULK1 fl/fl gene and the Cre gene; this was confirmed by routine genotyping. (Figure 2) ULK1 f/f Cre mice were fed doxycycline or PBS (as control) by oral gavage for 7 days. The loss of expression of ULK1 in the articular cartilage chondrocytes was confirmed by immunohistochemistry in 8 week old mice (Figure 2). 10 week old mice were subjected to OA surgery and kinetics of OA progression were assessed at 10 weeks post OA surgery using histopathology.

As expected, histological analysis of the sections stained with Safranin-O/Fast green staining showed some loss of chondrocyte cellularity, roughening of the articular cartilage and loss of proteoglycans in 10 week old control mice at 10 weeks post OA surgery. However, ULK1 KO mice at 10 weeks post OA surgery showed greater loss of chondrocyte cellularity, proteoglycan

loss and enhanced degree of cartilage degeneration (Figure 3 and 4). OARSI scores confirmed greater OA severity in ULK1 KO mice compared to control mice at 10 weeks post OA surgery (Figure 3).

Since OA does not involve changes in only articular cartilage but also other joint structures such as the synovium; histological analysis of synovium using H&E stained sections showed an enhanced degree of inflammation score in the synovium of ULK1 KO mice compared to control mice (Figure 5). These results showed accelerated cartilage degeneration and synovial inflammation in ULK1 KO mice compared to control mice.

**ULK1 KO mice exhibit increased apoptosis and increased expression of catabolic factors during OA**

Poly-ADP Ribose polymerase (PARP) immunostaining was performed to account for the degree of chondrocyte cell death (apoptosis) in control and ULK1 KO mice articular cartilage at 10 weeks post OA surgery. Results showed a greater number of PARP positive staining in ULK1 KO mice compared to control mice, suggesting an increased rate of apoptosis in ULK1 KO mice (Figure 6). Further, we observed increased expression of the major OA catabolic factor MMP-13 in ULK1 KO mice articular cartilage compared to controls, suggesting enhanced catabolic activity in the articular cartilage (Figure 6). These results show that genetic deletion of ULK1 is associated with enhanced cell death and catabolic activity within the articular cartilage, resulting in an accelerated OA phenotype.

**ULK1 modulates the expression of mTOR as well as OA catabolic factors**

We further determined if ULK1 modulates the expression of autophagy genes in chondrocytes. We extracted human OA articular cartilage chondrocytes and transfected these cells with ULK1 expression plasmid. Please note that our previous studies have shown that OA chondrocytes exhibit reduced ULK1 expression and reduced autophagy signaling as well as increased catabolic activity [212], therefore we hypothesize that transfecting OA chondrocytes with ULK1 expression plasmid will result in rescue of autophagy and reduction in catabolic activity. Our results showed that transfection of ULK1 plasmid resulted in increased expression of ULK1, decreased expression of mTOR (major inhibitor of autophagy) and decreased expression of OA catabolic factors including MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) and COX-2 (Figure 7 and Supp. Table 1). Our results also showed that ULK1 influences the expression of a variety of autophagy genes and autophagy-related genes (Table 1).

## Discussion:

The mechanisms associated with articular cartilage degeneration are not well understood. Chondrocytes are the only cell types present in the articular cartilage and are responsible for the maintenance of articular cartilage homeostasis. Chondrocyte cell death is one of the key events associated with degeneration of articular cartilage. Endogenous mechanisms that control chondrocyte survival and cell death are not well characterized. Recent studies by our group and others show that autophagy is essential in maintaining chondrocyte survival; loss of which results in reduced chondroprotection and cartilage degeneration [193, 195, 212, 220, 221]. It is still to be determined as to what endogenous mediators control autophagy in the articular cartilage.

ULK1 is the most upstream autophagy inducer. It is a serine/threonine protein kinase encoded by the ULK1 gene and is essential for the formation of the autophagosomes. ULK1 plays an important role in the development of the neurons and it also prevents tumor development before they are formed [179, 198, 199]. ULK1 is positively regulated by AMPK and negatively regulated by mTOR (major negative regulator of autophagy); and it phosphorylates and activates the regulatory proteins ATG13 and RB1CC1/FIP200 within the autophagy machinery.

In the present study, we demonstrate critical role of ULK1 in articular cartilage homeostasis. We created for the first time ULK1 cartilage-specific KO mice to study the specific *in vivo* role of ULK1 in articular cartilage homeostasis. We observed that ULK1 KO mice subjected DMM OA surgery (compared to control mice) exhibited accelerated cartilage degeneration associated with enhanced chondrocyte cell death and increased expression of catabolic MMP-13 in the articular cartilage at 10 weeks post OA surgery, suggesting that ULK1 is required for normal articular

cartilage homeostasis; loss of which results in accelerated cartilage degeneration.

In this study we used PARP staining to quantify apoptosis. Full length PARP protein is cleaved during apoptosis by caspase-3, and possibly other caspases, into smaller fragment. A specific antibody for that fragment of PARP that resulted from caspace cleavage is then used for IHC on fixed tissue. Therefore, the antibody detects apoptotic cells. To strengthen the apoptosis detection, another assay that could also have been used is the TUNEL (TdTmediated dUTP Nick-End Labeling) assay or the cell death detection ELISA kit.

Since OA is a disease of the whole joint; we identified that ULK1 KO mice not only exhibited accelerated cartilage degeneration but also exhibited enhanced synovial inflammation suggesting that ULK1-1 specific ablation in the cartilage also contributes to overall joint environment and also emphasize on the fact that changes in the cartilage can affect other surrounding joint structures.

Our previous studies have shown that cartilage-specific deletion of mTOR results in protection from cartilage degeneration in OA models [212]. In this study, we showed that loss of mTOR resulted in increased autophagy and protection from cartilage degeneration. Our study provides direct proof that suppression of autophagy by deleting ULK-1 results in acceleration in the OA phenotype. To further validate those results, we will next compare those results to another control group of ULK1 KO mice that did not receive any surgery. This type of control will validate that the effects observed in our study are caused by the loss of ULK1 and are independent of the OA mouse model used.

As shown by the figures some results did not show significance and this is due to the low number of animals used, to have more robust results and increase significance we will increase the

number of mice used for each experiment.

Using human OA chondrocytes transfected with ULK1 expression plasmid, we further show that ULK1 reduced the expression of mTOR. This is in line with previous studies that have shown that ULK-1 negatively regulates mTOR [207]. Furthermore our data shows that ULK1 is able to reduce the expression of key OA catabolic factors such as MMP-13, ADAMTS-5 and COX-2, all of these have been implicated in the process of articular cartilage degeneration[194, 212].

The relation between the ULK1 and mTOR is very complex. MTOR, a serine/ threonine protein kinase encoded by the mTOR gene, binds to RPTOR and MLST8 to form the MTOR complex 1 (MTORC1). MTORC1 acts upstream of ULK1 and phosphorylates it through the interaction of raptor to ULK1, leading to the inhibition of autophagy. But during starvation conditions, ULK1 is not bound to the complex leading to the activation of autophagy. But it has been also known that ULK1 phosphorylates Raptor on multiple sites and inhibits mTORC1 activity when nutrients are limited[205]. AMPK also activates ULK1 through phosphorylation of Ser 317 and Ser 777 leading to the activation of autophagy. Activated AMPK was thought to inhibit mTORC1 activity primarily in the opposite way as growth factors stimulate it, mainly by phosphorylation and activation of the negative regulator TSC2. Raptor has been identified as a direct substrate of AMPK[203].

Current therapies for OA rely on symptom control using a combination of pharmacological and non-pharmacological approaches as well as surgical approaches in end stage cases but an effective treatment that will prevent or stop the disease as well as its progression is yet to be discovered. Therefore, targeting autophagy and ULK1 may be a promising therapeutic strategy to achieve chondroprotection and correct the imbalance between catabolic and anabolic

processes to protect the articular cartilage during OA.

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## Figures:

**Figure 1**

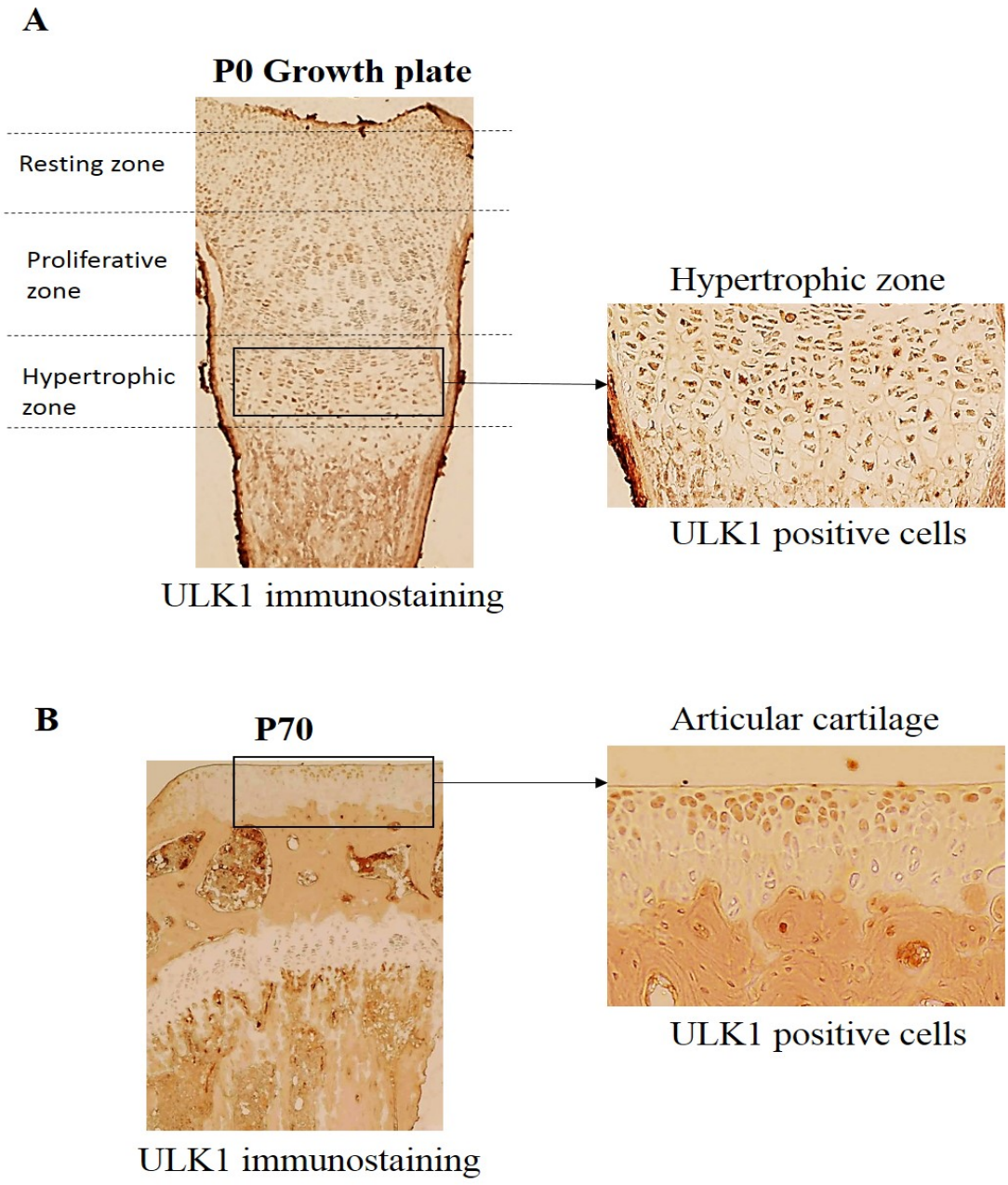



***Figure 1.*** *Expression of ULK1 in chondrocytes during early cartilage development (P0:time of birth) and adult mouse cartilage (P70): Immunohistochemistry using ULK1 antibody shows ULK1 is highly expressed in hypertrophic zone in P0 growth plates. (Magnifications: x10 and x20). In adult cartilage (P70), ULK1 is highly expressed in superficial and medial zones of articular cartilage. (Magnifications: x10 and x20).*

**Figure 2**

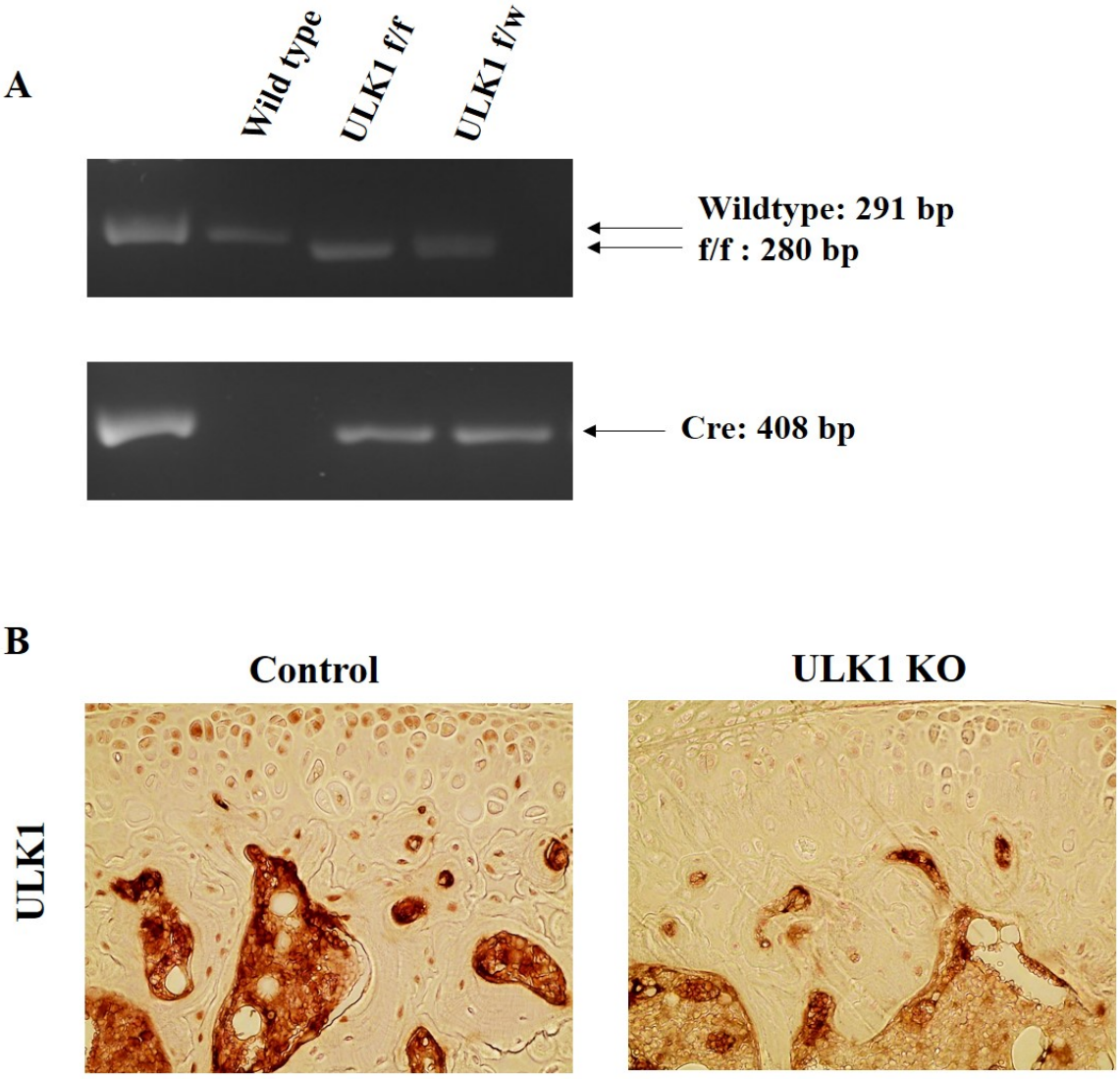



***Figure 2.*** *Generation of cartilage-specific ULK1 KO mice: (A) genotyping confirmed the presence of the Cre transgene in heterozygote (ULK1fl/w) and homozygote (ULK1fl/fl) mice and its absence in wild-type mice; (B) immunohistochemical staining for ULK1 confirmed the absence of its expression in the articular cartilage of ULK1fl/flCre mice treated with doxycycline compared with ULK1fl/flCre mice treated with saline (Magnification x40)*

**Figure 3**

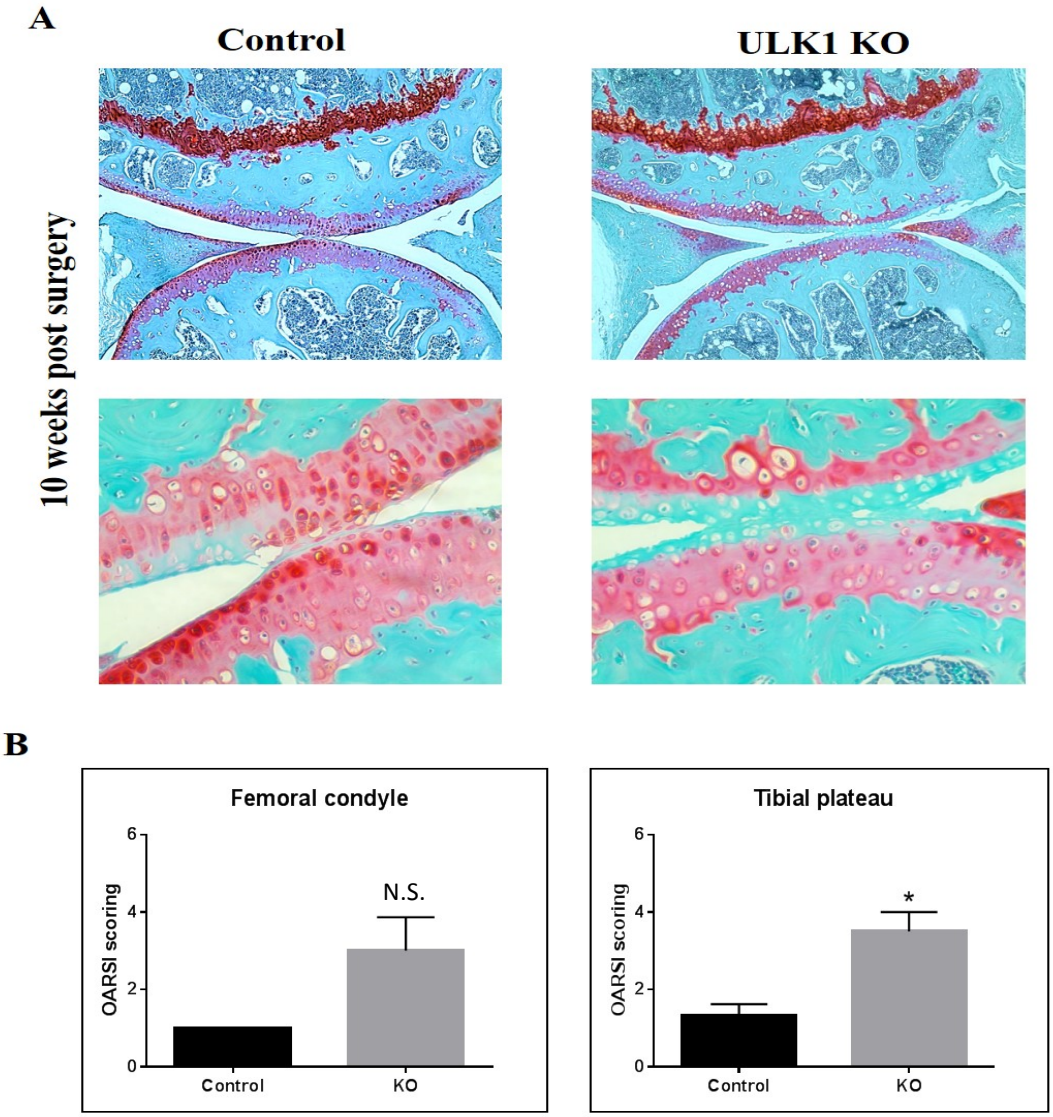



***Figure 3.*** *Inducible cartilage-specific ULK1 KO mice exhibit accelerated OA phenotype: (A) histological analysis using Safranin O/fast green staining of 10 weeks post-OA surgery knee joint sections demonstrate that ULK1 KO mice exhibit accelerated OA phenotype associated with greater cartilage degradation and loss of safranin O staining compared with control mice (Magnification x10 and x40); (B) Osteoarthritis Research Society International (OARSI) scoring of medial tibial plateau and medial femoral condyle showed a significant increase (*$p<0.05$) in the OARSI scores in 10 weeks post-surgery KO mice compared with control post-surgery mice($n=3$) .*

## Figure 4

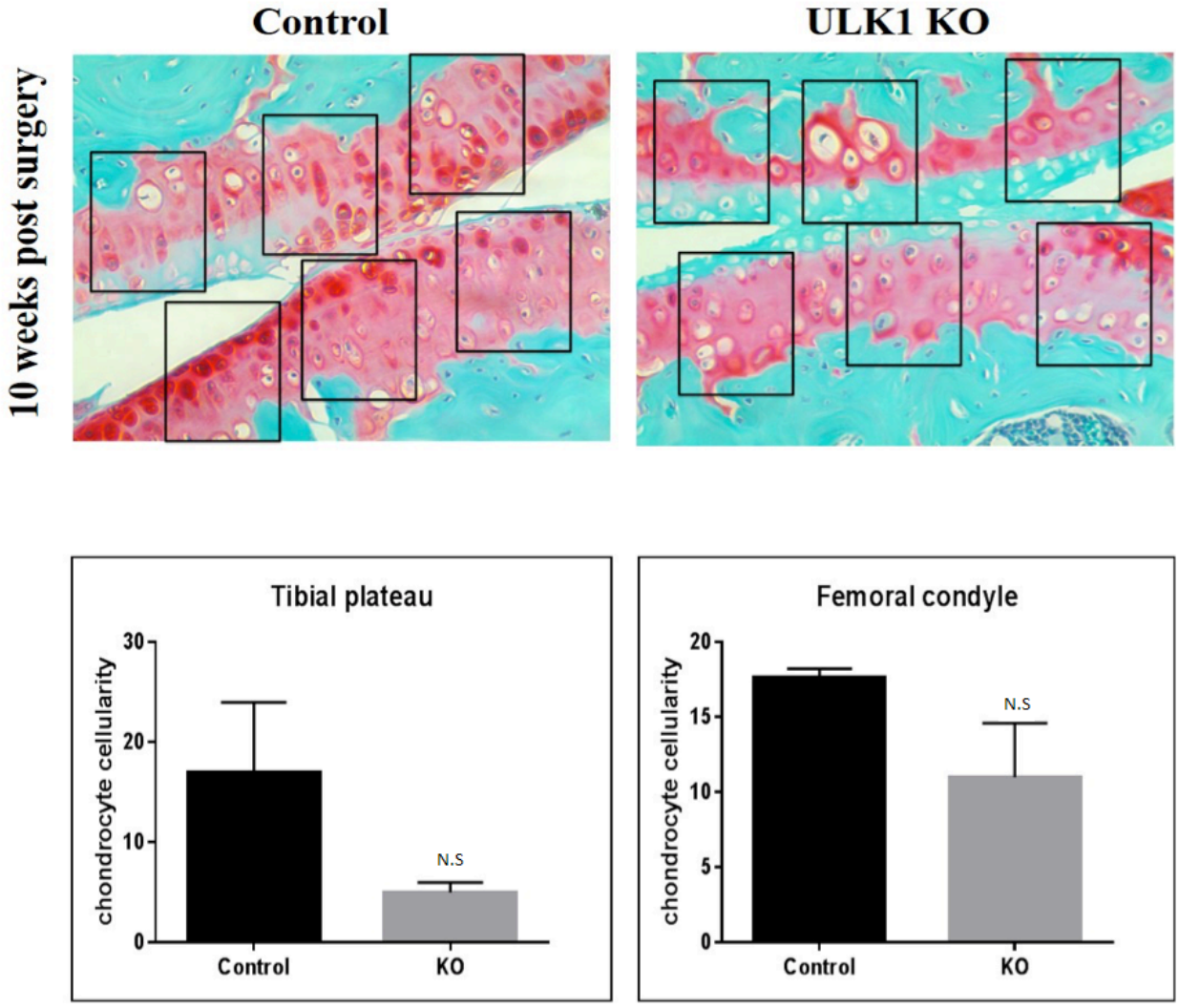



***Figure 4.*** *Loss of cellularity in ULK1 KO mice: quantification of articular chondrocyte cellularity revealed loss of chondrocyte cellularity in ULK1 KO mice (10 weeks post-OA surgery) compared with control (n=3), (magnification x40). Chondrocyte cellularity was assessed by calculating the average of three equal squares drawn on the articular cartilage, only cells with stained nuclei were counted.*

**Figure 5**

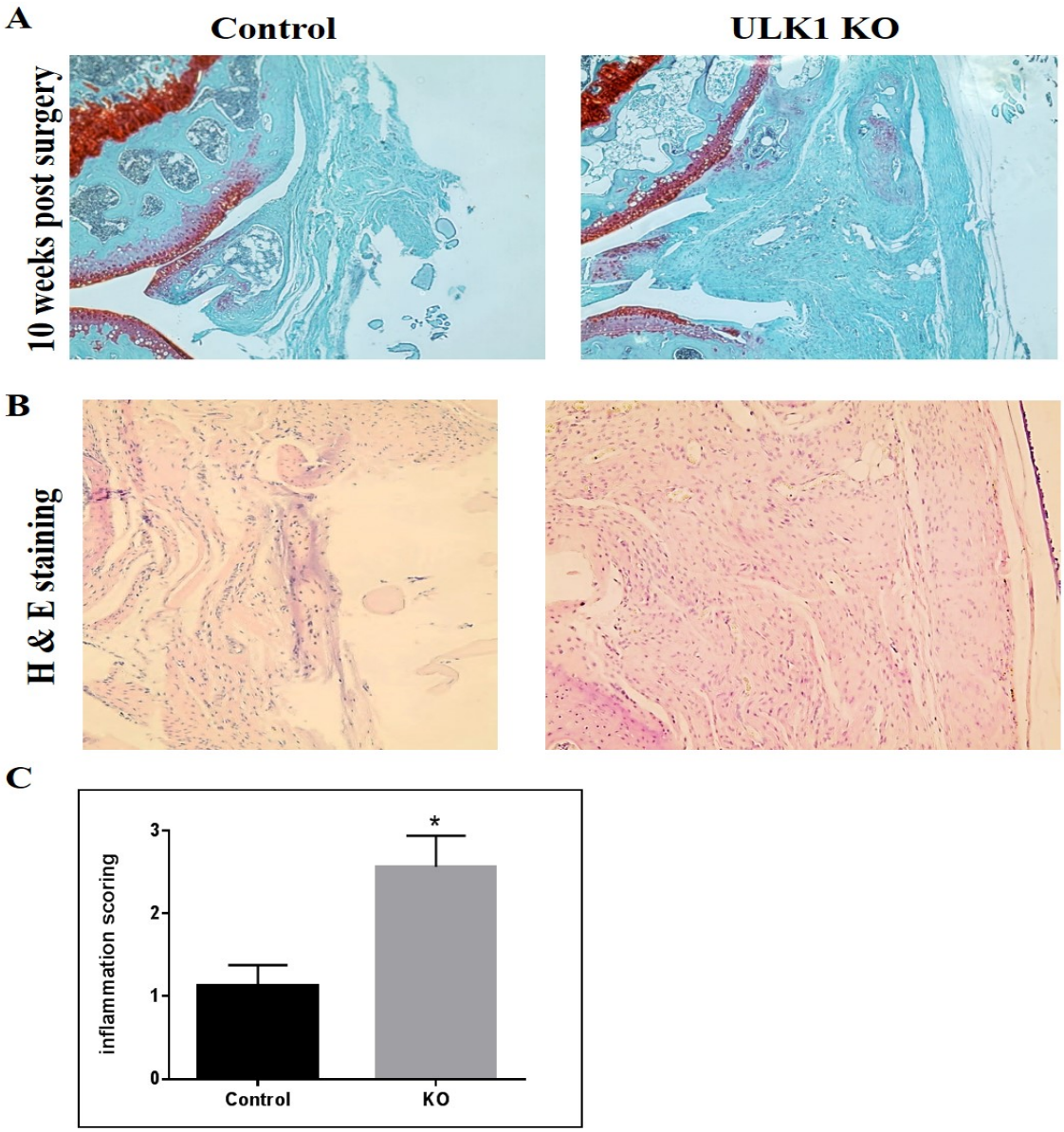
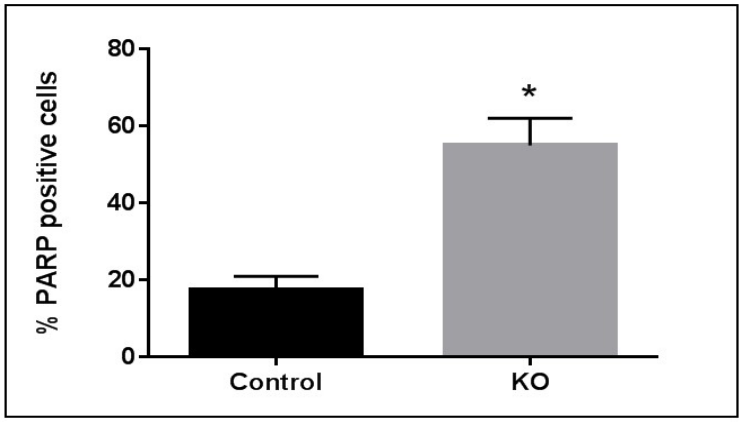



***Figure 5.*** *Increased synovium inflammation in ULK1 KO mice: (A) histological analysis using Safranin O/fast green staining of 10 weeks post-OA surgery knee joint sections demonstrate that ULK1 KO mice exhibit higher degree of inflammation in the synovium compared with control mice (magnification x10); (B) Hematoxylin and Eosin staining confirmed the higher degree of inflammation in the synovium of ULK1 KO mice at 10 weeks post surgery compared with control mice (magnification x20);*
*(C) inflammation scoring revealed significant (*$p<0.05$*) increase of inflammation in ULK1 KO mice (10 weeks post-OA surgery) compared with control mice (also 10 weeks post-surgery) (n=4)*

## Figure 6

**A**




**B**

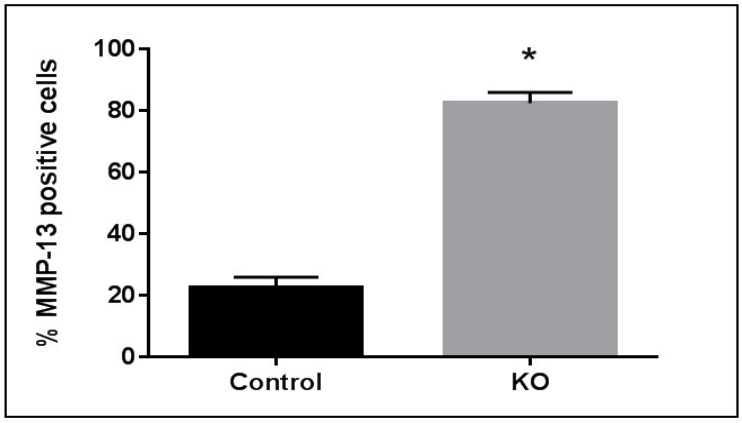



***Figure 6.*** *ULK1 KO mice exhibit increased apoptosis and increased expression of catabolic factors during OA*
*(A) PARP immunohistochemical analysis showed a significant (*p<0.05) increase in the percentage (%) of PARP positive cells in ULK1 KO OA mice compared with control OA mice at 10 weeks post-OA surgery. (n=3)*
*(B) MMP-13 immunohistochemical analysis showed a significant (*p<0.05) increase in the percentage (%) of MMP-13 positive cells in ULK1 KO OA mice compared with control OA mice at 10 weeks post-OA surgery. (n=3)*

**Figure 7**

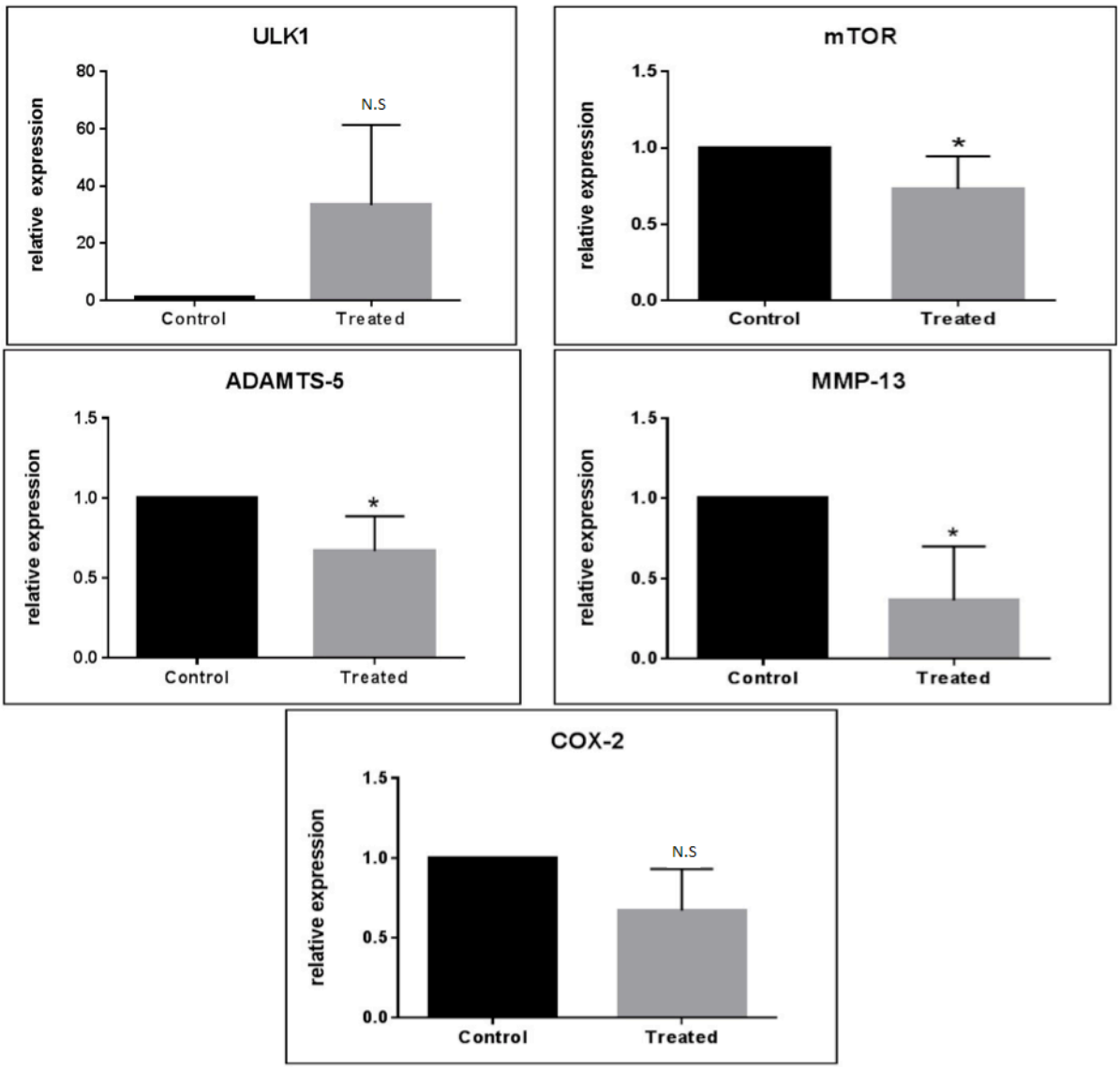



***Figure 7.*** *ULK1 modulates the expression of mTOR as well as OA catabolic factors*
*OA chondrocytes were treated with ULK1 plasmid and with an empty vector as a control. mRNA expression mTOR, MMP-13, ADAMTS5 and COX-2 was significantly increased (n=6; *p<0.05) in OA chondrocytes treated with ULK1 plasmid compared to control chondrocytes transfected with the empty vector. Transfection effeciency in the OA chondrocytes (n=6) was confirmed by qPCR. An increase in the expression of ULK1 was observed at the mRNA levels in ULK1 treated cells compared to control treated cells.*

***Table 1.*** *List of genes regulated by ULK1 using ULK1 plasmid in OA chondrocytes*

| Fold change of 1.5 | | Fold change of 1.2 | | Fold change of 0.8 | |
|---|---|---|---|---|---|
| Gene | Fold change | Gene | Fold change | Gene | Fold change |
| CLN3 | 1.5823 | AMBRA1 | 1.3398 | ATG16L1 | 0.2778 |
| CXCR4 | 1.8947 | BID | 1.3585 | FADD | 0.3396 |
| IFNG | 1.6841 | CASP3 | 1.2159 | TNF | 0.477 |
| ULK1 | 3.2535 | NFKB1 | 1.2675 | MTOR | 0.5366 |

***Supplementary table 1****. List of primers used for QPCR analysis during the study*

| Genes | 5'-primer | 3'-primer |
|---|---|---|
| GAPDH | CAGAACATCATCCCTGCCTCT | GCTTGACAAAGTGGTCGTTGAG |
| ULK-1 | AGTCTCAGACGCTGCTGGGGA | CGCATGGCGTGCAGGTAGTC |
| mTOR | GCCCCCAAAGGACTTCGCCC | ACTGCAGTGAGGGCAGGGCTTA |
| COX-2 | TCCACCAACTTACAATGCTGACTATG | AATCATCAGGCACAGGAGGAAGG |
| MMP-13 | TCCCAGGAATTGGTGATA | CTGGCATGACGCGAACAAA |
| ADAMTS-5 | GAACATCGACCAACTCTACTCCG | CAATGCCCACCGAACCATCT |

# GENERAL DISCUSSION

# 5. GENERAL DISCUSSION

Osteoarthritis (OA) is among the most prevalent chronic human health disorders and the most common form of arthritis. OA is characterized by cartilage deterioration/damage, inflammation, synovial fibrosis, subchondral bone remodelling and osteophyte formation [222-225]. Aetiology and pathogenesis underlying OA is poorly understood. Hence, there is no satisfactory treatment. Loss of chondrocyte cellularity within the articular cartilage is one of the critical events that initiate the degradation of articular cartilage during OA[35]. However, it's still uncertain which mechanisms control the fate of chondrocytes within articular cartilage during normal versus OA conditions. Understanding the exact chondrocyte cell death/survival mechanisms could lead to several promising OA therapeutic strategies. It was recently shown that the process of autophagy, a form of programmed cell survival[173], is impaired during OA and contributes to decreased chondroprotection resulting in degradation of articular cartilage. My group specifically identified some of the key autophagy regulators required for the normal induction of autophagy machinery are reduced in OA cartilage and this suppression of autophagy mediators is associated with increased chondrocyte cell death. This prompted me to further investigate the role of autophagy in articular cartilage homeostasis. I particularly got interested in ULK1 because it is the most upstream autophagy inducer and its expression is reduced in articular cartilage.

ULK1 acts upstream of many autophagy genes: LC3 which is responsible for the formation and the expansion of autophagosome, it executes autophagy. ATG5, which is a regulator of autophagy and Beclin 1, which forms a complex that allows the nucleation of the autophagic vesicles. Studies have showed that ULK1 directly phosphorylates AMBRA1, a Beclin1-

interacting protein and regulatory component of the PI3K class III complex. During starvation conditions, once ULK1 is activated, it phosphorylates AMBRA1; this leads to the release of the PI3K complex and its translocation to the ER, where it initiates autophagosome formation[203]. It has also been shown that activated ULK1 phosphorylates beclin-1 on S14 that further enhances the activity of ATG14L-containing VPS34 complexes. This phosphorylation of beclin-1 by ULK1 is required for full autophagic induction[204].

There are emerging studies indicating that the function of ULK1 is not restricted to the maintenance of energy metabolism but it can coordinate several housekeeping mechanisms, e.g. autophagocytosis of damaged structures and alleviate stress by increasing tissue stress resistance. ULK1 has been associated with neuronal development, neurodegeneration, metabolism and cancer; however specific *in vivo* role of ULK1 in cartilage development and OA pathogenesis is largely unknown.

Since the exact role of ULK1 in OA pathophysiology is unknown, I created for the first time ULK1 cartilage-specific KO mice to study the specific *in vivo* role of ULK1 in articular cartilage homeostasis. I observed that ULK1 KO mice subjected DMM OA surgery (compared to control mice) exhibited accelerated cartilage degeneration associated with enhanced chondrocyte cell death, greater proteoglycan loss and enhanced synovial inflammation at 10 weeks post OA surgery. Using PCR array analysis in human OA chondrocytes transfected with ULK1 plasmid, I identified a panel of genes involved in cell death/survival pathway that are regulated by ULK1. Specifically, I identified that ULK1 reduces the expression of mTOR (major negative regulator of autophagy) as well as reduces the expression of ADAMTS-5 (major OA catabolic mediator). Overall, my MSc research shows that ULK1 is required for normal articular cartilage

homeostasis; loss of which results in accelerated cartilage degeneration and accelerated OA phenotype.

**Future Studies**

Though beyond the scope of my thesis, future studies need to be directed towards understanding the exact signaling pathway through which ULK1 operates within the articular cartilage. Since, ULK1 genetic deletion results in acceleration in OA phenotype, one would expect that ULK1 therapy may enhance chondrocyte survival and protect cartilage for degeneration. Therefore, studies are currently underway in my lab where we are planning to intra-articularly deliver ULK1 in mouse knee joints subjected to OA surgery to determine if ULK1 can delay or stop the process of articular cartilage degeneration. If these studies show positive results, future studies using preclinical dog models of OA (to mimic clinical scenario) can be utilized to fully exploit therapeutic potential of ULK1 therapy.

After we looked at the negative regulators of autophagy, it would be interesting to further continue the study and look at the positive regulators and the effectors of autophagy. ULK1 is the main inducer of autophagy. Some of the main autophagy genes like LC3, Beclin1, ATG5 etc… are directly affected by the expression of ULK1. Those genes are essential for the proper autophagy function. My lab is currently working on identifying the effect of the overexpression of ULK1 on the expression of those autophagy genes and through which pathway they are regulated by ULK1 and how this affects autophagy.

Results from those experiments combined with the results from the experiments described above should give us a clearer picture of the autophagy pathway and how it is modulated in OA.

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