Université de Montréal

# Rôle de la méthylation des histones dans la régulation de l'expression des gènes de la COX-2, iNOS, et mPGES-1 dans les chondrocytes humains: Implication pour l'arthrose

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# Role of histone methylation in the regulation of COX-2, iNOS, and mPGES-1 gene expression in human chondrocytes: Implication for Osteoarthritis

By

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Faculty of Graduate and Postdoctoral Studies

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# Résumé

L'arthrose (OA) est une maladie articulaire dégénérative, classée comme la forme la plus fréquente au monde. Elle est caractérisée par la dégénérescence du cartilage articulaire, l'inflammation de la membrane synoviale, et le remodelage de l'os sous-chondral. Ces changements structurels et fonctionnels sont dues à de nombreux facteurs.

Les cytokines, les prostaglandines (PG), et les espèces réactives de l'oxygène sont les principaux médiateurs impliqués dans la pathophysiologie de l'OA. L'interleukine-1β (IL-1β) est une cytokine pro-inflammatoire majeure qui joue un rôle crucial dans l'OA. L'IL-1β induit l'expression de la cyclooxygénase-2 (COX-2), la microsomale prostaglandine E synthase-1 (mPGES-1), la synthase inductible de l'oxyde nitrique (iNOS), ainsi que leurs produits la prostaglandine $E_2$ ($PGE_2$) et l'oxyde nitrique (NO). Ce sont des médiateurs essentiels de la réponse inflammatoire au cours de l'OA qui contribuent aux mécanismes des douleurs, de gonflement, et de destruction des tissus articulaires.

Les modifications épigénétiques jouent un rôle très important dans la régulation de l'expression de ces gènes pro-inflammatoires. Parmi ces modifications, la méthylation/ déméthylation des histones joue un rôle critique dans la régulation des gènes. La méthylation/ déméthylation des histones est médiée par deux types d'enzymes: les histones méthyltransférases (HMT) et les histones déméthylases (HDM) qui favorisent l'activation et/ou la répression de la transcription. Il est donc nécessaire de comprendre les mécanismes moléculaires qui contrôlent l'expression des gènes de la COX-2, la mPGES-1, et l'iNOS.

L'objectif de cette étude est de déterminer si la méthylation/déméthylation des histones contribute à la régulation de l’expression des gènes COX-2, mPGES-1, et iNOS dans des chondrocytes OA humains induits par l'IL-1β.

Nous avons montré que la méthylation de la lysine K4 de l'histone H3 (H3K4) par SET-1A contribue à l’activation des gènes COX-2 et iNOS dans les chondrocytes humains OA induite par l'IL-1β. Nous avons également montré que la lysine K9 de l’histone H3 (H3K9) est déméthylée par LSD1, et que cette déméthylation contribue à l’expression de la mPGES-1 induite par IL-1β dans les chondrocytes humains OA. Nous avons aussi trouvé que les niveaux d'expression des enzymes SET-1A et LSD1 sont élevés au niveau du cartilage OA.

Nos résultats montrent, pour la première fois, l'implication de la méthylation/ déméthylation des histones dans la régulation de l’expression des gènes COX-2, mPGES-1, et iNOS. Ces données suggèrent que ces mécanismes pourraient être une cible potentielle pour une intervention pharmacologique dans le traitement de la physiopathologie de l'OA.

**Mots clés**: Osteoarthrite, chondrocyte, Interleukin-1ß, COX-2, mPGES-1, $PGE_2$, iNOS, NO, inflammation, méthylation/déméthylation, histone.

# Abstract

Osteoarthritis (OA) is a disabling disease classified as the most common form of arthritis worldwide. It is characterized by cartilage degeneration, synovium inflammation, and subchondral bone remodeling resulting in a loss of joint function. These structural and functional changes are due to numerous factors.

Cytokines, prostaglandins (PG), and reactive oxygen species are the major mediators implicated in the pathophysiology of OA. Interleukin-1β (IL-1β) is a major pro-inflammatory cytokine that plays a crucial role in OA. IL-1β induces the expression of Cyclo-oxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1), inducible nitric oxide synthase (iNOS), as well as their products prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO). These are critical mediators of the inflammatory response during OA causing pain, swelling, and joint tissue destruction.

The activation of these pro-inflammatory genes results from different changes at the level of chromatin known as epigenetic modifications. Epigenetic modifications such as DNA methylation and histone modifications play a crucial role in gene expression. Among these modifications, histone methylation/demethylation is the most critical one. Histone methylation/demethylation is mediated by two types of enzymes: histone methyltransferases (HMT) and histone demethylases (HDM) which can either activate or repress transcription. It is therefore necessary to understand the molecular mechanisms which underlie the regulation of COX-2, mPGES-1, and iNOS expression.

The objective of this study is to investigate whether histone methylation/demethylation can modulate COX-2, mPGES-1, and iNOS expression in IL-1β induced OA human

chondrocytes.

We demonstrated that histone H3 lysine K4 (H3K4) methylation by SET-1A contributes to IL-1β-induced COX-2 and iNOS expression in human OA Chondrocytes. We showed also that LSD1-mediated demethylation of histone H3 lysine 9 (H3K9) contributes to IL-1β-induced mPGES-1 expression in human OA chondrocytes. We found that levels of SET-1A and LSD1 expression are elevated in OA cartilage as compared with normal cartilage.

Our data demonstrates, for the first time, the implication of histone methylation/demethylation in COX-2, mPGES-1, and iNOS regulation suggesting that these mechanisms could be a potential target for pharmacological intervention in the treatment of the pathophysiology of OA.

**Key Words**: Osteoarthritis, chondrocyte, Interleukin-1ß, COX-2, mPGES-1, $PGE_2$, iNOS, NO, inflammation, Histone methylation/demethylation.

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# List of abbreviations

AA: Arachidonic Acid

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motif

AIA: Antigen induced arthritis

AOL: Amine oxidase-like

AP-1: Activating protein-1

ARE: Adenylate- and uridylate (AU)-rich elements

ATP: Adenosine triphosphate

cAMP: Cyclic adenomonophosphate

C/EBP: CCAAT/enhancer-binding protein

CHD: Chromo-helicase/ATPase DNA binding

CIA: Collagen-induced-arthritis

cPGES: Cytosolic prostaglandine E synthase

COX-2: Cyclooxygenase-2

CRE: Cyclic AMP response element

CREB: cAMP response element binding protein

CREBBP: CREB-binding protein

Col-II: Type II Collagen

CoREST: Corepressor to the RE1 silencing transcriptionfactor

DNA: Deoxyribonucleic acid

DNMTs: DNA methyltransferases

ECM: Extracellular matrix

Egr-1: Early growth response gene-1

eNOS: Endothelial nitric oxide synthase

EP: E prostanoid

ERK1/2: Extracellular signal-regulated kinase 1/2

FAD: Flavin adenine dinucleotide

GAG: Glycosaminoglycan

GI: Gastrointestinal

GRE: Glucocorticoid-responsive elements

H: Histone

HA: Hyaluronic acid

HAT: Histone acetyltransferases

HDAC: Histone deacetylases

HDACi: Histone deacetylase inhibitors

HDMs: Hsitone demethylases

HIF-1α: Hypoxia-inducible factor 1- alpha

HMT: Histone methyltransferases

$H_2O_2$: Hydrogen peroxide

K: Lysine

Kb: Kilobase

kDa: Kilodalton

KDMs: Lysine demethylases

KMTs: Lysine methyltransferases

KO: Knockout

ICE: IL-1-converting enzyme

IFN-γ: Interferon-gamma

IGF-1: Insulin growth factor

INO80: Inositol requiring 80

iNOS: Inducible nitric oxide synthase

IL: Interleukin

IL-1ß: Interlukin-1 beta

IL-1R: Interlukin -1-receptor

IL-1Ra : IL-1 Receptor antagonist

ISWI: Imitation switch

JNK: c-Jun N-terminal kinase

JHDM: JmjC domain-containing histone demethylase

L-NIL: N-iminoethyl-L-lysine

L-NMMA: N-monomethyl-L-arginine

LIF: Leukemia inhibitory factor

LOXs: Lipoxygenases

LPS: Lipopolysaccharide

LSD: Lysine specific demethylase

MAPEG: Membrane-associated protein involved in eicosanoid and glutathion metabolism

MAPKs: Mitogen-activated protein kinases

MBD : Methyl-CpG-binding domain

MGST1-L1: Microsomal glutathione transferase-1-like-1

MiRNAs: Micro RNAs

MMPs: Matrix metalloproteinases

mPGES-1: Microsomal prostaglandine E synthase type 1

m1: Mono-methylated

m2: Di-methylated

m3: Tri-methylated

MTA: 5'-deoxy-5'-(methylthio)adenosine

NAB-1: NGF1-A-binding proteins-1

NaBu: Sodium butyrate

NFAT: Nuclear Factor of Activated T-cells

NF-κB: Nuclear factor- kappa B

NO: Nitric oxide

nNOS: Neuronal nitric oxide synthase

NSAID: Non-steroidal anti-inflammatory drug

NSD: Nuclear receptor SET domain

NuRD: Nucleosome remodelling and histone deacetylase

NURF: Nucleosome remodeling factor

OA: Osteoarthritis

PAD: Peptidyl arginine deiminases

PARs: Protease-activated receptors

$PGE_2$: Prostaglandin $E_2$

$PGI_2$: Prostaglandin $I_2$

$PGD_2$: Prostaglandin $D_2$

$PGF_2\alpha$: Prostaglandin $F_2\alpha$

$PGG_2$: Prostaglandin $G_2$

PGs: Prostaglandins

PGES: Prostaglandine E synthases

PHF: PHD Finger

$PLA_2$: Phospholipase $A_2$

PPARγ: Peroxisome proliferator-activated receptor gamma

PRDM: PRDI-BF1 and RIZ homology domain containing protein family

PRMTs: Protein arginine methyltransferases

R: Arginine

RA: Rheumatoid Arthritis

ROS: Reactive oxygen species

SAM: S-adenosyl-L-methionine

siRNA: Small interfering RNA

SIRT: Sirtuin

Sox-9: Sex determining region- 9

Sp1: Specificity protein-1

STAT-1α: Signal transducer and activator of transcription-1 alpha

SWI/SNF: SWItch/Sucrose NonFermentable

TACE: TNF-α converting enzyme

TCP: Tranylcypromine

TGF-ß:Transforming growth factor-ß

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-alpha

TNFR: Tumor necrosis factor receptor

TSA: Trichostatine A

$TXA_2$: Thromboxane $A_2$

UTR: Untranslated region

VA : Valproic acid

Wnt: Wingless integration site

To my parents, my sister, my brother,

To my grandma and grandpa, my aunties,

To all my family and friends.

Thank you for believing in me, thank you for your unfading love, thank you for your confidence, continuous encouragement, and sacrifice throughout my studies

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# Part A-

## 1. Osteoarthritis:

### 1.1 Definition of Osteoarthritis:

Osteoarthritis (OA) is a degenerative joint disease that affects a wide range of population (1). OA is considered as a disease of the entire joint involving all tissues. It is a composite of pathologic contribution from cartilage, synovial membrane, bone and adjacent soft tissues. The pathophysiological changes of OA include degradation and erosion of the articular cartilage, inflammation of the synovial membrane “synovitis”, subchondral bone remodeling, marginal osteophytosis, joint capsule fibrosis, tearing and fibrillation of intra-articular ligaments and menisci (2, 3). These changes are important for the onset and OA progression (**Figure 1**).

OA is a group of overlapping distinct diseases, which may have different etiologies, but with similar biologic, morphologic, and clinical outcomes. It is the result of both mechanical and biologic events. OA occurs when the equilibrium between the breakdown and the repair of joint tissues becomes unbalanced. This happens often when the mechanical loads applied exceed those that can be tolerated by the joint tissues causing joint pain, tenderness, limitation of movement, occasional effusion, and variable degrees of inflammation. Knees, hips, feet, and spine are the most frequently affected joints. Others such as finger and thumb joints may also be affected (4).

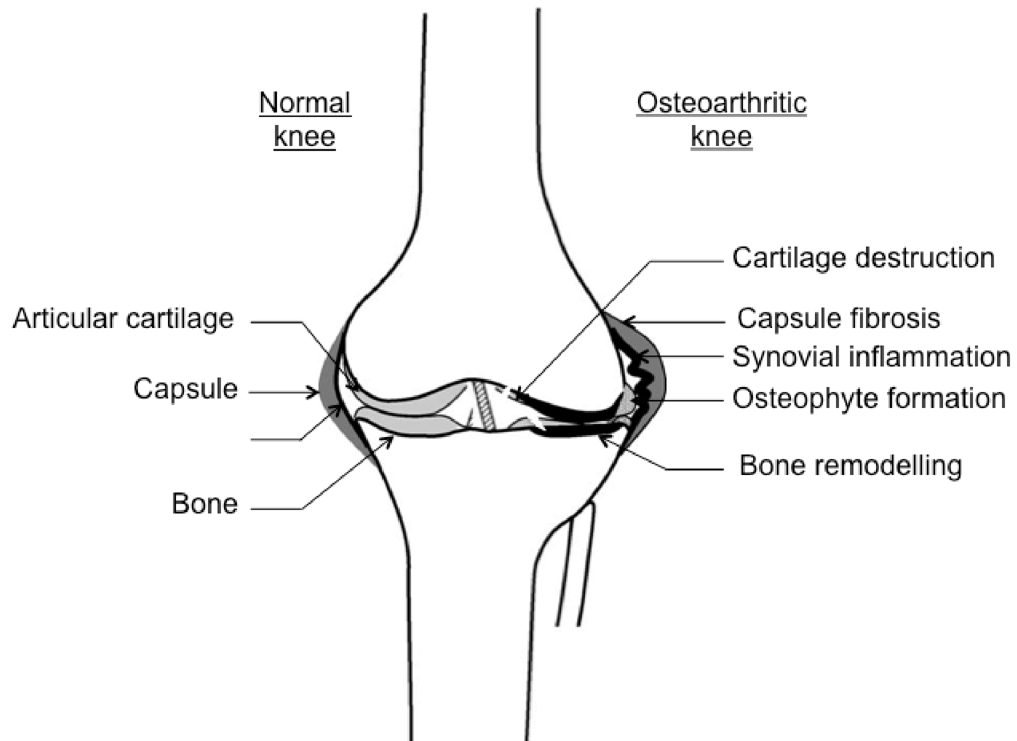


**Figure 1**: Schematic representation of the main constituents of a normal and osteoarthritic knee. OA is a disease of the whole joint with pathological changes occurring in all joint tissues. As shown, in the healthy (normal) cartilage, there is no degradation, no signs of synovial inflammation and no bone remodeling. However, in OA, cartilage is degenerated with lesions and fibrillation. This is accompanied by synovial inflammation, capsule fibrosis, and remodeling of bone leading to bony outgrowth and subchondral sclerosis (Adapted from 5).

## 1.2 Epidemiology of Osteoarthritis:

OA is the most common form of chronic arthritis. It is a highly prevalent disabling disease that has a large worldwide socioeconomic cost affecting approximately 15% of the population (6). In Canada, OA is one of the leading causes of disability and accounts for the majority of the disease burden for musculoskeletal disorders. Over 13% of Canadians are estimated to suffer from OA (7). In the United States, OA affects more than 10% of Americans (at least 27 million) (8, 9). People above the age of 65 years are more likely to develop OA. While only 7.6% of those 18-44 years of age and 29.8% of those 45-64 years of age, more than 50% of people older than 65 years are diagnosed with OA (10).

OA is classified into two groups: primary and secondary OA. Primary OA, called also idiopathic OA, is the most common form of OA. It is classified as primary when there are clear predisposing causes like age and heredity (11). Primary OA is a frustrating disease because both the cause and cure are unknown. However, OA is classified as secondary when it is obviously associated with a defined pathology most likely developmental disorders, trauma and metabolic diseases (11, 12).

## 1.3 Symptoms of Osteoarthritis:

Symptoms of OA vary overtime between joint sites and individuals. Clinically, OA is described by joint pain, dysfunction, stiffness, deformity and joint space narrowing. It tends to follow periods of inactivity, such as sleep or sitting. Pain is typically accompanied by stiffness at the morning (11). As the disease advances, the pain may occur even when the joint is at rest.

OA Patients describe the most distressing aspect of living as fatigue, disability and reduced quality of life produced by chronic joint pain (13). Chronic pain in OA patients depends

primarily on the activation of sensory neurons that innervate the affected joint (14). In the joint and surrounding tissues, nociceptin, an endogenous peptide with opoid-like-activity, can be produced and delivered to its appropriate receptors, the so-called silent nociceptors which are located in peripheral tissues like capsule and ligaments (11, 15). In healthy joints, nerve fibers are quiescent. However, due to tissue injury or induction of inflammation, these receptors become active and start sending nociceptive information to the central nervous system. Moreover, joint nerves become sensitized to mechanical stimuli through the actions of eicosanoids, proteinase activated receptors and several others molecules. Patients with OA may also experience sensation of instability or buckling.

OA symptoms also include loss of mobility and cracking noise with joint movement (16). These signs are often associated with significant functional impairment and result in considerable impact on ability to perform activities of daily living (15, 17).

Radiography like X-rays and magnetic resonance imaging remain the best tools for OA diagnosis. Osteophyte formation and sclerosis are the most critical signs detected with these tests. Some patients, with no symptoms, have showed severe radiographic changes. However, other individuals experience significant joint pain with only minimal radiographic changes (13). This might be due to joint space width, which is too insensitive to determine structural alterations (14). More than 60 % of people above the age of 75 years old present radiologic signs. Further studies have confirmed discordance between radiographically diagnosed knee OA and symptoms like pain (14, 17).

## 1.4 Risk factors of Osteoarthritis:

The exact etiology of OA is unknown; however, a variety of risk factors have been described. They are classified into systemic and local risk factors. Systemic risk factors for OA include age, gender, sex, race, genetic predisposition, and obesity, whereas local risk factors include certain physical activities, tissue injuries, and developmental deformities (18, 19).

### 1.4.1. Systemic factors:

a. Age:

Aging is one of the most prominent risk factor for OA development. It is considered as a strong predictor of OA (6). Numerous studies have found that increased age promotes the initiation and progression of OA. Mitotic and synthetic cell activities decline with age, resulting in a reduced cartilage hydration (20).

Women are associated with a higher prevalence and severity of OA. Females are more likely to suffer from severe knee OA than males, especially following menopause. This has led to investigate the role of oestrogen in OA (6, 21).

b. Genetics:

Genetic factors are strong determinants in the pathogenesis of OA; they account for at least 40% of knee OA (6). Many genes have been shown to play crucial role in OA pathophysiological pathways. This includes genes involved in the regulation of inflammatory responses such as cytokines, other pro-inflammatory mediators, and genes involved in cartilage and bone metabolism (22). For instance, vitamin D receptors, insulin-like growth factor-1, and type II collagen (Col-II) genes have been demonstrated to be implicated in the susceptibility and the severity of OA and may represent therapeutic targets (6).

c. Obesity:

Obesity is another pivotal risk factor for OA incidence and progression (6, 23). It is one of the major OA modifiable risk factors (18, 19, 24). It has become a major focus since the identification of the white adipose tissue, which secretes adipokines. These biological active substances are highly produced in overweight people and may affect cartilage homeostasis. Leptin, the most abundantly produced adipokine, is released by adipose tissue. Leptin receptors are present in cartilage (25). Adipocyte cells share a common mesenchymal stem-cell precursor with chondrocytes and osteoblasts (14, 26). Leptin regulates bone mass and mineralization via a neuroendocrine pathway implicating the sympathetic nervous system (27). It has been shown to increase levels of degradative enzymes like matrix metalloproteinases (MMPs), nitric oxide (NO) and pro-inflammatory cytokines (24, 28).

d. Diet:

Studies have emphasized the importance of early life nutrition (29). Low levels of vitamin D are associated with the pathological changes of OA. It has been reported that low levels of vitamin D may increase the incidence and the progression of knee and hip OA, predicting also loss of joint space and increased osteophyte growth in knee OA (30). In addition, low intake of vitamin C has been also shown to be associated with an increased risk of knee OA progression (6).

### 1.4.2. Local factors:

a. Occupation and physical activity:

Studies have found that people whose occupations require physical activity have twice the risk of developing knee OA than occupations that doesn't. In addition, workers in some occupations like athletics, coal miners and farmers have also increased risks of knee OA (6).

b. Tissue injuries:

Tissue injury is the second major risk factor of OA (18, 19). Athletics like soccer and football players are exposed to a high risk of OA due to the high incidence of menisectomy and cruciate ligament injuries (11).

## 2. Articulation components:

Joints provide support, stability, and protection. These are essential functions for normal and painless movement. The knee joint is a synovial joint that is composed of bones, cartilage, ligaments, tendons and joint capsule connecting the femur to the tibia. These bones, attached by ligaments, give strength and flexibility in the knee. The cartilage, the synovium, and the subchondral bone are the three basic elements that supply joint functions. Cartilage is a tissue that coats the ends of the bones; one of the few tissues in the body that does not have its own blood supply. Synovium is a membrane that surrounds the entire joint. It is filled with a lubricating liquid, the synovial fluid that supplies nutrients and oxygen to cartilage. The third element is the subchondral bone. The main role of subchondral bone is to provide structural support to the overlying articular cartilage (31) (**Figure 1**).

### 2.1. Articular Cartilage:

Cartilage is a specialized translucent connective tissue that covers the weight-bearing surfaces of articulating joints. There are three types of cartilage: the elastic cartilage, the fibro-cartilage, and the articular cartilage called also the hyaline cartilage. Articular cartilage is a hypocellular, aneural, and avascular tissue. This smooth lubricated tissue is derived mainly from

the synovial fluid, which consists of water and nutrients including electrolytes, small molecules and glucose (32).

The principal role of articular cartilage is reducing friction in the joint and absorbing the shock associated with locomotion (33). Articular cartilage affords a resistance to compressive forces. Thereby, it protects the underlying bones from mechanical damage during loading of the joint and allows for an efficient gliding motion during joint movement. This mechanical load is necessary for cartilage homeostasis. It induces fluid movement between the cartilage and the synovial fluid allowing the diffusion of molecules across cartilage and thus facilitating its nutrition (34). Thus, the main function of articular cartilage is the absorption and dissipation of mechanical load.

In its molecular composition, articular cartilage is composed of two main elements: the chondrocytes and the extracellular matrix (ECM) (**Figure 2**). Chondrocytes represent the unique cell type of cartilage that lies in the ECM. However, ECM is an extensive network of collagen fibrils, proteoglycan molecules, and water (35). While water represents about 75% of the wet weight, about 70% of the dry weight is collagen (9). Col-II is the principal type of collagen present in the articular cartilage.

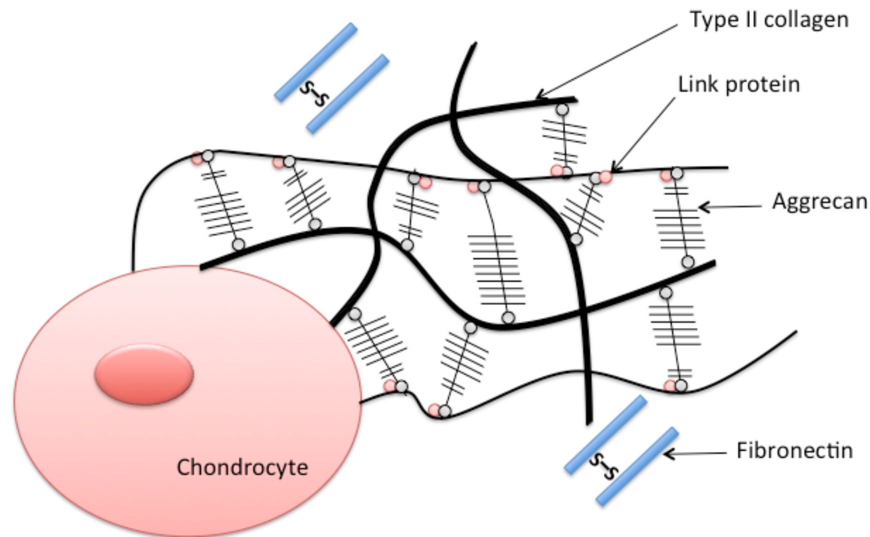


**Figure 2**: The molecular composition of normal articular cartilage. In healthy articular cartilage, chondrocytes are surrounded by the extracellular matrix (ECM). The ECM is composed of several types of collagens (Type II collagen), collagen-binding proteins (link protein), large molecules of proteoglycan (aggrecan), small molecules of proteoglycan (fibronectin), and sulfate (S-S).

Articular cartilage consists of four zones: the superficial, middle, deep and calcified zone. The superficial zone is the thinnest zone of articular cartilage. It is composed of a highly structured network of uniform collagen fibers, proteoglycans, non-collagenous proteins and other ECM proteins. This layer maintains a high water content. The middle layer, called also the transitional zone, is composed of larger rounded chondrocytes. The collagen fibers are randomly oriented within this layer. Unlike the middle zone, the deep zone is constituted of collagen fibers that are arranged perpendicularly. The chondrocytes are grouped in columns. While the

concentration of water is low in this layer, the proteoglycan content is high. The calcified zone is the last layer of articular cartilage; it is composed of calcified cartilage and hypertrophic chondrocytes. This zone is characterized by the absence of proteoglycans (32). Of importance, the two last layers, the deep and calcified zones, are separated by a thick bundle of collagen named the tidemark. The tidemark is a thin line that marks the mineralization front between the calcified and the non-calcified articular cartilage (34). Small gaps that may exist in the tidemark allow the passage of nutrients through channels (32) (**Figure 3**).

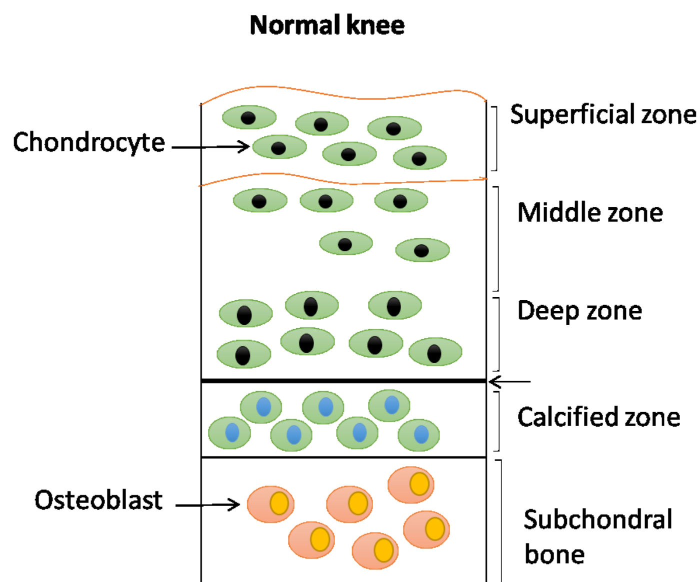


**Figure 3**: The anatomy of articular cartilage and subchondral bone in normal knee. Normal articular cartilage is divided into four zones: superficial zone, middle zone, deep zone, and calcified zone. Each zone is made of small number of chondrocytes embedded in collagen matrix. The calcified zone is separated from the deep zone by a tidemark (demarcation line). This calcified zone is located above the subchondral bone.

### 2.1.1. Chondrocytes:

Chondrocytes are the unique cellular component of articular cartilage (32). These cells represent 1-5 % volume of the articular cartilage (35). They receive their nutrition by diffusion through the matrix. The primary function of chondrocytes is to maintain cartilage homeostasis by the production of ECM components. They synthesize and degrade matrix components in response to environmental conditions like growth factors, cytokines, and biomechanical variations (9). Chondrocytes divide and produce new matrix in the peripheral zone. They produce collagens, proteoglycans, and non-collagenous proteins and organize all of these components in a highly ordered structure (32).

Number, size, and shape of chondrocytes vary depending on the layer of cartilage plate in which they are located. For instance, in the superficial zone, chondrocytes show a flattened ellipsoid form in parallel to the join surface. In this layer, they synthesize high concentration of collagen and low concentration of proteoglycans to provide the highest water content. However, in the transitional zone, chondrocytes are predominantly spheroid and the proteoglycan aggrecan concentration is higher. Chondrocytes in the superficial zone synthesize various relative amounts of proteoglycans than do cells in the deeper zone. Unlike the previous layers, the calcified cartilage zone contains small number of cells showing very low metabolic activities (35). Thus, most of the tissue contains water and inorganic salts such as sodium, calcium, and potassium chloride. The content of water and other molecules plays a crucial role in maintaining the resiliency of the tissue and contributing to the nutrition and lubrication system. These characteristics endow cartilage tissue with special properties like elasticity and ability to absorb and distribute loads.

Chondrocytes survive under hypoxic conditions (< 5% $pO_2$) possessing a low metabolic activity. Their metabolic activity differs in the various layers of cartilage. Chondrocytes maintain the balance between anabolism and catabolism mechanisms as well as a continual remodeling since there is replacement of matrix macromolecules lost through destruction. Together, the interaction between the chondrocytes and the ECM allows maintenance of the biological and mechanical properties of the articular cartilage.

#### 2.1.2. Extracellular matrix:

The extracellular matrix is primarily made of tissue fluid and macromolecules like collagen, proteoglycans and non-collagenous proteins in specific distribution depending on the articulation. This texture provides tensile strength and resistance to compressive load.

More than 90% articular cartilage's dry weight consists of two major components, Col-II and the large molecules of proteoglycan, aggrecan (36). Col-II is the most important type of the cartilage matrix. It is synthesized by chondrocytes. However, other collagen types such as VI, IX, XI, XII, and XIV that are contained in the ECM have also important structural and functional properties (37). While collagen forms a mesh to give support and flexibility to the joint, proteoglycans molecules are capable to ensure the high−fluid content in cartilage. Proteoglycan molecules are composed of glycosaminoglycan (GAG) subunits. They are bound to the protein core by means of sugar bonds. Due to link proteins, these chains are stabilized with a central hyaluronic acid (HA) chain (38) (**Figure 2**).

There are two major classes, large aggregating proteoglycan monomers, aggrecans, and small proteoglycans. Aggrecan is the main proteoglycan present in cartilage constituting 90% of the total cartilage proteoglycan mass. It is an elastic macromolecule that gives the tissue its ability to resist compression. The cartilage matrix contains also smaller proteoglycans like syndecans,

glypican, decorin, biglycan, versican, fibromodulin, lumican, and perlecan. These molecules are produced inside the chondrocytes and secreted in the matrix. They make up approximately 3% of the total proteoglycan mass (39, 40). Because of the hydrophilic nature of proteoglycans, the high water content of normal cartilage is maintained.

Transforming Growth Factor-β (TGF-β) is another important protein produced in the matrix. It plays a critical role in a variety of physiological processes like cell proliferation, differentiation, and apoptosis. Chondrocytes secrete TGF-β in an inactive form, which is covalently bound to TGF-β binding proteins. TGF-β is activated from the growth plate by factors such as MMPs in order to bind to its receptor (41, 42).

TGF-β has several regulatory mechanisms. It stimulates chondrocytes to induce aggrecan and Col-II production and also to intiate the first step of chondrogenesis. Of important, TGF-β acts against inflammatory cytokines like IL-1β, responsible for upregulation of MMPs like MMP-13. TGF-β promotes cartilage ECM synthesis through counteracting the effects of catabolic cytokines (43).

The specific distribution and functions of collagens, proteoglycans, link proteins, hyluronic acids, and other components provide an integrated hydroelastic suspension system capable of resisting compression. Thus, the uniqueness of articular cartilage lies in its remarkable elasticity and ability to withstand enormous physical forces. Such extraordinary features of cartilage tissue are related to the collagen network and the high water content that is tightly held within the extracellular matrix.

## 2.2. Synovial membrane

The synovial membrane is a soft tissue made of layers that line the spaces of diarthrodial joints, tendons, and bursae. It is a tissue that secretes a glairy fluid named the synovial fluid. The synovial membrane plays an important role in maintaining normal joint physiology and function (44).

The synovial membrane covers all the intra-articular structures. It is composed of two layers: the synovial lining layer and the connective sublining layer (45). The synovial lining layer is made up of two kinds of cells: macrophage-like type A and fibroblastic type B cells. While type B cells synthesize and modify ECM and synovial fluid components, type A cells predominantly eliminate degradation products, including fluid and fine particulate materials from the joint space and from their ECM. In addition to these two different cells populations (type A and B), several additional studies have identified a third type of intermediate synovial lining cells which express CD68, a macrophage marker, indicating that these cells share both phenotypic properties of macrophages and fibroblastic cell types. According to that, it is thought that these three types of synovial lining cells originate from the same cell lineage and differentiate under the influence of local conditions (46).

The synovial fluid of joints functions as a biological lubricant and provides low friction and low-wear properties to articulating cartilage surfaces in order to facilitate motion. These lubricants, secreted by synovial cells in the synovium, are concentrated in the synovial space (47). Moreover, hyaluronans are large polysaccharide molecules found naturally in the synovial fluid; they help to create a viscous environment cushioning joints and preserving normal function. A deficiency in this lubricating system may contribute to the erosion of articulating cartilage surfaces in OA conditions. Hyaluronans are extensively used in the management of OA (48).

## 2.3. Subchondral bone:

Bone is a vascularized tissue constituted of bone forming cells, osteoblasts, and osteocytes. Chemically, bone is made up of both organic and mineral components. While the organic component is primarily type I collagen, hydroxyapatite is the mineral component of bone. As bone matures, the size, crystallinity, and stoichiometry of the hydroxyapatite crystals change. These substitutions into the hydroxyapatite lattice are very important to bone strength and flexibility (49).

The subchondral bone is the epiphyseal bone located under the articular cartilage. It includes the subchondral bone plate and the underlying trabecular bone (4, 49). The subchondral bone provides structural support to the overlying articular cartilage. Several studies have demonstrated the potent role of abnormal subchondral bone cell metabolism in the initiation and progression of OA (11). In addition to the articular cartilage destruction, OA is characterized also by the increase of subchondral plate thickness and the formation of new bone at the joint margins, called osteophytes (50). It has been shown that subchondral bone changes may actually precede those of the synovial membrane and articular cartilage. The concept of crosstalk between subchondral bone tissue and articular cartilage that may be crucial for the initiation and/or progression of OA was highlighted (51).

# 3. The pathophysiological mechanisms of Osteoarthritis:

In addition to the pivotal role of cartilage destruction as a hallmark in OA, the synovium and subchondral bone are implicated in OA development and progression. The cartilaginous changes are accompanied by synovial inflammation and pathological remodeling in the subchondral bone (15).

Under normal conditions, chondrocytes maintain a dynamic balance between synthesis and degradation of matrix components. In such non-stressed steady states, chondrocytes are quiescent and there is very little low turnover of collagen network (34). However, In OA, a disruption of matrix equilibrium leads to progressive degeneration of cartilage tissue with an increase in matrix-degrading enzymes within the joint. A multitude of molecules drive cartilage breakdown and disrupt cartilage homeostasis (14). These changes are accompanied by a tremendous loss of proteoglycan from the upper zone followed by degradation of the collagen network (**Figure 4**). The metabolism of chondrocytes becomes unbalanced because of the excessive production of catabolic mediators with a down-regulation of anabolic mechanisms. Destruction of the ECM causes a gradual impairment of the articular cartilage accompanied with pain and physical disability (15). Further irregularities at the cartilage surface such as fibrillation are also features of cartilage damage in OA.

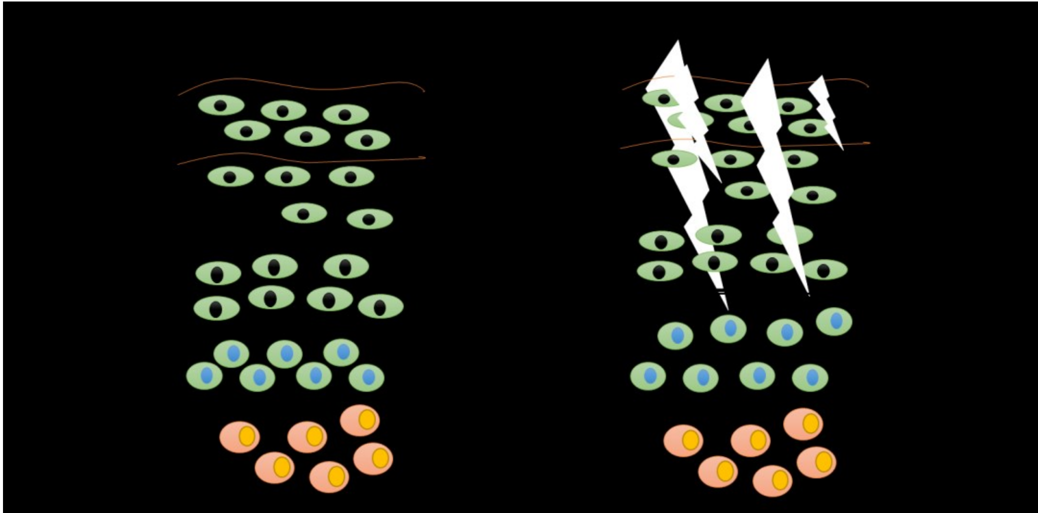

**Figure 4**: Comparison of the anatomy of articular cartilage and subchondral bone in normal and osteoarthritic knee. Normal articular cartilage is divided into four zones: superficial zone, middle zone, deep zone, and calcified zone. Each zone is made of small number of chondrocytes embedded in collagen matrix. The calcified zone is separated from the deep zone by a tidemark (demarcation line). This calcified zone is located above the subchondral bone. In OA, Fissured articular cartilage induces vascularization of cartilage, which leads to exposure of subchondral bone to external surface. Microcracks that go through the cartilage and the subchondral bone contribute to reactivation and upward shifting of the tidemark.

The excessive catabolic activity which results in an imbalance of cartilage homeostasis and matrix breakdown is largely mediated by pro-inflammatory mediators including cytokines, prostaglandins and other mediators. Chondrocytes produce mediators associated with inflammation like cytokines, chemokines (52), and proteolytic enzymes that can cause further

damage to the cartilage. Interleukin-1β (IL-1β), MMPs, growth factors and free radicals, are key contributors to cartilage destruction (14, 52) (**Figure 5**). The activation of these mediators causes an aberrant expression of inflammation related genes including IL-1β converting enzyme (ICE/caspase-1), type IL-1 receptor (IL-1R), and tumor necrosis factor-α (TNF-α).

Synovial inflammation, or synovitis, may be either a primary event that initiate OA or a secondary mechanism that happens due to the accumulation of cartilage degradation products within the joint. Synovitis can result from both acute and chronic inflammatory state. It involves infiltration of mononuclear cells into the synovial membrane as well as production of pro-inflammatory mediators like IL-1β, TNF-α, and chemokines. IL-1β and TNFα are able to excite and sensitize nociceptors thereby inducing pain (14). Studies have showed that following acute anterior cruciate ligament, high levels of inflammatory biomarkers can be detected in synovial fluids (53). As stated above, joint space is filled with synovial fluid that is abundantly composed of HA. In OA, the concentration and the molecular size of HA are diminished. These changes result in less efficiency of lubrication. Furthermore, synovial mast cells are particularly implicated and increase in number during OA (14).

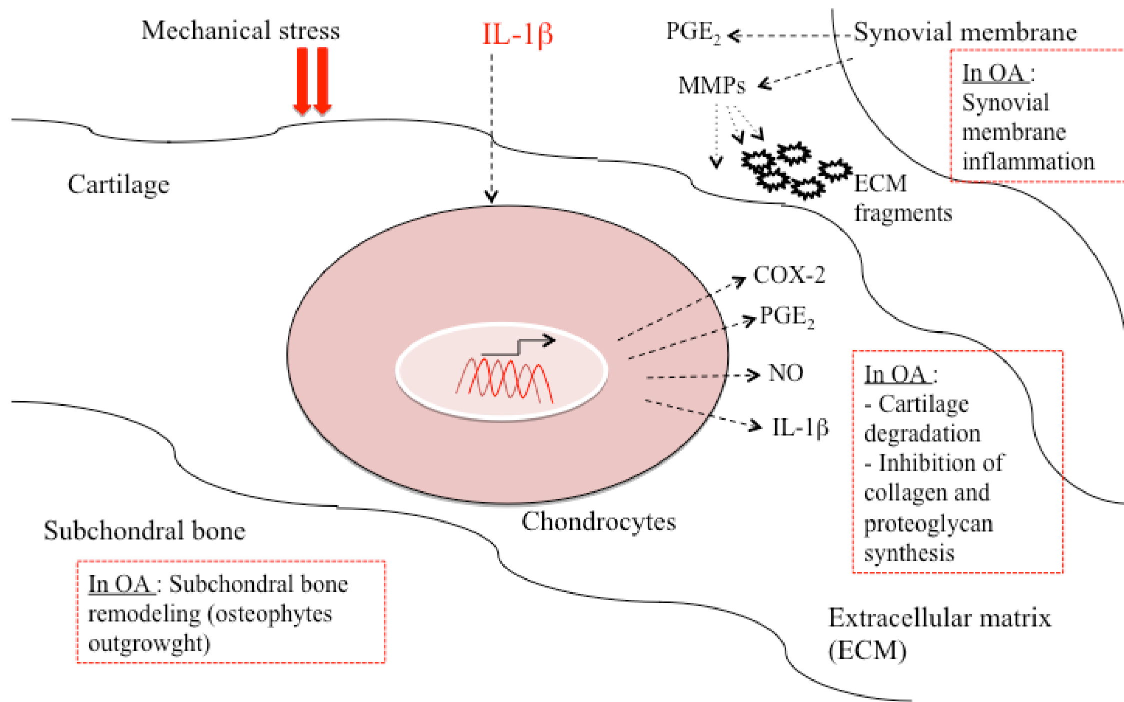


**Figure 5**: Molecular and cellular mechanisms in osteoarthritis. Mechanisms that drive cartilage destruction, synovial membrane inflammation as well as subchondral bone remodeling in osteoarthritis. Abbreviations: IL-1β, interleukin-1β; MMPs, matrix metalloproteinases; NO, nitric oxide; $PGE_2$, prostaglandin $E_2$; COX-2, cyclooxygenase-2; ECM, extracellular matrix.

The pathological structural changes that occur in the subchondral bone, both cortical and trabecular, are also one of the hallmarks of OA. The cortical subchondral plate becomes thick with irregularities at the trabecular bone (54). The role of subchondral bone in OA biology has been an interesting area of investigation. As the cartilage breaks down, changes occur in the underlying bone. Changes in subchondral bone mineralization and bone volume have been detected in samples with severe cartilage damage (55, 56). During the development of OA, subchondral bone undergoes adaptations like an increase in the subchondral plate thickness, sclerosis, reduced matrix mineralization, increased cancellous bone volume, osteophyte formation, and advancement of the tidemark associated with vascular invasion of the calcified cartilage (14, 57). However, it is still unknown whether these subchondral bone changes occur at the same time as changes in articular cartilage.

## 4. Role of inflammation in Osteoarthritis:

The inflammatory response is a series of local cellular and vascular mechanisms triggered in response to injuries and damage that a tissue may face. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli, whereas chronic inflammation is a prolonged response that leads to a progressive shift in the type of cells, present at the site of inflammation. Clinical manifestation of inflammation includes rubor (redness), tumor (swelling), dolor (pain), and fever (58). The inflammatory process is characterized by simultaneous destruction and healing of the tissue. A cascade of biochemical events propagates and matures the inflammatory response, involving the immune system, local vascular system, various cells, and different inflammatory mediators within the injured tissue.

Cytokines, prostaglandins (PGs) and reactive oxygen species (ROS) are the key players in the inflammatory process (59, 60, 61).

OA is now well recognized as an inflammatory arthropathy. It is shown to be associated with signs and symptoms of inflammation. The involvement of an inflammatory response is marked by symptoms such as joint pain, swelling and stiffness. Numerous studies have shown that inflammatory mediators are highly implicated in OA (61).

Together, the articular cartilage, the synovial membrane, and the subchondral bone undergo alterations in the pathophysiology of OA. In fact, there is a coordinated release of cytokines and other inflammatory mediators from these three tissues. Such network makes them in a situation of interdependence, evidence that was supported by the magnetic resonance imaging techniques (11). Synoviocytes are considered as the principal cells mediating joint inflammation. This occurs through secretion of effector molecules that act on a variety of cells to modulate joint inflammation and promote matrix degradation. Cytokines and growth factors are the best example of these effector molecules that can be released. For instance, within the synovium, the presence of cytokine networks involves complex interactions between lymphocytes, synovial fibroblasts and macrophages. The secretion of IL-1β or TNF-α by monocytes/ macrophages followed by activation of resident tissue cells, such as fibroblasts, triggers the inflammatory cascade (45).

It is believed that synovial inflammation is a factor that contributes to dysregulation of chondrocytes function causing an imbalance between the catabolic and anabolic activities of chondrocytes. Interestingly, chondrocytes in OA cartilage express IL-1β, ICE (caspase-1), and IL-1RI. IL-1β is synthesized by chondrocytes at concentrations that are capable to induce the expression of MMPs, aggrecanases, and other catabolic genes. It colocalizes with TNF-α, MMP-1, -3, -8, and -13, and Col-II cleavage epitopes in regions of matrix depletion in OA cartilage (62).

Cartilage breakdown products, resulting from mechanical or enzymatic destruction, can provoke, in turn, the release of collagenases and other hydrolytic enzymes from the synovial cells; thereby, leading to vascular hyperplasia in OA synovial membranes. This cascade sequentially results in the induction of synovial IL-1β and TNF-α, which further the inflammatory outcome (37). Thus, high levels of cytokines and proteinases may exacerbate the inflammatory process.

## 4.1. Cytokines in Osteoarthritis:

Cytokines play a pivotal role during the inflammatory process in the pathophysiology of OA. They cause a loss of metabolic homeostasis through promoting the catabolic process. They might be produced either spontaneously or following stimulation of the joint tissue cells (15, 63).

Cytokines are classified with respect to their biological pro-inflammatory and anti-inflammatory effect. While IL-1β, TNF-α, interleukin-6 (IL-6), interleukin-15 (IL-15), interleukin-17 (IL-17), and interleukin-18 (IL-18) are categorized as pro-inflammatory mediators, interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13) are anti-inflammatory cytokines that modulate the inflammatory response (**Figure 6**).

### 4.1.1. The pro-inflammatory cytokines:

Pro-inflammatory cytokines have a crucial role in OA development and progression. They induce degradation of matrix molecules by enhancing the production and activation proteolytic enzymes like collagenases and aggrecanases.

In OA, cytokine expression is suggested to result from the mechanical insult. This is associated with subsequent MMP expression. For instance, IL-1β and TNF-α, secreted by chondrocytes or other cells like synoviocytes, promotes the expression of matrix enzymes (32).

Either IL-1β or TNF-α, can recruit a unique set of receptor-associated proteins that transduce the stimulus into the cell upon ligand binding.

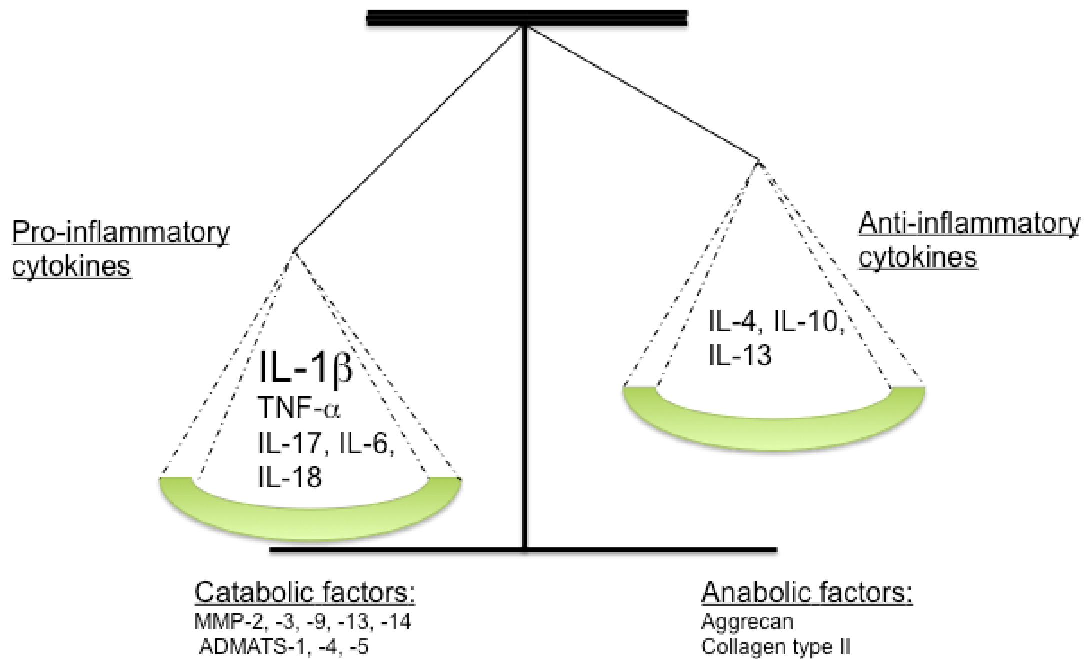


**Figure 6**: Imbalance of cytokine production in osteoarthritic cartilage. Chondrocytes are active players within the process of inflammation. The increased production of pro-inflammatory cytokines enhances the cartilage matrix turnover. Chondrocytes increase the catabolic activity through synthesizing most of the matrix degrading proteases and decrease the anabolic activity by down-regulating collagen and proteoglycan synthesis. Abbreviations: IL-1β, interleukin-1β; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-18, interleukin-18; TNF-α, tumor necrosis factor-α; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs.

Although their receptors are different, IL-1β and TNF-α elicit series of shared phosphorylation events within the cells that facilitate transcriptional induction of MMPs as well as a number of distinct inflammatory and catabolic factors (64). These phosphorylation events are mediated by specific group of kinases, the mitogen activated protein kinases (MAPKs).

IL-1β, TNF-α, and IL-17 play important roles in OA progression. These cytokines increase cartilage destruction, synovial inflammation, and also bone resorption. However, there are other pro-inflammatory cytokines that have been shown to be expressed in OA tissues and have been considered as essential contributing factors. IL-6 has been proposed as an amplifier of the IL-1β effects on the increased synthesis of MMPs (65), IL-8 for its chemotacic activity and ability of generating reactive oxygen metabolites (66), LIF that has diverse effects including the enhancement of IL-1β expression in chondrocytes (67).

**a. <u>Interleukin-1β:</u>**

- <u>Interleukin-1β expression and regulation:</u>

IL-1β belongs to the IL-1 family; it is primarily produced as a cytosolic precursor protein pro-IL-1β (60, 69). The active form of IL-1β then results from an intracellular proteolysis accomplished by the ICE and finally released in the extracellular space (60, 70). IL-1β has two membrane receptors: interleukin-1 receptor-1 (IL-1R1) and interleukin-1 receptor-2 (IL-1R2). The activation of cells by IL-1β is mediated by its interaction with these receptors. These receptors may bind to a receptor antagonist named interleukin-1 receptor antagonist (IL-1Ra); thereby blocking their interaction with IL-1β. In the joint, IL-1β is mainly produced by chondrocytes, osteoblasts, and cells of the synovial membrane (60, 71).

IL-1β induces its effect by activating several signaling pathways like nuclear factor-kappa B (NF-κB), p38MAPK, and c-Jun N-terminal kinase (JNK) initiated once bound to its receptor

(72). Induction of the NF-κB pathway by IL-1β or TNFα results in phosphorylation of the IκB kinase. The subsequent degradation of this kinase unmasks the latent NF-κB, which translocates into the nucleus. This promotes the expression of many genes like cytokines, chemokines, MMPs and other inflammatory mediators (60, 73) (**Figure 7**).

- Role of interleukin-1β in Osteoarthritis:

IL-1β is the main inflammatory mediator implicated in numerous pathological features of OA. Patients with OA have elevated levels of IL-1β in the cartilage, the synovial membrane, the synovial fluid, and the subchondral bone. It has been reported that IL-1β induces the inflammatory response during the course of OA. Immunohistochemical studies revealed that IL-1β is produced in the superficial zone of human OA cartilage in which the degenerative changes has been identified (60, 74).

Chondrocytes express not only IL-1β but also the receptor of this interleukin (IL-1RI). Higher levels of IL-1RI have been detected in OA patients (71). These destructive effects of IL-1β in OA mediate elevation of cartilage catabolism, both by targeting MMPs for cartilage destruction, decreasing ECM synthesis, and leading to a down-regulation of anabolic activities of articular cartilage. Because of this high level of IL-1β in OA cartilage, the correlation between the expression of IL-1β and the severity of cartilage damage can be understood.

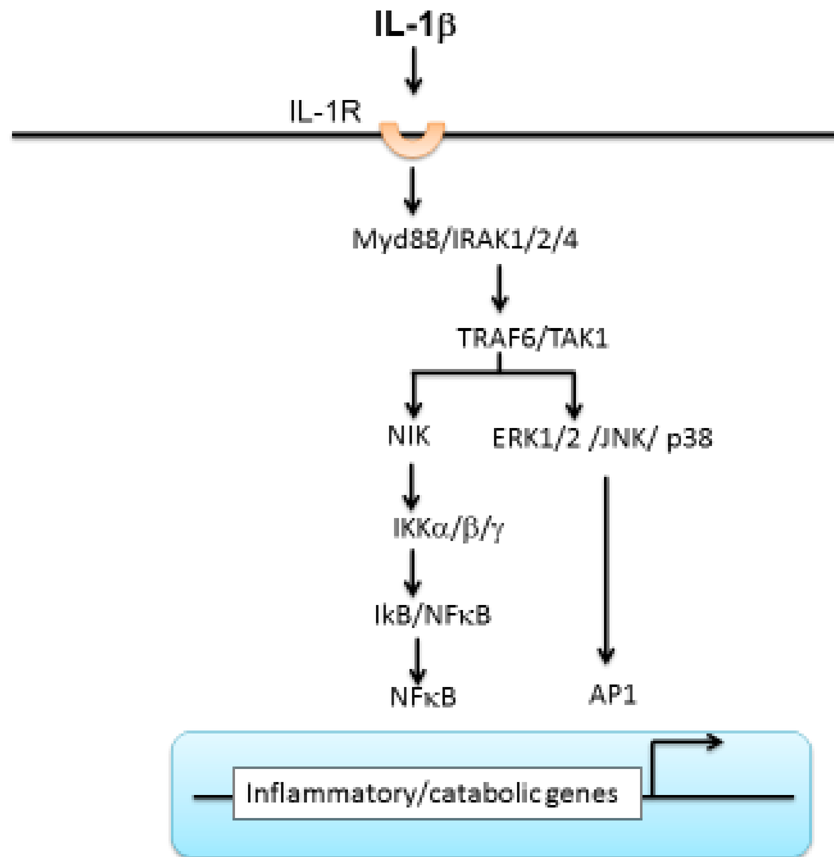


**Figure 7**: Signaling pathway of interleukin-1β in osteoarthritis. Binding of IL-1β to IL-1R activates either NF-κB (IKK complex) or AP1 (ERK/JNK/p38 pathways) transcription factors. IκB-NF-κB complex is inactive in the cytosol. After activation of IκB-NF-κB complex, free NF-κB transfers into the nucleus and induces the expression of inflammatory, catabolic, and anti-anabolic genes. Abbreviations: IL-1β, interleukin-1β; IL-1R, interleukin-1 receptor; IL-6, interleukin-6; IL-8, interleukin-8; COX-2, cyclooxygenae-2; NF-kB, nuclear factor-kappa B; IκB, inhibitor of κ B; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; AP1, activator protein 1.

As reported before, IL-1β has been shown to play a prominent role in cartilage degeneration. IL-1β has potent bioactivities in repressing the expression of essential ECM components like Col-II and aggrecan, and inducing a spectrum of proteolytic enzymes like MMPs. It up-regulates the synthesis of MMPs such as MMP-1; -3; and -13 which have a catabolic effect on cartilage components as well as A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), enzymes responsible for the proteolysis of aggrecans (40, 75, 76).

IL-1β induces its own secretion in cells of the joint in an autocrine way to stimulate the production of other cytokines such as TNF-α, IL-6, and IL-8 (60, 77). It promotes the secretion of numerous enzymes and mediators implicated in the pathophysiology of OA like inducible nitric oxide synthase (iNOS) producing NO, phospholipase $A_2$ ($PLA_2$), COX-2, and Prostaglandin E synthase generating $PGE_2$ (60, 78, 79) (**Table I**).

| Pathophysiological role of IL-1β in OA |
|---|
| - Inflammatory mediators: Upregulation of $PGE_2$ (COX-2 and mPGES-1) and NO (iNOS) |
| - Anabolic pathways: Downregulation of proteoglycan and collagen biosynthesis |
| - Cytokines: Upregulation of IL-1β, TNFα, IL-6, and IL-8 |
| - Proteases: Upregulation of MMP-1, -3, -13 and ADMATS4 |
| - Transcription factors: Nuclear translocation NF-κB and AP1 |

**Table I**: The various pathophysiological effects of interleukin-1β in Osteoarthritis.

**b. Tumor necrosis factor-α:**

- Tumor necrosis factor-α expression and regulation:

Tumor necrosis factor-α (TNF-α) belongs to the TNF superfamily. This cytokine is synthesized as a precursor protein in an inactive state and the proteolytic cleavage is done via a TNF-α converting enzyme named TACE. TNF-α has two receptors: TNFR55 or TNF57 (80).

The mechanism of regulation of TNF-α involves several signal transduction pathways including NF-κB activation and MAPK pathway. Expression level of iNOS, COX-2 and mPGES-1 as well as their produtcs NO and $PGE_2$ has been shown to be increased in chondrocytes treated with TNF-α (81).

- Role of tumor necrosis factor-α in Osteoarthritis:

TNF-α is implicated in maintaining the homeostasis of articular cartilage in combination with other cytokines and metabolic mediators like IL-1β, insulin growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β). In the course of OA, TNF-α has potent catabolic effects. Increased levels of TNF-α were observed in cartilage, synovial membrane, synovial fluid and subchondral bone (60, 74). The level of TACE is also increased in OA (80). Like IL-1β, TNF-α is a potent inducer of matrix degradation and synovial membrane inflammation. It initiates a cascade of inflammatory response through the production of IL-1β, IL-6 and IL-8 (82, 83). TNFR55 is the central receptor of TNF-α in articular cartilage during OA and its expression is increased in OA chondrocytes and synovial fibroblasts (83).

Inhibition of IL-1β and TNF-α block the amplification of the cleavage of Col-II and GAG in human OA cartilage (84). Anti-TNF-α treatments, with TNF-α antibodies, demonstrated a prolonged reduction of pain symptoms in OA (15).

**c. Interleukin-17:**

- Interleukin-17 expression and regulation:

Interleukin-17 (IL-17) is a pro-inflammatory cytokine secreted by T-cells. It belongs to the interleukin-17 family. The IL-17 family includes six ligands (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F), and five receptors (IL-17RA, IL-17RB/ IL-25R, IL-17RC, IL-17RD/SEF and IL-17RE) (85). The evolving IL-17 family of ligands and receptors may play an important role in the homeostasis of tissues in health and disease beyond the immune system.

IL-17 was thought to be secreted only by T cells. However, it is now known to be produced by a variety of cells like macrophages, dendritic cells, natural killer T and lymphoid tissue inducer cells (85).

- Role of interleukin-17 in Osteoarthritis:

IL-17 is another pro-inflammatory cytokine that has been shown to be involved in cartilage destruction. It has been demonstrated that IL-17 in combination with IL-1β enhances collagenase-3 in human OA chondrocytes through activator protein (AP)-1 (86). Furthermore, IL-17 can also increase the expression of NO in human OA chondrocytes (148 /faizeh). Studies on explants of human OA knee menisci has revealed that NO and $PGE_2$ production is increased by IL-17 (87).

**4.1.2. The anti-inflammatory cytokines:**

The anti-inflammatory cytokines are spontaneously elaborated by cartilage and synovial membrane. They are found in increased levels in OA patients. The anti-inflammatory and chondroprotective effects of these cytokines on cells of the articular cartilage and the synovium in OA have been well reported (60, 88, 89). The purpose of their production is to decrease the level of the pro-inflammatory cytokines, mainly IL-1β and TNF-α; thereby, downregulating MMP production.

IL-4, IL-10, and IL-13 are the main anti-inflammatory cytokines implicated in OA. Increased levels of IL-4 have been observed in the synovial fluid and synovial cells (60, 90). IL-4 has a strong chondoprotective effect. Numerous studies have reported that IL-4 inhibits the degradation of proteoglycans in the articular cartilage, inhibits the secretion of MMPs, and reduces the variation in the production of proteoglycans in the course of OA (91). Chondrocytes treated with IL-4 showed a decreased synthesis of inflammatory cytokines like IL-1$\beta$, TNF-$\alpha$, and IL-6 (60, 92, 93, 94). In addition, IL-4 has been found to decrease the secretion of other inflammatory mediators like $PGE_2$, COX-2, $PLA_2$, and iNOS (92, 93, 95, 96).

IL-10 is another major anti-inflammatory cytokine that shows chondoprotective effects in OA. Chondrocytes express both IL-10 cytokine and its receptor IL-10R (97). It has been shown that IL-10 is implicated in stimulating the synthesis of Col-II and aggrecan. IL-10 can also inhibit the production of MMPs (98). Both IL-10 and IL-4 inhibit chondrocyte apoptosis (60, 99). Like IL-4, IL-10 decreases the secretion of $PGE_2$, COX-2, and $PLA_2$.

Similar to IL-4 and IL-10, IL-13 has also chondroprotective effects. IL-13 showed inhibitory effects on the synthesis of pro-inflammatory IL-1$\beta$, TNF$\alpha$, and MMP-3. While IL-10 has been shown to modulate TNF-$\alpha$ production, IL-13 has been shown to inhibit the production of a wide range of pro-inflammatory cytokines and increase IL-1Ra production, a competitive inhibitor of IL-1R (89).

Thus, the effects of anti-inflammatory cytokines include increased proteoglycan synthesis, inhibited apoptosis of chondrocytes, decreased synthesis and secretion of MMPs, and decreased level of $PGE_2$.

In addition to cytokines, prostaglandins are also major inflammatory mediators. The prostaglandin $E_2$ ($PGE_2$), known to be abundant in a number of physiological fluids, can exert an inflammatory effect, can modulate the effect of other inflammatory mediators, and can contribute to joint damage by promoting MMPs production. Accumulating evidence implicates cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1)-derived $PGE_2$ in arthritis diseases. COX-2 and mPGES-1 levels are up-regulated in the superficial layers of human OA cartilage, areas where IL-1β was also found, suggesting its implication in increased mPGES-1 and COX-2 expression in OA cartilage (100).

In addition to these mediators, ROSs contribute to the inflammatory process during OA. NO, a major pro-inflammatory mediator, was also reported to play critical roles in the pathogenesis of OA. It contributes to the development of OA by inhibiting the synthesis of collagen and proteoglycans, enhancing inflammatory responses and mediating chondrocyte apoptosis (101).

Moreover, leukotrienes are also effector molecules resulting from the arachidonic acid produced by lipoxygenases' enzyme (LOXs). Those mediators play an important role in the development and persistence of the inflammatory process. In human tissue, there are three LOX isoforms: 5-LOX, 12-LOX, and 15-LOX (102).

Studies from our laboratory have shown, for the first time, the implication of 15-LOX enzymes in OA. We demonstrated that chondrocytes express isoforms, 15LOX-1 and -2. The metabolites of these enzymes, 13(S)-hydroxy octadecadienoic and 15(S)-hydroxyeicosatetraenoic acids, suppressed IL-1β-induced MMP-1 and MMP-13 expression through peroxisome proliferator activator receptor gamma (PPARγ) activation (103). We have recently shown that IL-1β down-regulates PPARγ expression in human OA chondrocytes indicating that induction and

recruitment of the transcription factor Early growth response gene-1 (Egr-1) contributed to the suppressive effect of IL-1β on PPARγ expression (104). Other effectors have been shown to be implicated in the inflammatory pathways like protease-activated receptors (PARs). These receptors belong to the family of seven transmembrane G-protein coupled receptors. PAR2 expression has been detected in human chondrocyts and synovial fibroblasts and was modulated by pro-inflammatory cytokines IL-1β and TNF-α (105).

Although OA is largely a biomechanical disease, its manifestations are mediated by the activity of pro-inflammatory factors; and thus a more active inflammatory response contributed to more severe OA. Various cytokines can be produced during the progression of OA depending on the duration and the severity of OA. This cytokine production disrupts the catabolism and anabolism balance. Therefore, current research is focused on the identification of factors responsible for the development of inflammatory mechanisms in OA.

## 5. Prostaglandins:

Arachidonic acid (AA) is released from cell membrane by enzymatic hydrolysis of phospholipids through $PLA_2$ activity. Among phospholipases, cytosolic $PLA_2$ is the main enzyme responsible for the production of AA. Once the AA is released, a metabolic cascade is initiated mediating generation of eicosanoids and particularly prostaglandins (106). Prostaglandins (PG) play a major role to maintain physiologic and pathophysiologic functions. They are implicated in a variety of biological systems like reproduction, cardiovascular system, renal system. On the other hand, they are involved in pathophysiological processes mainly the inflammatory response and pain (61, 107).

Prostaglandins are bioactive lipids derived from 20-carbon, polyunsaturated fatty acids. These are the products of a cascade of COXs and PG terminal synthases. To date, five prostanoids are known to be produced in mammals: prostaglandin $E_2$ ($PGE_2$), prostaglandin $I_2$ ($PGI_2$), prostaglandin $D_2$ ($PGD_2$), prostaglandin $F_2\alpha$ ($PGF_2\alpha$) and thromboxane $A_2$ ($TXA_2$) (**Figure 8**).

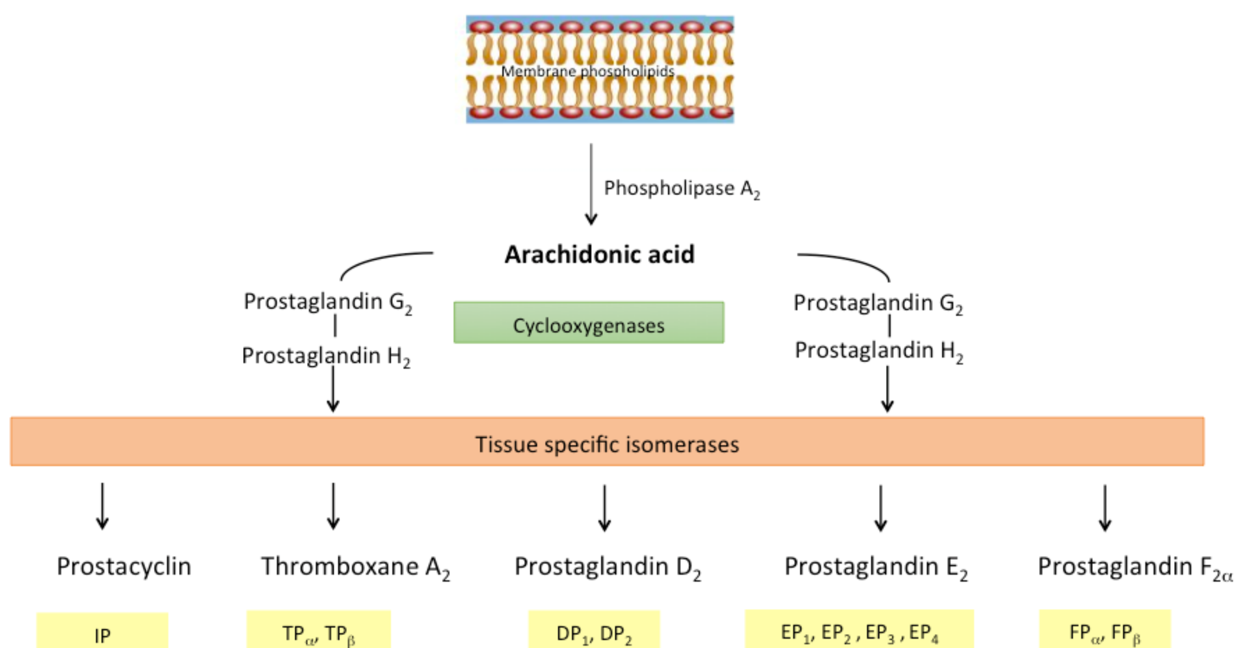


**Figure 8**: The prostaglandin biosynthetic cascade. Phospholipase $A_2$ ($PLA_2$) converts membrane-bound phospholipids into arachidonic acid (AA). AAs are converted into $PGG_2$ by COXs and then to $PGH_2$. Distinct terminal syntheses catalyze the conversion of $PGH_2$ to $TXA_2$, $PGF_2\alpha$, $PGD_2$, $PGE_2$, or prostacyclin ($PGI_2$). These prostaglandins activate distinct receptors: IP, TP, DP, EP, and FP receptors. Abbreviations: IP: $PGI_2$ receptors; TP, Thromboxanex$A_2$ receptors; DP, $PGD_2$ receptors; EP, E prostanoid receptors; FP: $PGF_2$ receptors.

## 5.1 Biosynthesis of Prostaglandin $E_2$:

Of all prostaglandins, $PGE_2$ is a pivotal PG produced by most mammalian tissues. Its biosynthesis is sequentially catalyzed by COX and PGE synthases (PGESs) (**Figure 9**). Three isoforms of COXs have been identified: COX-1, COX-2, and COX-3. Similarly, there are three forms of PGES that have been characterized. They are known as microsomal PGES-1 (mPGES)-1, microsomal PGES-2 (mPGES-2), and cytosolic PGES (cPGES). While COX-1, cPGES and mPGES-2 are constitutive isoforms, COX-2 and mPGES-1 are inducible synthases (81, 100).

$PGE_2$ regulates a variety of biological mechanisms under both normal and pathological conditions (61, 108). It plays a critical role in cellular physiological events like female reproduction, vascular hypertension, kidney function, gastric mucosal protection. On the other hand, it is implicated in pain hypersensitivity and inflammation. $PGE_2$ has a wide range of biological effects associated with inflammation. It elicits diverse effects on cell proliferation, angiogenesis and apoptosis (108). It is also supporting tumor growth by inducing angiogenesis (61, 109).

## 5.2. Cyclooxygenases: expression and regulation

Prostaglandin endoperoxide synthases, members of the fatty-acid oxygenases of the myeloperoxidase superfamily, are the cyclooxygenase (COX) isoenzymes (110). These key enzymes catalyze the rate-limiting step in the production of prostaglandins; they have been identified as bioactive compounds involved in different processes.

COX activity consists on converting AA into $PGG_2$. This later is then transformed into $PGH_2$. Finally, $PGH_2$ is converted into several prostanoids including $PGE_2$, $PGF_2$, $PGD_2$, $PGI_2$, and $TXA_2$. The production of a type of prostaglandin depends on synthases expression (61, 111).

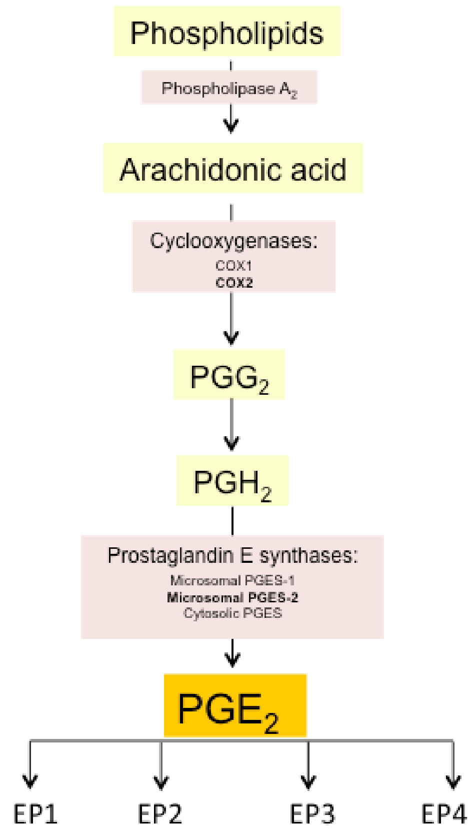


**Figure 9**: Pathway of $PGE_2$ biosynthesis.

In mammals, COX genes encode three types of COXs: two constitutive isoenzymes (COX-1 and COX-3) and an inducible isoenzyme (COX-2) (**Table II**). Both COX-1 and COX-2 are of significant pharmacological importance. They share approximately 60 to 65% amino acid of identity. Both are homodimers and largely located on the lumenal side of the endoplasmic reticulum membrane and the nuclear envelope. Because of the oxidative potential of the lumen, this localization is highly important; it allows a proper protein folding either for the structure or the function of COXs (112).

| Name | Molecular Weight (kDa) | Gene locus | Transcriptional regulation |
|---|---|---|---|
| COX-1 | 68 | 9q32-q33.3 (chromosome 9) | Constitutive |
| COX-2 | 72 | 1q25.2-q25.3 (chromosome 1) | Inducible |
| COX-3 | 30 | 9q32-q33.3 (chromosome 9) | Constitutive/ alternative splicing of COX-1 gene |

**Table II**: Characteristics of the cyclooxygenase synthases.

#### 5.2.1. Cycloxygenase-1:

COX-1 is localized at the 9q32-q33.3 chromosome. It is constitutively expressed in most of the tissues and regulates the production of PGs and thromboxanes. This isoform is activated upon physiological stimuli.

COX-1 regulates numerous physiological effects like tissue homeostasis. It is involved in the regulation of vascular, gastrointestinal and renal homeostasis. Mice lacking COX-1 gene have shown a protection against gastric pathologies. These mice are resistant to indomethacin-induced-ulcerations (113).

#### 5.2.2. Cycloxygenase-2:

COX-2 is an inducible isoform in the inflammatory response wherein promotes the synthesis of most prostaglandins (114). COX-2 gene regulation is controlled at various levels mainly gene transcription and post-transcriptional levels. COX-2 mRNA stability and translational efficiency is mediated by multiple regulatory elements within the 3'-untranslated region (UTR). Regulation of COX-2 involves alternative polyadenylation. Its transcriptional activation occurs quickly and transiently in response to a wide range of stimuli including cytokines, ROS stimuli like NO, and growth factors.

COX-2 gene contains binding sites for numerous regulatory transcription factors. The promoter of COX-2 contains two CREs (cAMP-response elements), C/EBP (CCAAT/enhance-binding protein), two AP-1 (activating protein) sites, two NF-κB sites and three Sp1 (specificity protein-1) sites as cis regulatory elements (115). Activated NF-κB and AP-1 transcription factors can bind to their binding sites in the promoter of COX-2 gene and initiate transcription. Depending on cell type and which activated regulatory pathway, transcription factors bind to these sites in a variety of combinations (**Figure 10**).

As mentioned before, expression of COX-2 is induced following stimulation with IL-1β, TNF-α, or lipopolysaccharide (LPS) involving the transcriptional factor NF-κB. However, it is inhibited by glycorticoids, IL-4 and IL-10 through NF-κB pathway inhibiting (116, 117).

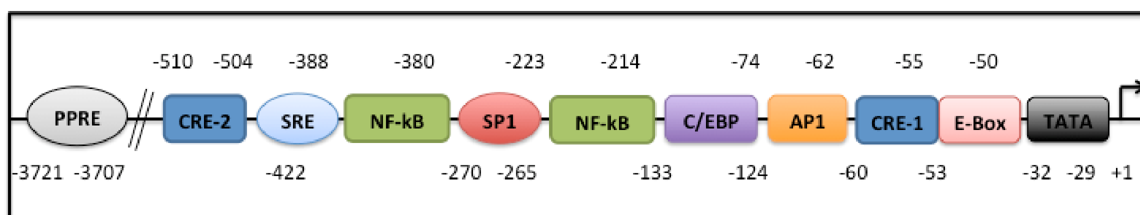


**Figure 10**: The structure of cyclooxygenase-2 promoter. Abbreviations: PPRE, PPAR-responsive elements; CRE, cAMP responsive elements; SRE. Sterol regulatory elements; NF-κB, Nuclear factor-kappa B; SP, Specificity protein; C/EBP, CAAT enhancer–binding protein; AP, Activating protein.

Analyses of 5’-UTR region have showed that COX-2 is an immediate early gene since it contains TATA box as well as other transcriptional elements that characterize early gene expression. COX-2 is regulated at the post-transcriptional level. Its 3’-UTR contains, in addition to regulatory elements, many sites of polyadenylation. This part contains as well a rich region of AU: ARE (adenylateand uridylate (AU)-rich elements) AU rich elements; these are multiple copies of AUUUA sequence (118).

COX-2 levels are elevated in pathological conditions. Several studies suggested that the degradation of COX-2 is programmed to control these amounts of COX-2 as a mechanism of regulation. Another alternative mechanism of regulation is chromatin remodeling and epigenetic events. These mechanisms may also be involved in transcriptional regulation of COX-2 (78, 119,

120).

#### 5.2.3. Cycloxygenase-3:

A third form of cyclooxygenases is COX-3. COX-3 has been recently identified as a novel COX isozyme. Studies have reported that this synthase is generated upon alternative splicing of COX-1 gene (121). This isoform is predominantly expressed in cerebral cortex and heart but its existence in humans is still unkown (122).

### 5.3. Prostaglandin E synthases: expression and regulation

After oxidation of AA into $PGH_2$ through COXs, the prostaglandin $PGH_2$ is converted, among others, into $PGE_2$ by prostaglandin E synthase (PGES). PGES enzymes are available in three isoforms: membrane-bound prostaglandin E synthase 1 (mPGES-1), membrane-bound prostaglandin E synthase 2 (mPGES-2), and cytosolic prostaglandin E synthase (cPGES) (**Table III**).

| Name | Molecular Weight (kDa) | Gene locus | Transcriptional regulation |
|---|---|---|---|
| mPGES-1 | 16 | 9q34.4 (chromosome 9) | Inducible |
| mPGES-2 | 33 | 9q33–q34 (chromosome 9) | Constitutive |
| cPGES | 23 | 12q13.13 (chromosome 12) | Constitutive |

**Table III**: Characteristics of the prostaglandin E synthases.

### 5.3.1. Microsomal Prostaglandin E synthase-1:

Microsomal prostaglandin E synthase-1 (mPGES-1), the first identified synthase (123), was original called microsomal glutathione S-transferase 1-like 1 (MGST1-L1). MPGES-1 is a member of the membrane-associated proteins in eicosanoid and glutathione (MAPEG) superfamily of transmembrane protein (100, 107, 124). It is localized at the perinuclear membrane as well as at the endoplasmic reticulum. MPGES-1 is a terminal enzyme that acts downstream of COXs and catalyzes the conversion of $PGH_2$ to $PGE_2$ (100, 125).

The gene encoding mPGES-1 in humans is located on chromosome 9q34.4 and has a size ~ 14.8 kb. It contains three exons and two introns (126) encoding for a protein of 152-153 amino acid residues with a ~ 16 kDa. MPGES-1 catalytic function is linked to a well-conserved residue, the Arg 110. Of interest, mPGES-1 requires glutathione as an essential cofactor for its activity (61, 127).

MPGES-1 has significant homology to the proteins of the superfamily MAPEG with a strong homology to MGST1 (38%) and a high homology (80%) with the mPGES-1 of other species (rat, mouse and rabbit) (128). Human, mouse, or rat mPGES-1 gene vary in critical amino acids allowing for conformational changes of the enzyme and its targeted binding compound (61, 129).

The human mPGES-1 promoter is composed of several potential transcription factor-binding sites. It contains two GC-boxes, two tandem barbie boxes and an aryl hydrocarbon response element. Unlike COX-2 promoter, mPGES-1 promoter lacks TATA box (130). The mouse mPGES-1 promoter contains, in addition to GC-boxes, C/EBP, AP-1, and GRE (glucocorticoid-responsive elements) (**Figure 11**).

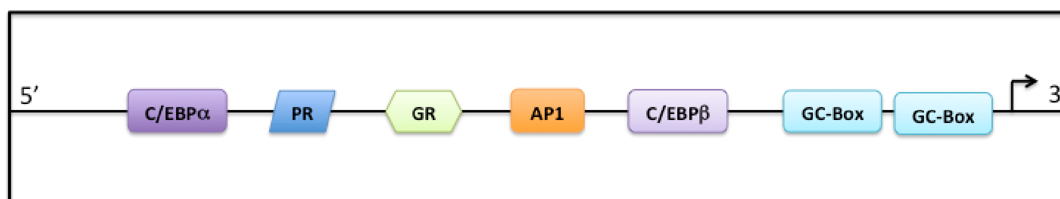


**Figure 11**: The structure of microsomal prostaglandin E synthase-1 promoter. Abbreviations: C/EBP, CAAT enhancer–binding protein; PR, Progesterone receptor; GR, Glucorcticoid receptor; AP, Activating protein.

Expression of mPGES-1 involves specific transcription factors that direct its regulatory expression. Binding of the transcription factor Egr-1 to the proximal GC box at mPGES-1 promoter is an essential mechanism for transcriptional regulation of mPGES-1 (131). In addition to Egr-1, PPARγ is a ligand specific transcription factor wish may contribute to mPGES-1 regulation. Studies from our laboratory showed that PPARγ ligands like 15d-$PGJ_2$ and troglitazone inhibit IL-1β-induced mPGES-1 expression in human synovial fibroblasts (132).

MPGES-1 expression is induced upon stimulation with pro-inflammatory cytokines, growth factors, and LPSs (107, 133) in a variety of cells including macrophages, fibroblasts, chondrocytes, synoviocytes and osteoblasts. Of importance, mPGES-1 is induced in coordination with COX-2 by various inflammatory stimuli and it is functionally coupled with COX-2 (125, 127). This functional coupling of mPGES-1 and COX-2 results in highly increased production of $PGE_2$ during the course of inflammation (107, 134). Immunohistochemical analyses confirmed the correlation between both enzymes since their subcellular localization overlap in the perinuclear membrane (135).

Furthermore, $PGE_2$ exerts various physiological functions through E prostanoid receptors (EP), which are: EP-1, -2, -3, and -4. EP-2 and EP-4 have been reported to regulate mPGES-1 expression. Selective agonists of EP-2 and EP-4 increase mPGES-1 expression through increasing cyclic adenomonophosphate (cAMP). During the inflammatory process, inflammatory mediators activate the transcription of target genes like mPGES-1 and COX-2 (107, 136, 137) and regulate $PGE_2$ formation by activating NF-κB. However, once $PGE_2$ stimulates its cognate receptor mostly EP-4, it can inhibit the formation of NF-κB by preventing the p65 translocation (107, 138, 139).

Several research studies demonstrated a major role of mPGES-1-derived $PGE_2$. COX-2 inhibition has been shown to be associated cardiovascular side effects attributable to $PGI_2$ inhibition (59, 140). Therefore, targeted inhibition of $PGE_2$ pathway, specifically through inhibition of mPGES-1, has received much attention to alleviate pain and inflammation suggesting that mPGES-1 can be a pharmacological target against inflammatory pathologies. Several compounds like MF-63, NS-398, and MK-866 were tested in invitro studies as mPGES-1 inhibitors. Arzanol was identified and detected as a major anti-inflammatory compound. It acts by inhibiting NF-κB activation, release of pro-inflammatory cytokines like IL-1β, TNF-α and pro-inflammatory mediators like $PGE_2$ and mPGES-1 (141, 142, 143). Thus, understanding the mechanism of regulation of mPGES-1 is a great area of investigation.

#### 5.3.2. Microsomal prostaglandin E synthase-2:

Microsomal PGES-2 is the second isoform and the most recently identified PGES; it was purified from bovine heart tissue (125) then subsequently cloned. This isoform has a unique N-terminal hydrophobic domain and glutaredoxin-like domain (125, 144). Synthesized as a golgi membrane-associated protein, the mature form is then generated upon the removal of the N-terminal hydrophobic domain (125, 145). MPGES-2 is ubiquitously expressed in diverse tissues

mediating both early and late $PGE_2$ production. This isoform is functionally linked to both COX-1 and COX-2. MPGES-2 is implicated in physiological and pathological conditions (145).

#### 5.3.3. Cytosolic prostaglandin E synthase:

Cytosolic PGES (cPGES) is a glutathione-dependent enzyme that was identified as heat shock protein 90 (Hsp90)-associated protein p23 (125, 146). It is constitutively expressed in the cytosol under basal conditions in different tissues. Cytosolic PGES is functionnally coupled to COX-1. This isoform promotes an immediate production of $PGE_2$ (100, 146, 147). Cytosolic PGES-genertaed-$PGE_2$ is implicated in numerous physiological processes like tissue homeostasis, gastrointestinal protection, reproduction, osteogenesis as well as other neuronal functions (148, 149).

### 5.4. Cycloxygenase-2/microsomal PGES-1/$PGE_2$ pathway in Osteoarthritis:

Evidence has showed the implication of COX-2 in arthritic diseases and OA in particular. The expression of COX-2 is increased in OA cartilage (150) and synovial membrane (151).

OA cartilage produces high amounts of $PGE_2$. However, it is inhibited when treated with protein synthesis inhibitors. It has been reported that human OA chondrocytes induce the expression of COX-2 following IL-1$\beta$, IL-17, TNF-$\alpha$, and LPS stimulation (152) and thereby produce elevated levels of $PGE_2$. However, the expression/production of COX-2/$PGE_2$ is inhibited when treated with anti-inflammatory cytokines like IL-4, IL-10, and IL-13 (95).

Furthermore, the super-induction of $PGE_2$ correlates with the up-reguation of COX-2 in OA cartilage. COX-2 inhibitors like dexamethasone and indomethacin inhibited the spontaneously released $PGE_2$ (150). Studies on animal model of arthritis have showed that inhibitors of COX

synthases can repress joint inflammation. This was concomitant with the attenuation of $PGE_2$ synthesis.

MPGES-1 plays a crucial role in various pathophysiological mechanisms related to pain (153), fever (154), bone resorption (155), angiogenesis (156) and inflammation (125). Interestingly, mPGES-1 has a main role in the development of chronic inflammation in patients with OA (157).

$PGE_2$ is the most produced prostaglandin in OA tissues. It is a key mediator of the inflammatory process during the course of OA. $PGE_2$ is induced by IL-1β and TNF-α in both OA chondrocytes and synovial fibroblasts (125). This overexpression contributes to the development of OA.

$PGE_2$ is one of the major catabolic mediator implicated in cartilage destruction (79). High levels of $PGE_2$ are released in OA cartilage in comparison to normal cartilage (158). $PGE_2$ contributes to OA by increasing the production of catabolic molecules like pro-inflammatory cytokines, MMPs, and ROSs that participate to cartilage tissue alterations. $PGE_2$ exerts these effects via various EP receptors (EP1-4) (15). It utilizes EP-2 and -4 to induce its downstream catabolic effects.

As mentioned before, IL-1β has been shown to induce the production of high levels of $PGE_2$. Several research studies have reported that IL-1β enhances the expression of both COX-2 and mPGES-1 at the mRNA and protein levels (159). During the inflammatory response, inflammatory mediators induce the transcription of target genes like COX-2 and mPGES-1 and $PGE_2$ synthesis by promoting NF-κB pathway (136, 137). However, it has been reported that $PGE_2$ could be implicated in the resolution of inflammation (160). $PGE_2$, by stimulating it cognate

receptors, mostly EP4, can inhibit the formation of NF-kB (p50 and p65) through the prevention of p65 translocation (138, 139) (**Table IV**).

| Effects of $PGE_2$ in OA cartilage |
| --- |
| - Induction of pain |
| - Increase of cartilage destruction and pro-inflammatory mediators:<br>- Increase of IL-1β expression<br>- Increase of COX-2 expression |
| - Increase of MMPs production and activation |
| - Increase of pro-apoptotic mechanisms (ROS) |
| -Increase of angiogenesis |
| -Inhibition of collagen and aggrecan synthesis |

**Table IV**: The various physiological and pathological effects of $PGE_2$ in osteoarthritic cartilage.

## 6. Reactive oxygen species:

Reactive oxygen species (ROS) play a major role in the regulation in both normal and pathological conditions, particularly, in articular joint tissues. Among the inflammatory mediators, both oxygen and nitrogen-derived free radicals play a critical role in the pathogenesis of OA. ROSs such as superoxide anion, hydrogen peroxide, and hydroxyl radicals directly promote chondrocyte apoptosis, most probably via mitochondrial dysfunction (161).

In normal states, chondrocytes are living in anaeorobic conditions. However, in pathological conditions, oxygen tension is subject to variations. As a response for such fluctuations,

chondrocytes produce altered (abnormal) levels of ROS. They are implicated in cartilage destruction and inflammation. ROS may reduce matrix components synthesis, induce apoptosis and activate latent MMPs (161).

Effects of ROS on intracellular signaling have been also studied. ROS, like Hydrogen peroxyde ($H_2O_2$) and NO have been shown to be implicated in the regulation of numerous intracellular signaling pathways, through the binding of cytokines and growth factors to different types of cell membrane receptors (161). Among these pathways, MAPK pathways are implicated in several systems, including extracellular signal-regulated kinase (ERK)1/2, Jun-NH2-terminal kinase and p38 MAPK Cascades. In addition, ROS may also regulate the activity of transcription factors through oxidative modifications like NF-κB, AP-1, Sp-1, Egr-1 and Hypoxia-inducible factor-1α (HIF-1α) (161). ROSs activate NF-κB and MAPK cascades-activated transcription factors AP-1 and CREB-binding protein (CBP/p300). These transcriptional factors lead to high expression of $cPLA_2$, COX-2 and mPGES-1, and thus to production of prostaglandins, particularly $PGE_2$ (162, 163, 164).

NO and superoxide anion generated-$H_2O_2$ are the major ROS. They are mainly produced by chondrocytes (161).

## 6.1. Nitric oxide:

### 6.1.1. <u>Biosynthesis of nitric oxide:</u>

Nitric oxide (NO) is an important intracellular and intercellular signaling molecule implicated in a diversity of physiological and pathophysiological mechanisms. This biological mediator has crucial physiological roles in cardiovascular, nervous, homeostatic and immunological systems. On the other hand, NO is a free radical that exhibits a wide range of

pathophysiological actions particularly in the pro-inflammatory response (165). NO production is induced by IL-1β, TNF-α, interferon (IFN)-γ and LPS, and inhibited by TGF-β, IL-4, IL-10 and IL- 13 (161, 164, 166).

NO is produced endogenously from the amino acid L-arginine in a number of tissues by a family of NO synthases (NOSs). There are three types of NOS enzymes that has been isolated and cloned: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The inducible synthase can form much large amounts of NO (**Figure 12**).

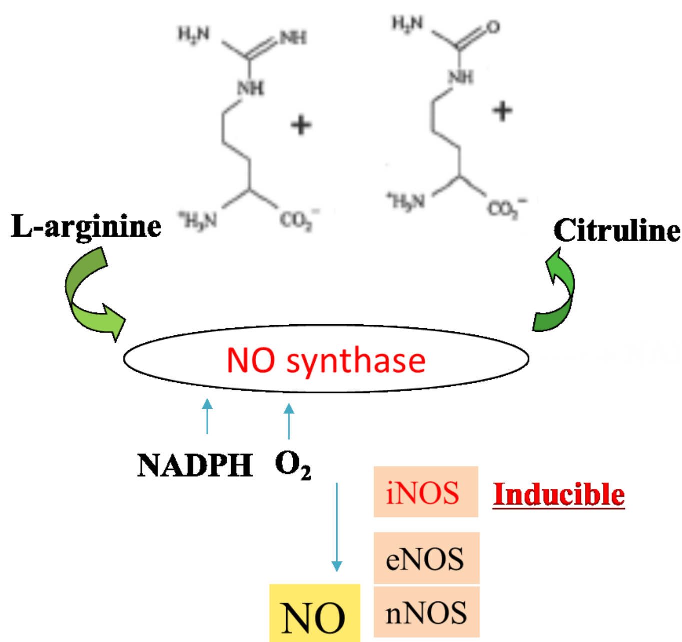


**Figure 12**: The biosynthesis of nitric oxide (NO). NO is synthesized endogenously by the conversion of L-Arginine to Citrulline by NO synthases (iNOS, eNOS, and nNOS). Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; $O_2$, oxygen; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

These subtypes are encoded by different genes, which share between 50-60% sequence homology (165). Neuronal NOS (nNOS) and endothelial NOS (eNOS) mediate the constitutive synthesis of NO from L-arginine and show little association with the development of inflammatory process. However, iNOS, a distinct calciumindependent isoform of NOS (130kDa protein), plays an essential role in the inflammatory response and injury repair (167) (**Table IV**).

| Name | Molecular Weight (kDa) | Gene locus | Transcriptional regulation |
|---|---|---|---|
| iNOS | 131 | 17cen–q11.2 (chromosome 17) | Inducible |
| eNOS | 133 | 7q35–7q36 (chromosome 7) | Constitutive |
| nNOS | 161 | 12q24.2–12Q24.3 (chromosome 12) | Constitutive |

**Table V**: Characteristics of the nitric oxide synthases.

### 6.1.2. <u>Nitric oxide synthases: expression and regulation</u>

a. <u>Inducible nitric oxide synthase:</u>

Inducible NOS was originally purified and cloned from a macrophage cell line (168, 169). It was first isolated from primary hepatocytes (169, 170) and then cloned and characterized in chondrocytes (171). In humans, iNOS gene is located on chromosome 17q11.2-q12 and extends over ~ 37 kb. It encodes two transcripts of which one is composed of 27 exons however the second one is still unknown.

In mouse, iNOS promoter gene is composed of several binding sites for transcription factors like NF-κB, Jun/Fos heterodimers, CREB and STAT (172, 173). Human iNOS promoter has sequence homologous to mouse promoter (174) (**Figure 13**).

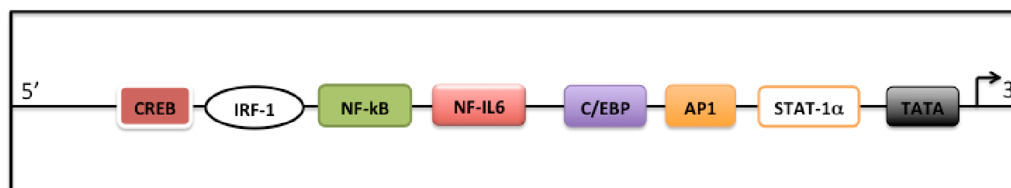


**Figure 13**: The structure of the inducible nitric oxide synthase promoter. Abbreviations: CREB, c-AMP response element binding; IRF-1, Interferon regulatory factor-1; NF-κB, Nuclear factor-kappa B; NF-IL6, Nuclear factor for IL-6 transcription; C/EBP, CAAT enhancer–binding protein; AP, Activating protein; STAT-1α: Signal transducer and activator of transcription-1α.

Inducible NOS is regulated at the transcriptional, posttranscriptional, translational, and posttranslational level (175). In resting cells, iNOS is expressed at low levels. However, inflammatory stimuli like LPS, IL-1β, TNF-α, and IFN-γ induce iNOS expression. Regulation of iNOS transcription depends on activation or inhibition of the NF-κB and or the JAK/STAT pathway (169, 172). In macrophage, iNOS is regulated by cellular receptor molecules like Toll-like receptors (TLRs) and CD14, which is a LPS receptor. This later plays a critical role in the pro-inflammatory response through activation of the NF-κB pathway (176). In addition, INF-γ induces iNOS through the Jak-STAT signaling pathway resulting in NO production (174, 177).

Inducible NOS expression is also regulated by PPARs, which may antagonize the activities of transcriptional factors like AP-1, STAT and NF-κB. PPAR activity is modulated by TNF-α and IFN-γ, leading to a modified activity on p65/RelA subunit of the NF-κB signaling pathway (178). PPAR-α agonists have been particularly shown to inhibit IL-1-induced NO production by promoting iNOS protein degradation in chondrocytes (179, 180).

Expression of iNOS may be related to the post-transcriptional regulation. The 3´ UTR of iNOS mRNA has AU-rich sequences, known to destabilize iNOS mRNA (181). It has been reported that TGF-β inhibits iNOS induction causing iNOS mRNA destabilization (182).

Furthermore, iNOS expression is controlled at the translational and posttranslational levels. Recently, dexamethasone and sodium salicylate modulate iNOS mRNA translation (174). Both TGF-β and dexamethasone (180) have showed an inhibitory effect on NO production through enhanced iNOS protein degradation. Inducible NOS can promote phosphorylation of the transcription factore IF-2α which is related to protein synthesis at the translational level (183). The reduction of protein synthesis results in reduction of iNOS activity. Posttranslational regulation of NO synthesis controls iNOS protein availability via effects on protein stability, dimerization, phosphorylation, and cofactors binding. Additionally, enhancement or blockade of degradation of iNOS protein is another posttranslational regulatory mechanism.

b. Endothelial nitric oxide synthase:

Endothelial NOS (eNOS) is a constitutive isoform of NOSs. The eNOS, named also NOS III, is predominantly expressed in vascular endothelial cells (184) and is located in regions of the plasma membrane called caveolae, structures which are associated with the accumulation of receptors for agents that regulate endothelial cell activity (185).

The synthesis of physiologically vital amounts of NO from this constitutive isoforms is Ca2+ /calmodulin-dependent (186). Endothelial NOS is implicated in various physiological processes like the regulation of blood flow and pressure and the inhibition of platelet activation (167).

c. Neuronal nitric oxide synthase:

Neuronal NOS (nNOS) is a constitutive calcium-dependent enzyme. The nNOS (or NOS I) is predominantly expressed in neuronal tissue. It is expressed constitutively in resting cells and produce small amounts of NO in response to a receptor-mediated increase in the intracellular free calcium concentration (169).

Neuronal NOS has several physiological functions of which its role as neurotransmitter in gastrointestinal tract and blood flow is considered as one of the most important functions. This isoform is also implicated in synaptic plasticity (167).

### 6.1.3. Inducible NOS/NO pathway in Osteoarthritis:

An aberrant expression of iNOS has been shown to be majorly implicated in arthritic diseases and chronic inflammation leading, in turn, to an excessive production of NO. In inflammatory conditions, NO generated by iNOS has pro-inflammatory and destructive effects (169).

NO has an important role in the inflammatory response during OA cartilage degradation (187). Elevated levels of NO have been detected in OA joints (169). In OA chondrocytes, pro-inflammatory cytokines like IL-1β and TNF-α induces the production of iNOS-generated-NO. Once produced, NO exerts a number of catabolic effects that promotes the degeneration of articular cartilage, reduces collagen and proteoglycan synthesis, promotes the inflammatory reaction (107), and creates ECM damage in OA by enhancing MMPs activity. Importantly, NO

shifts the cytokine balance towards the pro-inflammatory and destructive direction by down-regulating the synthesis of anabolic mediators like TGF-β and IL-1RA (169, 188). Moreover, NO up-regulates indirectly the production of pro-inflammatory cytokines in inflamed joints by increasing TNF-α by the synovial cells.

NO can mediate different other processes like cell apoptosis through mitochondrial mechanism (189). It can be considered as a pro-apoptotic factor when present at high concentrations (190). Apoptosis is a complex mechanism that results from the imbalance between apoptotic and non-apoptotic factors and implicates complex processes (161, 191). NO has long been considered as the first inducer of chondrocyte apoptosis mediated by caspase-3 and tyrosine kinase activation (161) (**Table VI**).

| Effects of NO in OA cartilage |
| --- |
| - Increase of MMPs production and activation (MMP-3 and 13) |
| - Increase of COX-2 expression |
| - Increase of NF-κB activation |
| - Increase of chondorcyte death |
| - Increase of cytokine imbalance:<br>- Increase of IL-1β production<br>- Increase of TNFα production<br>- Decrease of TGFβ and IL-1Ra production<br>- Decrease of IL-10 production |
| - Increase of other oxidants |
| - Decrease of type II collagen and aggrecan synthesis |

**Table VI**: The various physiological and pathological effects of NO in osteoarthritic cartilage.

However, contrasting evidences claim that NO and its redox derivatives may play protective roles in the joint. In response to the same IL-1β stimulation, not all chondrocytes respond the same way. This was shown to be dependent on the different zones in which the chondrocytes may exist (187).

Furthermore, PPARγ agonists inhibit IL-1-induced-NO production by interfering with the activation of NF-κB and AP-1 (169, 180, 192).

Elevated levels of NO have been found in synovial fluid, of patients with OA. Animal studies have further emphasized on the implication of ROS in cartilage degradation. Our colleagues, Pelletier and collaborators have showed that N-iminoethyl-L-lysine (L-NIL), a specific inhibitor of inducible ·NOS, inhibits cartilage degradation in a dog model of OA (161, 193).

In rabbit articular chondrocyte, NO is involved in the IL-1 inhibition of aggrecan synthesis. NOS inhibitor N-monomethyl-L-arginine (L-NMMA) reduces the response to IL-1 and restores proteoglycan synthesis in cartilage (194). IL-1β can also inhibit the production of Col-II, an effect which is partially supressed by L-NMMA (195).

Selective iNOS inhibitor 1400W reduces IL-1-induced MMP-10 production and promotes anti-inflammatory IL-10 production in OA cartilage (196). In addition, The NO synthase inhibitor, HN monomethyl-L-arginine monoacetate inhibited the production of NO in OA cartilage. Studies on animal model of arthritis have showed that inhibitors of NO synthases can repress joint inflammation. This was concomitant with the attenuation of NO synthesis. (194). In experimental model of OA, the selective iNOS inhibitor L-NIL significantly reduced chondrocytes apoptosis.

In response to IL-1β and LPS, chondrocytes produce ·NO. The inhibition of ·NO production by L-NMMA induces an increase of IL-6 and IL-8 (197).

### 6.2. Hydrogen peroxyde:

Hydrogen peroxide ($H_2O_2$) is produced by the enzyme complex NADPH, which catalyzes the reduction of molecular oxygen to superoxide anion radicals.

Oxidative stress may results also in degradation of proteoglycans and collagens. All of these degradation products contributes to the process of inflammation. It may also cause cell death. $H_2O_2$ is implicated in matrix synthesis. $H_2O_2$ inhibits also proteoglycan and DNA synthesis in chondrocytes (161, 198, 199).

Together, ROS are involved as signaling intermediates for cytokines in articular chondrocytes like IL-1β and TNF-α (161, 200). ROS has showed their contribution to the breakdown of ECM in joint diseases like OA and RA.

## 7. Extracellular matrix proteinases:

### 7.1. Matrix metalloproteinases:

Matrix metalloproteinase (MMP) family members are the major enzymes that degrade the components of the ECM. MMPs belong to the metzincin superfamily. They contribute to pathological processes, mainly inflammation.

In the human genome, there are more than 25 type of MMP genes (201). They all share a common domain structure with a signal peptide, a pro-peptide, a catalytic domain, a hinge region, and a hemopexin C-terminal domain (202). All are active at neutral pH, require $Ca^{2+}$ for activity. Most MMPs are secreted into the extracellular space in a latent pro-form, and require proteolytic cleavage for enzymatic activity. However, few MMPs are activated intracellularly by a furin-like mechanism. When these enzymes reach the extracellular space, they show full activity (203). MMPs are classified into four groups based on their primary structure, substrate specificity and

cellular location: collagenases, stromelysins, gelatinases, and membrane-type MMP (MT-MMP) (204).

Most cells in the body express MMPs, even though some enzymes are often associated with a particular cell type and function. The principle substrate of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) is the type IV collagen in basement membrane. These enzymes are usually expressed by endothelial cells, although they might be expressed by other cells such as stromal fibroblasts, macrophages, and tumor cells. MMP-1, an interstitial collagenase, and MMP-3 are among the most ubiquitously expressed MMPs. In contrast, MMP-13 (called also collagenase-3) has a more restricted pattern of expression within connective tissue (205).

In normal cells, expression of MMPs is low and these low levels allow for healthy connective tissue remodeling. However, in pathologic conditions, the level of MMP expression increases considerably, resulting in aberrant connective tissue destruction. In OA pathophysiology, connective tissue destruction is primarily mediated by different classes of MMPs (201, 206). The secreted collagenases (MMP-1 and MMP-13) have the major role in destructing the interstitial collagens. These MMPs are induced in response to the cytokines and growth factors usually found in arthritic joints (207). MMP-1 is largely responsible for the destruction of the articular joint tissue, mainly cartilage type I, II and type III. The level of MMP-1 is elevated in human OA cartilage. Its production is up-regulated by growth factors and pro-inflammatory cytokines such as IL-1β and TNF-α.

Collagenases including MMP-1 or collagenase-1 and MMP-13 or collagenase-3, degrade native collagens of types I, II, III, V, and XI (208). MMP-13 degrades type IV, IX, X, and XIV collagens, fibronectin laminin, aggrecan core protein, fibrillin-1, and serine proteinase inhibitors, which are abundantly expressed in OA (208).

Research has proved that the expression of the MMP-1 gene can be regulated. In fact, ligands of PPARγ receptors as 15d PGJ2 inhibited IL-1β-induced MMP-1 in a dose dependant manner in synovial fibroblasts (209). MMP-13 has also a particular role in cartilage degradation not only because it is expressed by chondrocytes but also because it hydrolyzes specifically Col-II more efficiently than the other collagenases (210). Animal mouse models that overexpress MMP-13 in cartilage have shown to display OA-like characteristics. The in vivo role of MMP-13 has been further demonstrated in OA. MMP-13 KO mice, in which OA was surgically induced, demonstrated significant inhibition of cartilage structural damage, showing that damage is dependent, at least in part, on MMP-13 activity (211). Furthermore, it has been reported that TGF-β, as anabolic cytokine, induces MMP-13 production in OA human cartilage (212).

MMP-3 and MMP-9 are matrix proteases that have been widely reported. Elevated levels of MMP-3 are produced in OA cartilage. MMP-3 cleaves the core protein of aggrecan and link protein, telopeptides of Col-II (208). MMP-3, in particular, has pro-apoptotic activity on epithelial cells (208, 213). MMP-9 is also an inducible MMP, but its role in connective tissue destruction in arthritis, appears to be secondary since it contributes to the degradation of collagen only after the chains of the triple helix have been cleaved by the interstitial collagenases. In contrast, MMP-2 and MMP-14 are constitutively expressed.

## 7.2. ADAMTS:

Aggrecan is also one of the most critical constituents of the articular cartilage ECM and its degradation is critical for OA devolepment and progression. A disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) family that belong to zinc MMPs, exhibit an aggrecanase activity. ADAMTS are constituted of signal sequence, prodomain, catalytic domain, spacer

domain, trombospondin motifs and submotifs (214).

ADAMTS contain two disintegrin loops and three C-terminal TS motifs. The most important members of this family are ADAMTS-4 and ADAMTS-5. These ADAMTS types are responsible for the degradation of the aggrecan structure in the ECM. Both of them are characterized by the presence of only thrombospondin motifs (TSP). ADAMTS-4 and -5 have become of considerable interest as potential therapeutic targets in OA. Both are expressed in normal human cartilage.

Mice studies suggest that ADAMTS-5 is particularly implicated in OA. ADAMTS-5 KO mice subjected to surgically-induced OA showed a protection against cartilage erosion (215, 216). However, when deficient mice of ADAMTS-4 were subjected to surgically-induced OA, they showed no effect on the progression or severity of OA (217). Unlike mice, both ADAMTS-4 and -5, were shown to be implicated in aggrecan degradation in human chondrocytes and cultured human cartilage explants (218). Studies using chondrocytes and cartilage explants reveal that ADAMTS-4 can be induced by IL-1β, TNF-α or TGF-β (219).

## 8. Therapeutics of Osteoarthritis:

OA, a major cause of disability, is characterized by joint pain and impairment. The aims of treatment of OA patients are to reduce pain, improve mobility, ameliorate functional capacity, and optimize the quality of life (16).

Current treatment of OA includes non-pharmacological and pharmacological interventions. However, cases with severe OA, who are unresponsive to such moderate therapy, are exposed to joint replacement surgery, which is a cost effective intervention (**Figure 14**).

## 8.1. Non-pharmacological interventions:

The principle non-pharmacological interventions include weight loss, exercise and various physical therapies like massage and acupuncture. These interventions are characterized by low or no side effects.

Overweight people are at higher risk of symptomatic and progressive OA. This is associated with increased load on weight bearing joints. Weight loss is considered as a primary preventive strategy for knee OA (220). It can significantly reduce pain and delay progression of joint damage and disability (24).

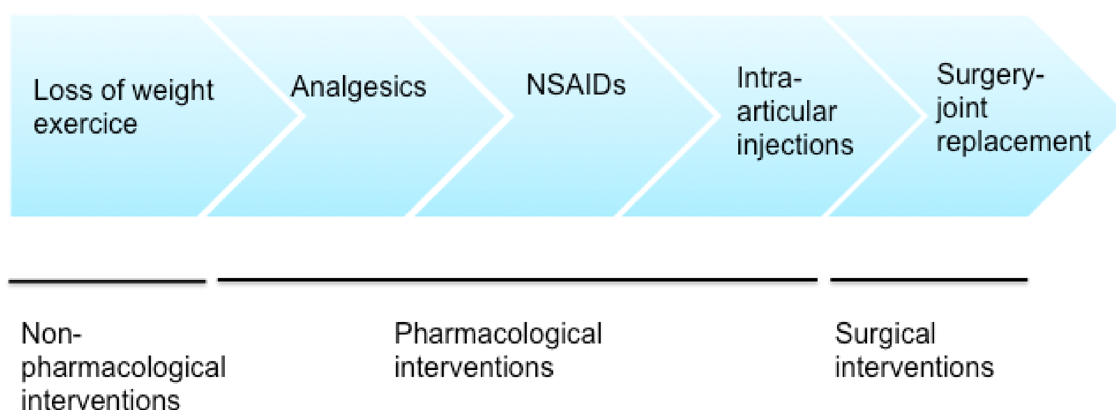


**Figure 14**: Osteoarthritis treatment options. According to the guidelines from the American College of Rheumatology, non-pharmaceutical measures such as weight loss and physical exercise are the first line of treatment. As a second line of treatment, pharmacological measures are prescribed including analgesics, non-steroidal anti-inflammatory drugs (NSAIDs) or intra-articular administration of steroids or hyaluronic acid. Surgical interventions like joint replacement are recommended when previous strategies fail to manage OA pain and progression.

Exercise is another important preventive strategy to manage OA. Physical activity ameliorates physical health, helps to control weight, and improves the quality of life (220). It has been shown that adequate exercise reduces signs and symptoms of OA.

There are many approaches to the management of OA. Non-pharmacological treatment has an important role in the patient care; however, it is often under-utilized. These non-pharmacological interventions are, most of the time, used in conjunction with a pharmacological treatment to reduce pain and improve functioning of the joint and quality of life.

## 8.2. Pharmacological interventions:

Pharmacological interventions have been widely studied and showed different level of effects on pain in OA patients. There is a combination of treatment options that aims to improve the functioning and the quality of life of OA patients by relieving pain and decreasing inflammation. This class includes analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular injections, and viscosupplementation care.

### 8.2.1 Analgesics:

Acetaminophen is the most prescribed type of analgesics. It relieves pain by inhibition of prostanoid production in the central nervous system with a limited peripheral anti-inflammatory activity (59). Acetaminophen is very safe when given in recommended doses. It is prescribed as a first-line of oral analgesic for knee OA (14, 221). Because of its minimal effect on inflammation, Acetaminophen has been recommended for mild to moderate pain OA unless contraindicated (220).

Alternatively, aspirin has anti-inflammatory, analgesic, and anti-pyretic effects. It is also used worldwide due to its antiplatelet effect that results from the irreversible of $TXA_2$ production (222, 223).

Unfortunately, analgesics that are currently available have limited efficacy. When non-pharmacological treatments and analgesics are ineffective, NSAIDs are prescribed in the treatment of OA.

#### 8.2.2. NSAIDs:

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs worldwide due to their anti-inflammatory, analgesic, anti-nociceptive, and antipyretic effects. They are widely used for treatment of acute pain. NSAIDs act by inhibiting prostanoids biosynthesis via the COX pathway (223).

These pharmaceuticals are used for the treatment of inflammatory conditions like musculoskeletal diseases including OA and RA and painful conditions (223). NSAIDs should be considered for patients who do not respond to acetaminophen (14, 221).

NSAIDs are classified into two groups: traditional NSAIDs (non-selective NSAIDs) that can target both COX isoforms, COX-1 and COX-2; selective NSAIDs that specifically target COX-2 isoform. The analgesic and anti-inflammatory benefits of NSAIDs derive from inhibition of prostaglandin synthesis. Unfortunately, the use of these drugs is associated with a wide range of side effects. The most common adverse effect of NSAIDs is gastrointestinal (GI) toxicity. Traditional NSAIDs have particularly a significant risk for gastrointestinal events.

The development of COX-2 selective inhibitors like Rofecoxib, Valdecoxib, Ibuprofen, Ketprofen, and Naproxen, and Celecoxib has helped to maintain the therapeutic actions of these drugs while avoiding their gastrointestinal toxicity (223, 224). Although they have lower risk of

GI adverse effects, COX-2 selective NSAIDs like Ibuprofen, Ketprofen, and Naproxen have been reported to be associated with increased cardiovascular risk and heart failure. It was also suggested that the cardiovascular risk is highest with Rofecoxib and Valdecoxib. For this reason, Rofecoxib and Valdecoxib were withdrawn voluntarily from the worldwilde market (225).

Due to the adverse effects related to these medications, the lowest dose should be prescribed for the shortest duration. NSAIDs may reduce pain for short term and should be used with caution particularly in patients with high GI risks and elderly patients (221, 226).

#### 8.2.3. Intra-articular injections:

Another form of treatment in OA is intra-articular injection. Intra-articular injection of corticosteroid is indicated to manage symptoms like acute pain and joint swelling in OA. These injections seem to be efficient and safe (227). However, the number of corticosteroid injections is limited to three or four times per year due to possible cartilage damage from repeated intra-articular injections (220).

Viscosupplementation is another alternative of treatment. It is based on the administration of synthetic HA into the joint via intra-articular injections. HA is a physiological component of synovial fluid and cartilage. HA contributes to the elasticity and lubrication of synovial and cartilage within the joint. This therapeutical strategy is indicated for pain relieve and mobility improvement.

#### 8.2.4. Glucosamine and Chondroitin sulfate:

Glucosamine and chondroitin are dietary supplements that have received a lot of attention for their potential benefit in reducing pain and in slowing the progression of OA.

Glucosamine has been used as analgesic in the management of OA because of its restorative properties. It is one of the important building blocks of cartilage. This naturally

occurring amino monosaccharide has been reported to exhibit protective properties in OA joint tissues (228). It can be taken as a tablet as a diet supplement, or sometimes as an injection (229).

Chondroitin sulfate has a role in preventing degradation of articular cartilage (215). Chondroitin alone appears to provide little benefit for OA patients without significant side effects. Therefore, it is generally taken in conjunction with glucosamine.

## 8.3. Surgical interventions:

The most widely used therapies are generally quite modest including analgesics like acetaminophen, traditional NSAIDs, and COX-2 selective inhibitors. Pharmacologic therapies that exist for OA help to reduce symptoms but are only moderately effective because it leaves a substantial pain and functional burden.

Research regarding surgical interventions for knee OA demonstrated surgery is most of the time reserved for severe OA that significantly limits people activities and that does not respond to other treatments. It used to replace a damaged joint with an artificial joint. However, surgery is recommended before arthritis causes complications such as muscle loss and joint deformities. Furthermore, those who undergo surgery should be in the best possible physical condition and should be prepared for rehabilitation after surgery.

There are no treatments capable of markedly altering OA progression. Current therapeutic interventions are palliative and fail to address pathophysiological and biochemical mechanisms implicated in cartilage destruction and pain induction (15). Therefore, OA remains an incurable disease and there is a large unmet need for desirable therapeutic interventions.

# Part B-

## 1. Chromatin structure:

Eukaryotic Deoxyribonucleic acid (DNA) is packaged into a nuclear highly structured entity termed chromatin (230). With a diameter of up to 10 microns, genomic DNA is compacted more than 10 000-fold by proteins known as histones (231).

Chromatin has a dynamic structure; it plays a pivotal role in different biological mechanisms including DNA transcription, replication, repair and recombination. Chromatin exists in two distinct functional forms: a condensed form called “heterochromatin”, which is an inaccessible form during mitosis and meiosis, and a loosely packed (decondensed) form in resting cells called “euchromatin” in which chromatin is kept accessible to the transcriptional apparatus leading to transcriptional activity (232, 233).

It is constituted of nucleic acids and a series of acid soluble proteins called histones (234). Histones as well as other chromosomal proteins are responsible for the proper packaging of DNA into chromosomes. These proteins that constitute the basic building blocks of chromatin are identified as “nucleosomes”. The nucleosome, a fundamental unit of chromatin, is composed of 146 base pairs of DNA wrapped around an octamer consisting of two of each of the core histones H2A, H2B, H3 and H4 (233, 235, 236, 237). However, histone H1 represents the linker histone. It is not a part of the nucleosome but it binds to the linker DNA and seals off the nucleosome (238). Each of these core histones contain two separate functional domains; a histone fold motif able to mediate both histone/histone and histone/DNA interactions within the nucleosome and $NH_2$ and COOH terminal tail domains (**Figure 15**).

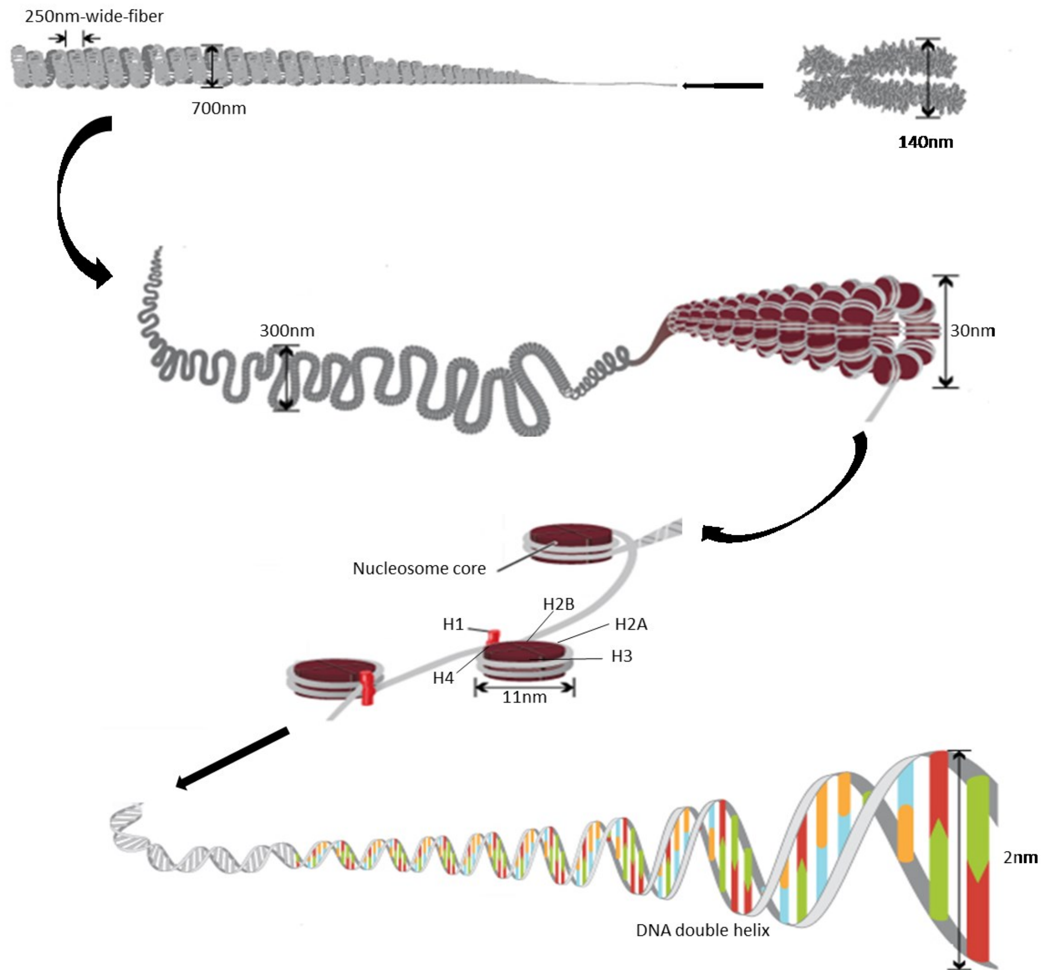


**Figure15**: The chromatin structure. Chromosomes are constituted of compact DNA tightly-wound around nucleosomes. DNA winds around a single nucleosome, composed of eight histone proteins: two of each of the core histones H2A, H2B, H3 and H4. At the simplest level, chromatin is a double-stranded helical structure of DNA. It is complexed with histones to form nucleosomes. The nucleosome core consists of eight histone molecules rounded by the DNA plus the H1 histone. These nucleosomes fold up to produce fibers that are tightly coiled, thereby generating the chromatic of a chromosome (adapted from 226 & 227).

The structure of chromatin fulfills a variety of important cellular processes, not only by protecting DNA while condensed but also by controlling gene expression and preserving genetic information. On the other hand, the relaxed state of chromatin allows access of cellular machineries to DNA and this is how chromatin can be remodeled. Of importance, numerous cellular mechanisms that allow an appropriate reorganization of chromatin are undergoing; these are epigenetic modifications (232).

## 2. Epigenetic mechanisms:

**Definition:** Epigenetics is a combination of the words "genetics" and "epigenesis". The later describes the differentiation of cells from their initial state in embryonic development (239). Epigenetics refers to all heritable changes in gene expression that are not dependent on modifications of the underlying DNA nucleotide sequence (233, 240, 241, 242).

Epigenetic marks include histone modifications, DNA methylation, non-coding RNAs, and chromatin remodeling (243). Conversion between heterochromatin and euchromatin states is basically controlled by two epigenetic processes. The first one includes covalent histone and DNA modifications. The second one is chromatin remodeling and it includes sliding of nucleosomes to new positions on genomic DNA, dynamic loss and gain of histones in nucleosome disassembly and reassembly, and conformational changes in histone-DNA interactions (233, 244, 245). To sum up, epigenetic regulation of genes is implicated in a variety of biological processes like cellular differentiation, development and regeneration of tissues (233, 246, 247).

## 2.1. Histone modifications: The histone code

Histone modifications occur through covalent changes either by additions or removals of specific groups. These modifications impact chromatin structure and function; they are named “the post-translational modifications” (236). The histone amino-terminal tails are the most frequently targeted spots. These spots are exposed on the nucleosomal surface where they are subjected to a wide range of enzyme-catalyzed modifications (248).

The post-translational modifications of N-terminal tails are major mechanisms implicated in the regulation of a variety of cellular processes. As mentioned before, they have fundamental roles in various biological processes such as DNA replication, transcription, cell cycle progression, alternative splicing, DNA repair, and chromosome condensation (233, 236, 238, 249, 250).

There are at least eight distinct types of modifications found on histones: acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization (236, 239, 243, 251) (**Figure 16**).

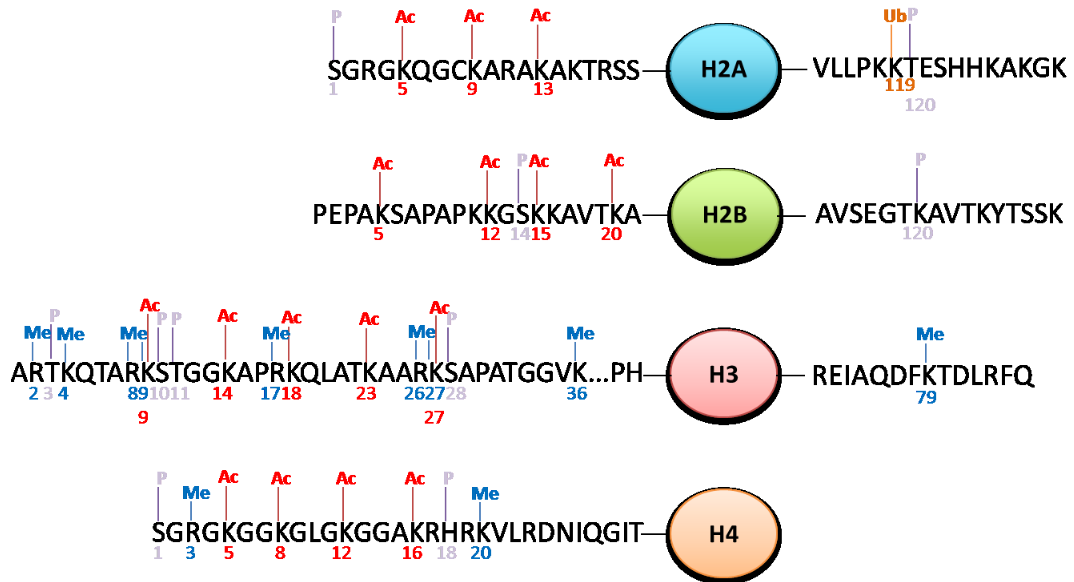


**Figure 16**: Histone modifications. Histones are subjected to a variety of post-translational modifications. These modifications include acetylation (Ac) on lysine residues, methylation (Me) on arginine and lysine residues, phosphorylation (P) on serine and threonine residues and ubiquitination (Ub) on lysine residues and target specific residues of histone H2A, H2B, H3, and H4.

Each of these modifications influences the chromatin structure depending on the site, the degree and the type of modification. These modifications are combinatorial and interdependent (237, 243). The dynamic interplay between histone modifications is culminated in the term of ``histone code``, which describes the role of modifications that enable DNA to function for a given task (237). Therefore, combination of different modifications may results in distinct and consistent cellular outcomes (232) (**Table VII**). Histone post-translational modifications orchestrate chromatin transcriptional regulation through a complex 'histone code' (240, 252) that dictate the chromatin state and extends the genetic code (237, 253, 254, 255).

| Epigenetic modification | Residue specificity | Function |
|---|---|---|
| Acetylation | K-Ac | Transcription, reparation, replication, condensation |
| Methylatin (Lysine) | K-me1, K-me2, K-me3 | Transcription, reparation |
| Methylation (Arginine) | R-me1, R-me2a, R-me2S | Transcription |
| Phosphorylation | S-ph, T-ph | Transcription, reparation, condensation |
| Ubiquitination | K-ub | Transcription, reparation |
| Sumoylation | K-su | Transcription |
| ADP ribosylation | E-ar | Transcription |
| Deimination | R >Cit | Transcription |
| Proline isomerisation | P-cis > P-trans | Transcription |

**Table VII**: Overview of different types and functions of identified histone modifications.

Histone methylation is the most critical type of histone modifications involved in numerous cellular mechanisms. Of particular, histone methylation can be associated either to activation or repression of genes. It is implicated in different molecular mechanisms to control gene expression. Histone modification is mediated by histone methyltransferases (HMTs) and histone demethylases (HDM). More characteristics of this modification will be discussed in detail in the coming parts.

Acetylation is another type of histone modifications; it is the most abundant type of modifications and the most widely studied one on histone tails (235). It is commonly associated to active chromatin. Histone acetylation is a dynamic modification mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (233, 256, 257).

To assign a clear distinct function, for each histone post-translational modification, is elusive. These modifications are highly related to each other and some of them are co-occurring while others are not, instead they are anti-correlated (251). Additionally, the same histone modifications may function differently in different moments of cell cycle (251, 254). Thus, understanding the mechanisms of epigenetic modifications is an exciting area of research.

## 2.2. DNA methylation:

DNA methylation is a critical mechanism implicated in gene regulation. It acts as a genomic response to physical and social signals from the environment at different time points in life resulting in a stable alteration of gene expression (239).

DNA methylation correlates with repression of gene transcription. It consists of the addition of a methyl group to a cytosine residue with a CpG dinucleotide in a DNA chain to form 5-methylcytosine (258). These CpG nucleotides are often clustered in islands that are located in

the promoters of approximately 30 % of genes (243, 259). DNA methylation is catalyzed by DNA methytransferases (DNMTs): DNMT1, DNMT3A, and DNMT3B (233, 243). DNMT1 is assigned for hemi-methylated DNA; it transfers patterns of methylation to newly synthesized strand after DNA replication. Whereas, DNMT3A and DNMT3B are de novo methyltransferases (260, 261)

DNA methylation plays a key role in genomic imprinting and X-chromosome inactivation (238, 262). It has also an important function, which is the silencing of transposons (261, 263). DNA methylation also plays a major role in normal development (233, 263, 264).

DNA methylation can inhibit gene expression by different mechanisms. It prevents transcription by blocking the access of transcriptional machinery like transcription factors to gene promoters and altering chromatin structure by recruiting repressive chromatin remodeling complexes (265). Methylated DNA can also enhance the recruitment of methyl-CpG-binding domain (MBD) proteins. These MBD proteins in turn recruit chromatin remodeling complexes and histone modifying to methylated sites (266). DNA methylation can also inhibit transcription directly by preventing the recruitment of DNA binding proteins from their target sites (267). However, unmethylated CpG islands form a chromatin structure allowing gene expression (268).

## 2.3. Micro RNAs:

Another epigenetic regulatory mechanism majorly implicated in gene regulation is non-coding RNAs. Micro RNAs (miRNAs) or small non-coding RNAs are important regulators of gene expression. These short strands are composed of 20- to 23-nucleotide-long single-stranded non-coding RNAs. They interact with target mRNA via complementary base pairing with the 3´untranslated region of the messager RNA (mRNA) (233, 269), the mRNA is then degraded.

However, incomplete base pairing between the mRNA and miRNA leads to gene silencing (265, 269).

In the human genome, there is approximately 1000 identified miRNA. They are transcribed from either intergenic regions with their own promoters or intragenic regions. They are major regulators in development and play a significant role in various diseases (265, 269).

## 2.4. Chromatin remodeling:

Chromatin remodeling is another powerful mechanism implicated in transcriptional regulation. This mechanism is mediated by specific remodelers called complex remodeling factors that are functionally based on ATP activity. Four remodeling complexes have been identified: the SWItch/Sucrose NonFermentable (SWI/SNF), the imitation switch (ISWI), the chromo-helicase/ATPase DNA binding (CHD), and the inositol requiring 80 (INO80) complex.

The SWI/SNF complex is the best characterized one. It is an ATP dependent complex. The recruitment of SWI/SNF to DNA activates the process of transcription by enhancing the recruitment of transcription factors and co-activators (270). Although it consists of about 10 sub-units, the activity of this complex relies essentially on two ATP-dependent-sub-units: Brahma (hBrm) or Brahma-related gene-1 (Brg-1) (271).

While the ISWI complex is capable of promoting chromatin assembly and repressing transcription, the CHD complex can either promote or repress transcription (272). However, the last family of complexes, The INO80complex, can participate in numerous cellular processes like transcriptional activation, DNA replication, DNA repair, chromosome segregation, and telomere regulation (273).

# 3. Histone methylation/demethylation:

Histone methylation is a highly dynamic modification that can particularly trigger either gene activation or inactivation depending on the target amino acid residues and the degree of methylation, mono- (m1), di- (m2), or tri-methylated (m3).

Methylation occurs on both the α-amino group of lysine and the guianidino group of arginine and catalyzed by enzymes using S-adenosyl-L-methionine (SAM) as a methyl group donor (274). Lysine (K) and arginine (R) residues of histone tails are methylated by histone lysine methyltransferases (KMTs) and protein arginine methyltransferases (PRMTs) respectively (243, 275). Histone modification nomenclature includes histone, residue and position of that residue followed by type and number of modifications. For instance, H3K4m2 means di-methylation of histone H3 on lysine 4 (233, 276).

As mentioned above, histone methylation is mediated by specific catalyzing enzymes. Unlike histone acetylation and phosphorylation, histone methylation was considered as an enzymatically irreversible modification. It was relatively thought to be permanent and could only be removed by histone exchange or dilution during DNA replication until the discovery of the first histone demethylase lysine-specific demethylase-1 (LSD1) (277). This discovery has showed that histone methylation is a reversible modification. Afterwards, a flow of other histone demethylase enzymes was discovered.

Histone methylation has been associated with many important biological processes such as regulation of chromatin structure and transcription, stem cell differentiation, X-inactivation, and DNA damage response (278, 279, 280). On the other hand, histone demethylation is a crucial mechanism implicated in diseases such as cancer. Of particular, histone lysine methylation is

reported to be widely implicated in a variety of cellular processes. It can be associated either with activation or repression of genes.

These modifications can be divided into those that correlate with activation and those that correlate with repression. In other words, any given modification has the potential to activate or repress under different conditions.

### 3.1. Histone lysine methylation:

First reported by Murray and by Allfrey and colleagues in the mid-1960, histone lysine methylation is a dynamic modification that plays a pivotal role in gene regulation (281).

Histones contain numerous lysine residues, of which many are methylated in vivo. Histone lysine methylation may take place on different lysine residues with opposite effects. Six of the lysine residues, H3K4, -9, -27, -36, and -79 as well as histone H4K20, have been studied extensively and linked to chromatin and transcriptional regulation as well as DNA damage response (231, 281). Three lysine methylation sites on histones are associated with activation of transcription: H3K4, H3K36, and H3K79. In contrast, H3K9, H3K27, H4K20 sites are implicated in transcriptional repression (236) (**Figure 17**).

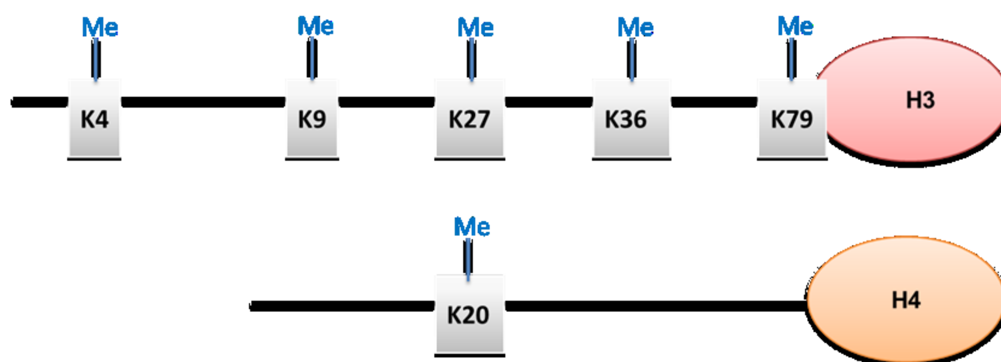


**Figure 17**: Histone lysine methylation. As shown, there are targeted lysine (K) residues on H3 (K4, K9, K27, K36, K79) and H4 (K20).

Lysine can be mono-, di-, and tri-methylated unlike arginine that can be only mono- and dimethylated (282). The numerous lysine on the histone tails, in conjunction with the various methylation levels that can be generated at each of these sites, provide tremendous regulatory potentials for chromatin modifications (249). The degree of lysine methylation is associated with potentially different functional outcomes. For instance, while H3K4m2 and H3K36m2 are activating marks, H3K27m2/m3 and H3K9m2/m3 are repressive marks (240, 252). Evidence showed that H3K4m3, but not H3K4m2, at the promoter of a gene is involved in active transcription (283, 284). In addition, an increase of H3K9m3 within the body of a gene is linked to active gene expression. However, H3K9m1 and H3K9m2 are associated with silent domains in the euchromatic regions. Furthermore, H3K36 methylation has been shown to have a negative effect when it is found on the promoter but a positive effect when in the coding region. H3K9

methylation has also been demonstrated to have a negative effect in the promoter but a positive effect in the coding region (285).

### 3.2. Histone arginine methylation:

Like histone lysine methylation, histone arginine methylation can be associated with both activation and repression of transcription. Histone arginine methylation is involved in different cellular processes like transcriptional regulation and DNA damage repair (282).

Arginine (R) residues can also be subjected to methylation mark on both histone H3 and H4. Arginine R2, R8, R17, R26 on histone H3 (H3R2, H3R8, H3R17 and H3R26) and R3 on histone H4 (H4R3) are target residues for histone metyhylation. This modification is mediated by protein arginine methyltransferases (PRMTs) that are classified as type I, type II, type III, or type IV (286). Most of the time, the methylated arginine residues are located near to other modified histone residues allowing crosstalk between this type of modification and other histone modifications.

Arginine methylation is reported as a very stable mark; it is not clear whether this modification can be reversed. To date, the sole histone demethylase reported to demethylate histone arginine reside is the histone demethylase JMJD6. It was reported to demethylate specifically H3Rme2 and H4R3me2. It could demethylate both, symmetrically and asymmetrically dimethylated residues. However, recent findings showed that JMJD6 is actually a lysine-hydroxylase without detecting a demethylase activity on either H3R2me2 or H4R3me2 peptides. In addition, structural analyses of JMJD6 suggest that it is not an arginine demethylase (287). Thus, whether an arginine demethylase exists or not remains unknown.

Arginine is also exposed to a deamination process (287). The peptidyl arginine deiminases (PADs) catalyze the deimination of arginine to citrulline thereby blocking methylation of arginine residues. PADs can prevent subsequent methylation but they are not considered as demethylases (286, 288).

However, this has raised questions regarding their implication in removing methyl marks. PADI4, in particular, targets the same arginine residues on histones H3 and H4 as PRMTs. Recent findings showed that PADI4 is recruited to the pS2 promoter region just prior to the loss of H3R17me2a, suggesting that it is responsible for removing this methyl-mark (287).

## 3.3. Histone methyltransferases:

Histone methylation is catalyzed by histone methyltransferases (HMTs). Both lysine and arginine residues from histones H3 and H4 can be methylated by enzymes belonging to different protein families. Several families have been found to be able of catalyzing the addition of methyl groups to histones using SAM as methyl donor (289).

Histone methyltransferases are classified into three different classes: the lysine-specific SET domain containing histones methyltransferases; the non-SET domain containing histones methyltransferases (Dot1/DOT1L family); and the arginine methyltransferases. While arginine can either be mono- or di-methylated, lysine can be mono-, di-, or tri-methylated (271).

In 2000, the first histone lysine methyltransferase, mammalian SUV39H1, was discovered. This discovery led to the identification of the SET domain KMTs as well as the non-SET domain KMTs. While the SET domain KMTs methylate the N-terminal tails of histone H3 at K4, K9, K27, and K36 and histone H4 at K20, the non-SET domain KMTs DOT1L (Dot1-like protein) methylate K27 of histone H3 (290, 291, 292) (**Figure 18**).

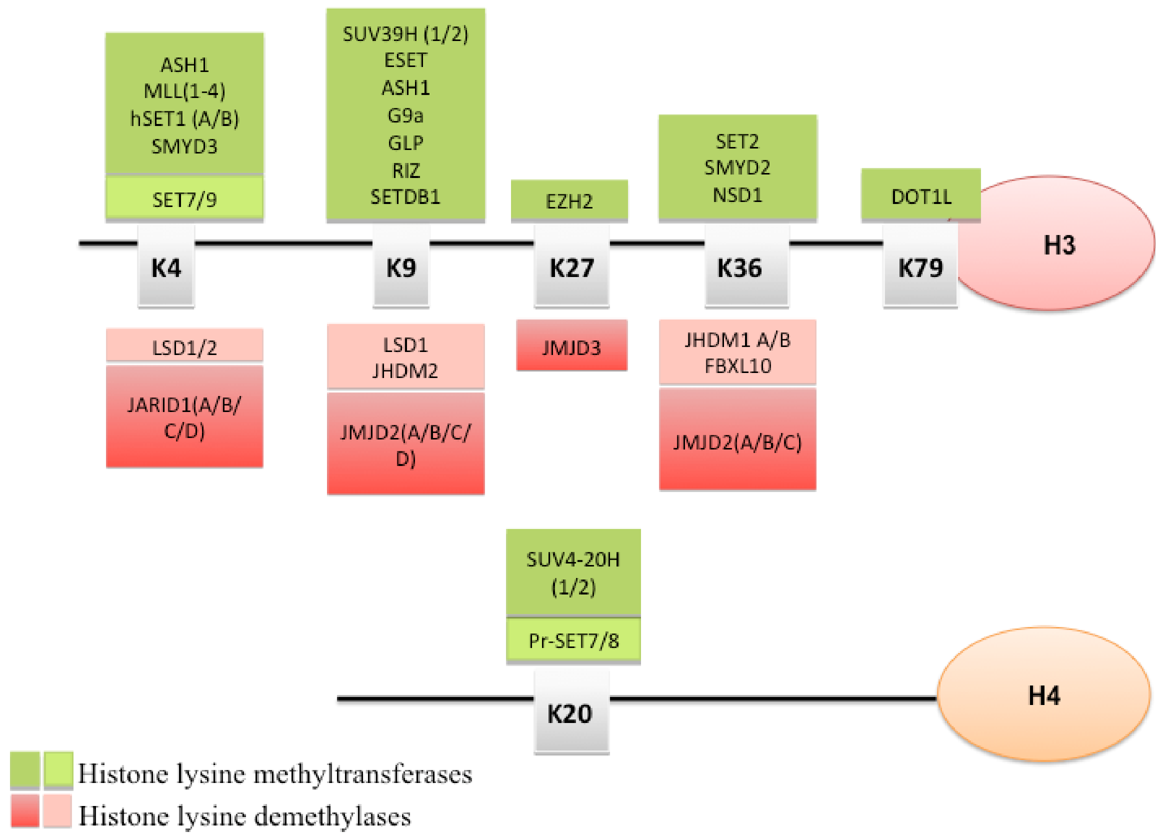


**Figure 18**: Histone lysine methyltransferases/demethylases and their target sites. These enzymes are grouped according to the specific lysine residue targeted for this modification. The histone methyltransferases for various lysine residues are indicated on green, and the corresponding demethylases are listed on red.

Unlike the disruptor of telomeric silencing 1/ Dot1-like protein (Dot1/DOT1L) family, histone lysine methyltransferases (KMTs) contain a SET domain composed of approximately 130 amino acids. This domain was originally identified as a shared domain in three Drosophila proteins that are implicated in epigenetic mechanisms: the suppressor of position-effect variegation [Su(var)3-9]; an enhancer of the eye colour mutant zeste which belongs to the PcG proteins [E(Z )]; and the homeobox gene regulator trithorax [TRX]. Mammalian homologues of Drosophila Su(var)3e9, Suv39h1 and Suv39h2, were the first KMTs characterized (271, 293).

The KMTs are classified into several different families according to sequence similarities within their SET domain, other adjacent domains, as well as other structural features like the presence of other defined protein domains (261) (**Table VIII**).

#### 3.3.1. The lysine specific SET domain family:

a. The SET1 family:

The SET1 family of proteins is composed of the TRX homologues MLL1 and MLL4, the related proteins MLL2 and MLL3, and the two proteins highly similar in their SET domain to the yeast Set1 protein, SET1 and SET1L. This family is characterized by a SET domain at the carboxyl terminus of the protein, followed by a Post-SET region. The Post-SET motif contains three conserved cysteine residues, which are essential for KMT activity (294).

SET1 KMTs, like the mammalian MLL, specifically methylate H3K4, and generate an epigenetic imprint for active euchromatin. It might also play a functional role of transcriptional co-activators and interact with other transcriptional co-activators like CREB-binding protein (CREBBP) (295).

| Name | Substrate specificity | Function |
|---|---|---|
| SUV39H1 | H3K9 | Heterochromatin formation/ silencing |
| SUV39H2 | H3K9 | Heterochromatin formation/ silencing |
| G9a | H3K9 | Heterochromatin formation/ silencing |
| EuHMTase/GLP | H3K9 | Heterochromatin formation/ silencing |
| ESET/SETDB1 | H3K9 | Transcription repression |
| CLL8 | H3K9 | --- |
| MLL1 | H3K4 | Transcription activation |
| MLL2 | H3K4 | Transcription activation |
| MLL3 | H3K4 | Transcription activation |
| MLL4 | H3K4 | Transcription activation |
| MLL5 | H3K4 | Transcription activation |
| hSET1A | H3K4 | Transcription activation |
| hSET1B | H3K4 | Transcription activation |
| ASH1 | H3K4 | Transcription activation |
| SET2 | H3K36 | Transcription activation |
| NSD1 | H3K36 | --- |
| SMYD2 | H3K36 (p53) | Transcription activation |
| DOT1L | H3K79 | Transcription activation |
| Pr-SET7/8 | H4K20 | Transcription repression |
| SUV4-20H1 | H4K20 | DNA-damage response |
| SUV4-20H2 | H4K20 | --- |
| EZH2 | H3K27 | Polycomb silencing |
| SET7/9 | H3K4 (p53 and TAF10) | Transcription activation |
| RIZ1 | H3K9 | Transcription repression |

**Table VIII**: Histone methyltransferases (HTMs): Specificity and transcriptional effects.

b. The SUV39 family:

The SET domain of SUV39 family is localized at the carboxy terminus of the protein and is flanked by Pre-SET and Post-SET regions. These domains are mandatory for KMT activity of the SET domain. Two proteins of the SUV39 family, SETDB1 and SETDB2, have, in particular, an expanded SET domain that results in the large insertion of amino acids. While these methyltransferases contain a MBD domain, other members of this family like SUV39H1 and SUV39H2 have a chromodomain (296).

The SUV39 family specifically methylates H3K9. G9a is the major eukaryotic KMT responsible for demethylation of H3K9 (H3K9me2) at active sites (297) targeting genes where it represses transcription (298). However, the G9a/BAT8 methylates, in addition to H3K9, the lysine 27 on H3 (H3K27) in vitro (299).

c. The SET2 family:

The SET2 family includes the three highly related proteins, the nuclear receptor SET domain-containing proteins (NSD): NSD1, NSD2 and NSD3, the homolog of the Drosophila TrxG protein ash1 (absent, small, or homeotic discs 1), ASH1L, and the huntingtin interacting protein SETD2/HYPB. The methyltransferase activity of SET2 family is not restricted to a single residue but various residue like H3K36, H3K4, H3K9, and H4K20 and members of this family are specific for some (300, 301).

d. The EZH family:

The EZH family includes two related members that are homologous to the PcG protein Enhancer of zeste (E(Z)). E(Z) is part of the Polycomb repressor complex 2 (PRC2) that methylates histone H3K9 and H3K27. The human EZH2 methylates histone H1K26 mediating transcriptional repression (295).

e. The SMYD family:

The SMYD family contains five related proteins that harboura a MYND-type zinc finger. The SMYD3 SET domain methylates specifically H3K4 however the MYND finger directly binds to specific DNA sequences (302).

f. The PRDM family:

The PRDI-BF1 and RIZ homology domain containing protein (PRDM) family is a vast family of proteins that harbour toward the amino terminus a PR domain having 20e 30% of sequence identity to the SET domain. The PR domain is classified as a subclass of SET domains (303). The PR/SET domain of PRDM proteins (PRDM2/RIZ1) has a catalytic activity, which is associated to H3K9-specific methyltransferase activity (304). However, other member of this family such as PRDM1/BLIMP1 and PRDM6/PRISM were shown to interact with G9a, the H3K9 specific methyltransferase (305). PRDM proteins are most of the time associated with gene repression (306, 307).

### 3.3.2. The non-SET HKMTs:

The human homolog of Dot1 (DOT1L) does not contain a SET domain, and it specifically acts on nucleosomal histones. It was identified as disruptor of telomere silencing in *Saccharomyces cerevisiae*. These histone methyltransferases methylate H3K79 (308, 309).

### 3.3.3. The protein arginine methyltransferases:

The protein arginine methyltransferases (PRMTs) catalyze monomethylation and both symmetric and asymmetric dimethylation of the arginine residue (310, 311). They are classified into four types. Both type I and II catalyze monomethylation of the guanidinium nitrogen of specific arginine residues. However, they differ in the dimethylarginine type they generate. Type I PRMTs catalyse asymmetric dimethylarginine, while type II PRMTs mediate the formation of

symmetric dimethylarginine. Type III enzymes are responsible only for the monomethylation of arginine residues (312). Finally type IV PRMTs have been characterized to catalyze arginine methylation only in *Saccharomyces cerevisiae*. In humans, nine PRMTs have been identified: PRMT1, PRMT3, PRMT4, PRMT6, and PRMT8, belonging to type I PRMTs as well as PRMT5, PRMT7, and PRMT9, members of type II (313).

Lysine methylation had raised intriguing questions regarding the existence of lysine demethylases that reverse this modification. In 2004, the first lysine demethylase LSD1 (KDM1) was discovered (277).

## 3.4. Histone demethylases:

Histone demethylases (HDMs) are classes of epigenetic enzymes, which can remove both activating and repressive histone methylation marks (**Figure 18**).

There are two histone demethylase families with distinct domains: the lysine specific demethylase (LSD) domain and the JmjC domain (314) (**Table IX**).

### 3.4.1 The lysine specific demethylase family:

The lysine specific demethylase (LSD) family is composed of two members: LSD1 and LSD2. This family of enzymes is characterized by the presence of both an amine oxidase-like (AOL) domain and a SWIRM (swi3, rsc8, and moira) domain. The catalytic activity resides in the AOL domain that uses an oxidation mechanism dependent on the flavin adenine dinucleotide (FAD) cofactor to catalyze the methyl groups from histone lysines (315, 316, 317). It involves the oxidation of an amine via the oxidative cleavage of the $\alpha$-CH bond of the substrate to form an imine intermediate with concomitant reduction of the flavin cofactor.

| Name | Substrate specificity | Function |
|---|---|---|
| LSD1/BHC110 | H3K4me1/2, H3K9me1/2 | Transcription activation and repression, heterochromatin formation |
| JHDM1a/FBXL11 | H3K36me1/2 | --- |
| JHDM1b/FBXL10 | H3K36me1/2 | --- |
| JHDM2a | H3K9me1/2 | Androgen receptor gene activation, spermatogenesis |
| JHDM2b | H3K9me | |
| JMJD2A/JHDM3A | H3K9, K36me2/3 | Transcription repression, genome integrity |
| JMJD2B | H3K9, H3K36me2/3 | Heterochromatin formation |
| JMJD2C/GASC1 | H3K9, K36me2/3 | Putative oncogene |
| JMJD2D | H3K9me2/3 | --- |
| JARID1A/RBP2 | H3K4me2/3 | Retinoblastoma-interacting protein |
| JARID1B/PLU-1 | H3K4me1/2/3 | Transcription repression |
| JARID1C/SMCX | H3K4me2/3 | X-linked mental retardation |
| JARID1D/SMCY | H3K4me2/3 | Male-specific antigen |
| UTX | H3K27me2/3 | Transcription activation |
| JMJD3 | H3K27me2/3 | Transcription activation |

**Table IX**: Histone Demethylases (HDMs): Specificity and transcriptional effects.

a. LSD1:

LSD1 is the first identified histone demethylase. It catalyzes demethylation of H3K4me1 and H3K4me2 and can also demethylates H3K9me1 and H3K9m2. LSD-1 can only demethylates mono- and dimethylated lysine residues but not trimethylated ones (318, 319). LSD-1 demethylates specifically H3K4m1/2 to favor gene silencing. However, when it is present in a complex with the androgen receptor (320), it demethylates H3K9m1/2 to activate transcription. In addition, LSD1 may be implicated in regulating the activity of enhancers. It is found associated with the nucleosome remodeling and histone deacetylase (NuRD) complex on enhancers of transcribed genes in embryonic stem (ES) cells. During the ES cells differentiation, LSD1 demethylates H3K4me1 at these enhancers, decommissiong the enhancers, silencing the associated genes, and contributing to efficient differentiation (321).

b. LSD2:

A second Flavin-dependent demethylase that has been later identified in mammals is the LSD2. LSD2 is also constituted of a conserved SWIRM domain which is required for its catalytic activity. It demethylases specifically H3K4me1 and H3K4me2. However, its repressive activity seems to be not associated with the demethylase function (322, 323). Unlike LSD1, LSD2 doesn´t contain a tower domain which prevent it to form active complex with Corepressor to the RE1 silencing transcription factor (CoREST) whereas it can bind to euchromatin histone methyltransferases (G9a and NSD3) and cellular factor implicated in transcription elongation (324). Recently, it has been shown that LSD2 enhance H3K9me2 in addition to H3K4me2 demethylation leading to control of NF-κB recruitment and activation of inflammatory genes like IL-12B promoter (325).

### 3.4.2. The Jumonji C family:

The second class of histone lysine demethylases is the Jumonji C family. These demethylases belong to a larger superfamily named the 2-OG oxygenases and mediate a diversity of oxidation reactions whose members use 2-OG, molecular oxygen, and $Fe^{2+}$ as co-substrates/co-factors. Unlike LSD, the JmjC family drives demethylase reaction of tri-methylated histones (310).

Within the human genome, there are 27 different JmjC domain proteins of which 15 have been shown to demethylate specifically H3 lysines while one to demethylate arginine (326). The catalytic domain that defines this group is the JmjC domain. This domain is essential for the oxidative lysine demethylation reaction (327).

a. The JHDM1 family:

The JmjC domain-containing histone demethylase 1 (JHDM1) family includes two related proteins JHDM1B/FBXL10 and JHDM1A/FBXL11. The first JmjC domain demethylase described was JHDM1A/FBXL11. JHDM1 specifically demethylates H3K36me1 and H3K36me2 using a -ketoglutarate and Fe(II) as co-factors (327, 328).

b. The JHDM2 family:

The JHDM2 family contains three related proteins, JMJD1A/JHDM2A, JMJD1B/JHDM2B and JMJD1C/ JHDM2C/TRIP8. JMJD1A/JHDM2A specifically acts on H3K9me1 and H3K9me2 (329).

c. The JHMD3 family:

The JHMD3 family consists of the four related proteins JMJD2A/JHDM3A, JMJD2B, JMJD2C/GASC1 and JMJD2D. JMJD2A and its three homologues JMJD2B, JMJD2C and JMJD2D showed that this subfamily catalyzes the demethylation of H3K9me2/me3 and

H3K36me2/me3 (327, 328, 330, 331). The JMJD2 proteins play a role in transcription and inhibition of JMJD2A expression resulting in increased H3K9me3 levels (310). However, another study has reported JMJD2C to be a co-activator for the androgen receptor. These data suggest that the JMJD2 proteins can act both as co-activators and co-repressors of transcription.

d. The JARID family:

The JARID family is composed of four related proteins, JARID1A/ RBP2, JARID1B/RBP2-like, JARID1C/SMCX and JARID1D/SMCY, as well as the more distant protein JARID2/JUMONJI. None of these demethyalse has been associated with an HDM activity, however they all have DNA-binding and/or chromatin-associated domains. JARID1 mediates the demethylation of H3K4me2/me3. JARID1 members seem to function as transcriptional repressors (310).

e. The PHF family:

The PHF (PHD Finger) family includes PHF8 and PHF2 related proteins. The PHF8 protein, that contains a PHD finger, in addition to the Jumonji C domain, has been shown to exert a positive effect on rDNA transcription. It interact with the RNA polymerase I transcriptional machinery and with WD repeat-containing protein 5 (WDR5)-containing H3K4 methyltransferase complexes. PHF8 demethylates H3K9me1/2 and its catalytic activity requires adjacent H3K4me3 (332).

f. The UT family:

Finally, the UT family is the last group of putative JmjC domain-containing HDMs. This group is composed of two similar proteins, UTX and UTY (271). UTX functions as transcriptional activator through removing the repressive H3K27me2/me3 marks. Studies have shown that binding of UTX to HOX genes correlates with transcriptional activation and inhibition

of UTX expression prevented the HOX genes activation (333).

The JmjC family can also function as demethylases for arginine demethylation through a hydroxylation dependent demethylation reaction mechanism. JMJD6, particularly, can demethylate H3R2me2 and H4R3me2 both in vitro and in vivo (334).

## 4. Histone acetylation / deacetylation:

Histone acetylation is the widely studied type of histone modifications. It plays a crucial role in transcriptional activation and repression. Histone acetylation and deacetylation are mediated by the activity of two opposing family of enzymes: histone acetylases (HAT) and histone deacetylases (HDAC). Acetylation of histone residues is conducted by the addition of acetyl groups from acetyl-coA enzyme to lysine residues on the N-terminal histone tails (335). Thus, if HATs are associated with an activation of gene expression, HDACs removes acetyl groups to mediate transcriptional repression (336) (**Figure 19**).

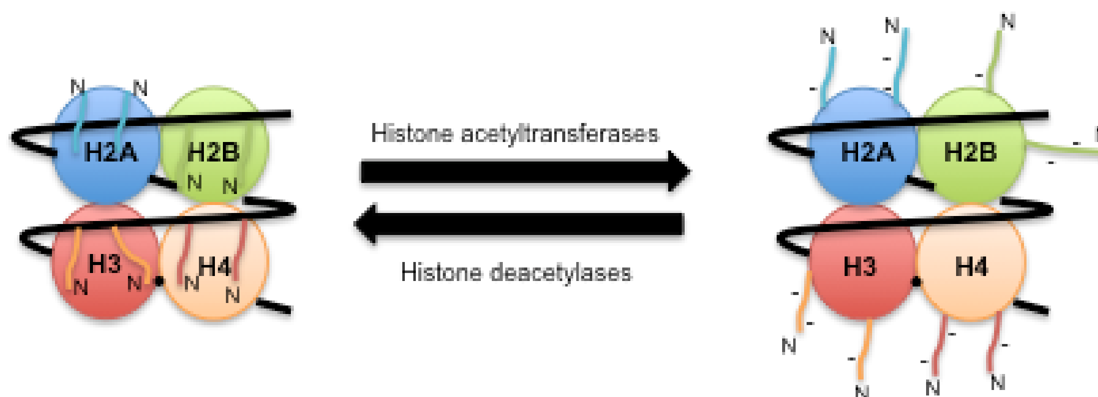


**Figure 19**: Histone acetylation/deacetylation.

### 4.1. Histone acetyltransferases:

Histone acetyltransferases (HATs) play a major role in epigenetic regulation and gene expression. They mediate the catalysis of an acetyl group from acetyl-CoA to ε-amino group of a histone lysine residue thereby allowing binding of chromatin remodeling complexes.

HATs are classified based on the structural and the functional similarities of their catalytic domains. In humans, there are about 30 HATs assembled into five families: the Gcn5-related N-acetyltransferases (GNATs), the MYST HATs, the p300/CBP HATs, the steroid receptor co-activators (SRC), and the nuclear receptor co-activators (NCoA).

GNATs are constituted of four conserved motifs forming HAT domain, and unusually having a bromodomain or chromodomain (337). However MYST HATs are characterized by the MYST domain, which contains an acetyl-CoA binding motif and a zinc finger (338). The three other families are smaller than the previous ones. The p300/CBP HATs, for instance, is characterized by the presence of the TAF250 domain (339) (**Table X**).

### 4.2. Histone deacetylases:

Histone deacetylases (HDACs) control the transcriptional activity of genes by removing acetyl groups from histone lysine residues resulting in a condensed form of chromatin and thereby reducing the accessibility for transcription factors.

| Name | Substrate specificity | Function |
|---|---|---|
| HAT1 | H4 (K5, K12) | Histone deposition, DNA repair |
| hGCN5 | H3 (K9, K14, K18)/H2B | Transcription activation |
| PCAF | H3 (K9, K14, K18)/H2B | Transcription activation |
| CBP | H2A (K5); H2B (K12, K15) | Transcription activation |
| P300 | H2A (K5); H2B (K12, K15) | Transcription activation |
| TAF1 | H3 > H4 | Transcription activation |
| TIP60/PLIP | H4 (K5, K8, K12,K 16) H3 (K14) | Transcription activation, DNA repair |
| MOZ/MYST3 | H3 (K14) | Transcription activation |
| MORF/MYST4 | H3 (K14) | Transcription activation |
| HBO1/MYST2 | H4 (K5, K8, K12) > H3 | Transcription, DNA replication |
| ELP3 | H3 | |
| TFIIIC90 | H3 (K9, K14, K18) | Pol III transcription |
| SRC1 | H3/H4 | Transcription activation |
| ACTR | H3/H4 | Transcription activation |
| P160 | H3/H4 | Transcription activation |
| CLOCK | H3/H4 | Transcription activation |

**Table X**: Histone acetyltransferases (HATs): Specificity and transcriptional effects.

In human genome, there are 18 genes encoding for HDACs. This family is divided into four groups based on structural and functional characteristics: HDAC class I, II, III, and IV. HDAC class I contains HDAC 1, 2, 3, and 8 enzymes. HDAC class II is constituted of HDAC 4, 7, and 9. However, HDAC class III is composed of sirtuin enzymes (SIRT 1, 2, 3, 4, 5, 6, and 7). Finally HDAC 11 is the unique member of HDAC class IV (340). While members of class I are widely expressed in nuclei, class II HDACs have limited expression (**Table XI**).

| Name | Substrate | Function |
|---|---|---|
| HDAC1 | p53, MyoD, Androgen receptor, STAT3 | Transcription, cell proliferation |
| HDAC2 | Bcl-6, STAT3, Glucocorticoid receptor | Transcription, cell proliferation |
| HDAC3 | GATA-1, STAT3 | Cell cycle, DNA-damage response |
| HDAC4 | GATA-1, HP-1 | Transcription, chondrocyte hypertrophy |
| HDAC5 | Smad7, HP-1 | Transcription, cardic stress response |
| HDAC6 | HSP90, $\alpha$-Tubulin | Transcription, cell migration |
| HDAC7 | FLAG1, 2 | Transcription , cell-cell adhesion, cell devolopment and metabolism |
| HDAC8 | --- | Transcription |
| HDAC9 | --- | Transcription |
| HDAC10 | HSP90? | Transcription |
| HDAC11 | --- | --- |
| Sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7) | H3K9, H4K16 | Transcription, ADP ribosylation |

**Table XI**: Histone deacetylases (HDACs): Specificity and transcriptional effects.

# 5. Epigenetics in Osteoarthritis:

OA is a complex pathophysiology with potent genetic components. Numerous studies have identified the major role that epigenetic events play in OA progression including DNA methylation, histone modifications, micro RNAs, and chromatin remodeling.

## 5.1. Histone modifications in Osteoarthritis:

Histone modifications have been shown to be implicated in the progression of OA pathophysiology. Histone modifications regulate the expression of catabolic mediators in cartilage. The most studied histone mark in OA pathology is histone acetylation. In OA chondrocytes, several HDACs are up-regulated including HDAC1, HDAC2 and HDAC7 (341, 342). It has been shown that HDACs inhibitors-treated-chondrocytes reduce the expression of catabolic genes like MMP-3 and ADMATS-5 (343). IL-1β-induced expression of MMP-1, -3, -13 was reduced upon trichostatin (TSA) and vaproic acid (VA) treatment of chondrocytes (344).

HDAC inhibitors have been also reported to modulate the expression of ECM components. The duration of treatment with these inhibitors play an important role in such regulation. Short-term chondrocytes treatment (less than 24h) promotes anabolic cartilage gene expression like Col2A1 (Col-IIa1), Col9A1, and ACAN (341, 345). However, extended treatment prevents the expression of the same transcripts (346, 347). The early positive effect may results from HDAC inhibition while extended inhibition may be due to the up-regulation of repressive factors like Wingless integration-5A (Wnt-5A) or NGF1-A-binding proteins-1 (NAB-1) (348).

Furthermore, the HDAC sirtuin, SirT1, is crucial for chondrocyte survival and apoptosis. Levels of SirT1 decrease during chondrocytes dedifferentiation, both in OA cartilage and in

cartilage exhibiting OA-like damage (349). SirT1 inhibition results in an increase in chondrocyte apoptosis, while treatment with resveratrol, an activator of sirtuin, protect chondrocytes from death (349). SirT1 also promotes cartilage matrix gene expression like Col2A1, Col9A1, and ACAN through deacetylation of sex determining region 9 (SOX9) gene (350) while preventing ADAMTS-5 expression (351). Interestingly, SirT1 up-regulates MMPs and cartilage destruction through deacetylation and activation of the catabolic transcription factor hypoxia-inducible factor-2α (HIF-2α) (352).

Small molecule inhibition of these enzymes is an intriguing therapeutic option; they have shown efficacy in small animal arthritis models including OA. The beneficial effects of HDAC inhibitors have been also reported in animal models of Rheumatoid Arthritis (RA). HDAC inhibitors can suppress and reduce the levels of inflammatory cytokines (353).

### 5.2. DNA methylation in Osteoarthritis:

DNA methylation has been well reported to play a critical role in OA. This epigenetic mark has been widely studied in OA pathology (354). DNMT1 and DNMT3A are highly expressed in chondrocytes suggesting that these methylases contribute to DNA methylation. However, the expression of DNMT3B is low in cartilage (355).

Methylation of specific CpG sites at the promoter of genes like MMP-13 and ADAMTS4, has been shown to be reduced in end stage of OA chondrocytes. This hypomethylation results in increased expression of these genes (356, 357). Further studies have similarly reported that increased MMP-13 expression was associated with demethylation of CpG sites in MMP-13

promoter (358). Numerous other MMP promoters, like MMP-3 and -9, showed decreased methylation sites at single CpG loci in OA cartilage compared to normal (259).

In human articular chondrocytes, a region of IL-1β promoter is demethylated and this demethylation correlates with an increased expression of IL-1β (359). Recently, studies have reported that inhibitors of NF-κB prevent cytokine-induced-demethylation of a specific CpG site in the IL-1β promoter resulting in a decreased expression of IL-1β in human OA chondrocytes (359).

Two CpG promoter sites were identified as possible binding sites for the transcriptional factor HIF-2α. This cartilage catabolic transcription factor may bind to the -110 site in the MMP-13 promoter which is hypomethylated in OA cartilage, and the -299 site in the IL-1β promoter in stimulated chondrocytes. The MMP-13 site has been claimed as a methylation modulated HIF-2α binding site while the IL-1β site is not (357, 360).

Leptin expression is also regulated by DNA methylation. OA chondrocytes were associated with hypomethylation of leptin promoters and high-leptin mRNA expression (243). It is further down-regulated by RNA interference resulting in decreased MMP-13 expression (361).

## 5.3. Micro RNAs in Osteoarthritis:

In addition to histone modifications and DNA methylation, the contribution of micro RNAs to the pathophysiological mechanisms of OA has been noted.

Abnormal expression of miRNAs has been reported in OA (362). It has been demonstrated that miR-140 is more abundant in human articular cartilage. It increases during chondrogenesis but decreases in OA (363). The expression of several genes is regulated by miR-140 (364). MiR-140 decreases ADAMTS-5 and other genes like IGFBP-5; however, it increases ACAN

expression (364). In antigen induced arthritis (AIA) model, an overexpression of miR-140 protects against cartilage damage and degradation (364). However, miR-140 knockout mice develop more severe OA changes in both aged and surgically induced models of OA compared to wild type mice (365).

Numerous other mirco RNAs contribute to the regulation of key genes in OA. Overexpression of miR-101 can abrogate ECM degradation induced by IL-1β (366, 367). This indicates the critical role of miRNAs in IL-1β-cultured-chondrocytes. An altered expression of miR-9, miR-98, miR-146, and miR-27 has been also reported in OA cartilage indicating that the overexpression of these miRNAs reduced IL1-induced-TNF-α production. There is, particularly, an increased expression of miR-9 and down-regulation of miR-146 and miR-27b in OA cartilage (364, 366, 368). An inhibition or overexpression of miR-9 modulated the secretion of MMP-13 (367). In chondrocytes stimulated with IL-1β, expression of miR-146 is increased and thereby suppresses expression of matrix enzymes like MMP-13. Expression of MMP-13 is also suppressed by miR-27 (363, 369). Furthermore, the expression of miR-146a decreases with increased OA severity (365). IL-1β induces miR-146 expression in chondrocytes, which via a feedback loop, represses IL-1β-induced gene expression (370). MiR-146a has been recently shown to be linked to the pain-related pathophysiology of OA (371). In contrast, IL-1β represses miR-27b expression in chondrocytes. The expression of miR-27b is reduced in OA cartilage where it inversely correlates with MMP-13 (369). Additionally, miR-22 has been also shown to be altered in OA cartilage. MiR-22 targets PPARα and bone morphogenic protein 7 (BMP7) with indirect effects on IL-1β, MMP-13 and ACAN expression.

Epigenetics of OA is still in its infancy but current data suggests that these mechanisms may play a crucial role in the pathophysiology of the disease of OA and may lead to new

therapeutics. Up to now, there is no data showing the involvement of histone methylation in OA. The role of histone methylation /demethylation in the regulation of inflammatory genes implicated in OA pathophysiology initiation and progression remains questionable. Therefore, the aim of this thesis is to investigate the role of histone methylation and demethylation in OA.

# Thesis proposal

The pathogenesis of OA has been associated with increased levels of pro-inflammatory cytokines mainly IL-1β. Provided evidences have reported that this inflammatory cytokine induce the production and release of prostaglandins and ROSs such as $PGE_2$ and NO. The biosynthesis of $PGE_2$ and NO is catalyzed by COX-2/mPGES1 and iNOS enzymes, respectively. COX-2, mPGES1, and iNOS are early response genes that are rapidly induced by IL-1β.

Gene expression is highly dependent on chromatin assembly and remodeling. These changes are known as epigenetic mechanisms either at the posttranscriptional or the posttranslational level. Epigenetics play a critical role in gene transcription and expression. Among all, histone modifications were shown to have a pivotal role in gene regulation. Histones are now recognized as active effectors of gene expression involving different modifications that functionally defines genomic landmarks. They are crucial for maintaining the integrity of the genomes' expression profiles and any disruption of theses profiles no doubt contributes to pathologies and diseases. Recent studies establish histone methylation as a critical process in the regulation of several biological processes. **Rationnel**: Histone lysine methylation, in particular, was reported to play important roles in the transcription of numerous genes including inflammatory and tumor genes. Our research team has demonstrated for the first time the contribution of histone acetylation in COX-2, mPGES-1 and iNOS regulation in OA. However, the implication of histone lysine methylation in the mechanisms of regulation of these genes in OA is still unknown.

In order to achieve efficient therapeutic interventions with potentially less adverse effects, it is essential to understand the mechanism of regulation of these genes. Therefore, for the first

time, this study will investigate the role of histone lysine methylation in the regulation of these inflammatory genes during OA.

**Hypothesis**: COX-2/mPGES-1/$PGE_2$ and iNOS/NO pathways play a critical role in OA development and progression. The upregulation of these genes induces the inflammatory and catabolic response in OA resulting in enhanced destruction of the articular cartilage. Our hypothesis is that histone lysine methylation/demethylation contributes to the regulation of COX-2, mPGES-1, and iNOS expression.

**Aim**: In this regard, we designed this study to investigate the implication of H3K4 and H3K9 in the regulation of COX-2, mPGES1, and iNOS genes in IL-1-induced human OA chondrocytes. We aimed also study whether KMTs and HDMs contribute to theses mechanisms.

The results of this study will provide to our knowledge mechanisms of regulation of these enzymes in OA cartilage tissue and may therefore represents novel therapeutic interventions for OA and possibly other arthritic conditions.

# Résumé: Article 1

**Objectif** : Étudier le rôle de la méthylation de la lysine K4 au niveau de l´histone H3 (H3K4) dans l´expression de la cyclooxygenase-2 (COX-2) et l´oxyde nitrique inductible (iNOS) induite par l´interleukine-1β (IL-1β) dans les chondrocytes arthrosiques humains.

**Méthodes** : Les chondrocytes ont été stimulées par l´IL-1β, et l'expression de l'ARN messager et de protéines de COX-2 et iNOS a été évaluée par la PCR en temps réel (analyse inverse d'amplification en chaîne par polymerase –transcriptase) et transfert de Western, respectivement. La méthylation de H3K4 et le recrutement des histones methyltransférases SET-1A et MLL-1 au niveau des promoteurs COX-2 et iNOS ont été évalué en utilisant des analyses d'immunoprécipitation de la chromatine. Le rôle du SET-1A a été évalué en utilisant à la fois l´inhibiteur méthylthioadénosine (MTA) et des siRNA pour une répression génique. Les niveaux de SET-1A ont été déterminés par des expériences d´immuhistochimie.

**Résultats** : l´induction de l´expression de la COX-2 et iNOS par l´IL-1β est associée à la di- et triméthylation des histones H3K4 au niveau des promoteurs COX-2 et iNOS. Ces changements corrèlent temporairement avec le recrutement de l´histone méthyltransférase SET-1A, ce qui suggère une implication de SET-1A dans ces modifications. Le traitement avec le MTA inhibe la méthylation des histones H3K4 ainsi que l’expression de COX-2 et iNOS induite par l’IL-1β. De même, la répression de SET-1A par les siRNAs inhibe la méthylation des histones H3K4 induite par l’IL-1β au niveau des promoteurs COX-2 et iNOS ainsi que l’expression de COX-2 et iNOS. Enfin, on a trouvé que l’expression de SET-1A est élevée dans le cartilage OA versus le cartilage normal.

**Conclusion** : Ces résultats indiquent que la méthylation des histones H3K4 par SET-1A contribue à l'expression de COX-2 et iNOS induite par l'IL-1β. Ceci suggère que cette voie peut représenter une stratégie thérapeutique potentielle pour le traitement de l'OA et autres maladies arthritiques.

# Paper 1

**Contribution of H3K4 Methylation by SET-1A to Interleukin-1–Induced Cyclooxygenase 2 and Inducible Nitric Oxide Synthase Expression in Human Osteoarthritis Chondrocytes**

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All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Fahmi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. El Mansouri, Fahmi Ezzahra.

Acquisition of data. El Mansouri, Chabane, Zayed, Fahmi.

Analysis and interpretation of data. El Mansouri, Chabane, Zayed, Kapoor, Benderdour, Martel-Pelletier, Pelletier, Duval, Fahmi

**ABSTRACT**

**Objective:** To investigate the role of histone H3 lysine 4 (H3K4) methylation in interleukin-1β (IL-1β)–induced cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in human osteoarthritic (OA) chondrocytes.

**Methods:** Chondrocytes were stimulated with IL-1, and the expression of iNOS and COX-2 messenger RNA and proteins was evaluated by real-time reverse transcriptase–polymerase chain reaction analysis and Western blotting, respectively. H3K4 methylation and the recruitment of the histone methyltransferases SET-1A and MLL-1 to the iNOS and COX-2 promoters were evaluated using chromatin immunoprecipitation assays. The role of SET-1A was further evaluated using the methyltransferase inhibitor 5'-deoxy-5'-(methylthio)adenosine (MTA) and gene silencing experiments. SET-1A level in cartilage was determined using immunohistochemistry.

**Results:** The induction of iNOS and COX-2 expression by IL-1 was associated with H3K4 di- and trimethylation at the iNOS and COX-2 promoters. These changes were temporally correlated with the recruitment of the histone methyltransferase SET-1A, suggesting an implication of SET-1A in these modifications. Treatment with MTA inhibited IL-1–induced H3K4 methylation as well as IL-1–induced iNOS and COX-2 expression. Similarly, SET-1A gene silencing with small interfering RNA prevented IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 expression. Finally, we showed that the level of SET-1A expression was elevated in OA cartilage as compared with normal cartilage.

**Conclusion:** These results indicate that H3K4 methylation by SET-1A contributes to IL-1–induced iNOS and COX-2 expression and suggest that this pathway could be a potential target for pharmacologic intervention in the treatment of OA and possibly other arthritic diseases.

## INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and is a leading cause of disability in the elderly (1). Clinical manifestations of OA may include pain, stiffness, and reduced joint motion. Pathologically, OA is characterized by progressive degeneration of articular cartilage, synovial inflammation, and subchondral bone remodeling. These processes are thought to be largely mediated through excess production of proinflammatory and catabolic mediators. Among these mediators, interleukin-1β (IL-1β) has been demonstrated to be predominantly involved in the initiation and progression of the disease (2-4). One mechanism through which IL-1 exerts its effects is by up-regulating the expression of genes encoding for inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) and the release of nitric oxide (NO) and prostaglandin E2 ($PGE_2$) (2-4).

The production of NO is an important component in the pathogenesis of OA, and increased levels of nitrite/nitrate have been observed in the synovial fluid and serum of arthritis patients (5). The biosynthesis of NO is catalyzed by a group of enzymes known as NO synthases (NOS). There are 3 distinct NOS. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while the iNOS is expressed following stimulation with a variety of inflammatory agents, such as endotoxins or cytokines (6). NO participates in the pathogenesis of arthritis by inducing chondrocyte apoptosis (7) and matrix metalloprotease (MMP) production (8) and by suppressing the synthesis of collagen and proteoglycans (9). In addition, NO enhances the production of inflammatory cytokines (5) and $PGE_2$ (10) and reduces the synthesis of endogenous IL-1 receptor antagonist (IL-1Ra) (11). The important role of NO in the pathogenesis of OA is further supported by the finding that selective inhibition of iNOS in an experimental model of OA reduces the structural changes and the expression of several inflammatory and catabolic factors

(12).

Like NO, $PGE_2$ contributes to the pathogenesis of arthritis through several mechanisms, including upregulation of MMP (13) and IL-1 (14) production, enhancement of the degradation of cartilage matrix components (15), and promotion of chondrocyte apoptosis (16). In addition, $PGE_2$ mediates pain responses and potentiates the effects of other mediators of inflammation (17). COX is the key enzyme in the biosynthesis of $PGE_2$, and 2 isoforms have been identified. COX-1 is constitutively expressed in a wide variety of tissues and is responsible for housekeeping functions. In contrast, COX-2 is undetectable in most normal tissues, but is rapidly induced by growth factors and proinflammatory cytokines, such as IL-1 and tumor necrosis factor α (TNFα) (17). COX-2 expression and activity are increased in cartilage from OA patients, and this is thought to play a primary role in the pain and inflammation associated with the disease (18). Moreover, COX-2 inhibitors have been extensively used in the treatment of OA.

Posttranslational modifications of nucleosomal histones, including acetylation, methylation, phosphorylation, and sumoylation, play important roles in the regulation of gene transcription through remodeling of chromatin structure (19,20). To date, histone acetylation and methylation are among the most studied and best characterized modifications. Unlike acetylation, which is generally associated with transcriptional activation, histone-lysine methylation is associated with either gene activation or repression, depending on the specific residue modified (21-24). For instance, methylation of the histone H3 lysine-4 (H3K4) is commonly associated with transcriptional activation, whereas methylation of H3K9 correlates with transcriptional repression (2124). In addition, H3K4 can be mono-, di-, or trimethylated, with the di- and trimethylated forms being the most positively correlated with transcriptional activation (21-24).

H3K4 methylation is catalyzed by the action of a family of histone methyltransferases (HMTs) that share a conserved SET domain, which was named for its presence in diverse Drosophila chromatin regulators: Su(var)3–9, Enhancer of Zeste (E[z]) and Trithorax (Trx). Several specific H3K4 methyltransferases have been identified and characterized, including SET-1A, SET-1B, and 4 mixed-lineage leukemia (MLL) family HMTs (MLL-1, MLL-2, MLL-3, and MLL-4). Among them, only SET-1A and MLL-1 are able to di- and trimethylate H3K4 (25-28).

Although the induction of iNOS and COX-2 expression by IL-1 in chondrocytes is well documented (2-4), the role of histone methylation in their regulation remains undefined. In this study, we examined the role of H3K4 methylation in IL-1–induced iNOS and COX-2 expression in chondrocytes.

## MATERIALS AND METHODS

### Reagents and antibodies

Recombinant human IL-1 was obtained from Genzyme. Aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, and 5_-deoxy-5_-(methylthio)adenosine (MTA) were from Sigma-Aldrich Canada. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal calf serum (FCS), and TRIzol reagent were from Invitrogen. Antibodies against iNOS and COX-2 were purchased from Cayman Chemical. Antibody against β-actin was from Santa Cruz Biotechnology. Antibodies against histone H3 and against mono-, di-, and trimethylated H3K4 were from Upstate/Millipore. Anti–SET-1A and anti–MLL-1 antibodies were from Bethyl Laboratories. Polyclonal rabbit anti-mouse IgG coupled with horseradish peroxidase (HRP) and polyclonal goat anti-rabbit IgG coupled with HRP were from Pierce.

### Specimen selection and chondrocyte culture

Normal human cartilage (from femoral condyles) was obtained at necropsy, within 12 hours of death, from donors who had no history of arthritic diseases (n =14; mean $\pm$ SD age 59 $\pm$ 13 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically. Only those found to be free of alterations by both methods were further processed. OA cartilage was obtained from patients undergoing total knee replacement surgery (n = 48; mean $\pm$ SD age 63 $\pm$ 19 years). All OA patients were diagnosed with knee OA according to the criteria developed by the American College of Rheumatology (29). At the time of surgery, the patients had symptomatic disease requiring medical treatment in the

form of nonsteroidal anti-inflammatory drugs or selective COX-2 inhibitors. Patients who had received intraarticular injection of steroids were excluded.

The Clinical Research Ethics Committee of Notre- Dame Hospital approved the study protocol and the use of human articular tissues. Informed consent was obtained from each donor or from an authorized third party.

Chondrocytes were released from cartilage by sequential enzymatic digestion, as previously described (30). Cells were seeded at 3.5 x $10^5$/well in 12-well culture plates (Costar)

or at 6–7 x $10^5$/well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 hours. Cells were washed and incubated for an additional 24 hours in DMEM containing 0.5% FCS before stimulation with IL-1.

**Protein extraction and Western blot analysis**

Histones were extracted from the cells as previously described (31). Briefly, cells were washed with phosphate buffered saline (PBS) and lysed with ice-cold lysis buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 1.5 mM PMSF, 1 mM Na3VO4, and 10 µg/ml of aprotinin, leupeptin, and pepstatin. Sulfuric acid was added to a concentration of 0.2N, and the resultant supernatant was collected and dialyzed twice against 0.1M acetic acid and 3 times against sterile water. Whole-cell lysates were prepared and analyzed as previously described (30).

**RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) analysis**

Total RNA from stimulated chondrocytes was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To remove contaminating DNA, the isolated RNA

was treated with RNase-free DNase I (Ambion). The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes), dissolved in diethylpyrocarbonate-treated-$H_2O$ and stored at –80°C until used. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Fermentas) as detailed in the manufacturer's guidelines. One-fiftieth of the RT reaction was analyzed by real-time PCR as described below. The following primers were used: for iNOS, 5´-ACATTGATGAGAAGCTGTCCCAC-3´ (sense) and 5´-CAAAGGCTGTGAGTCCTGCAC-3´ (antisense); for COX-2, 5´-TGTGTTGACATCCAGATCAC-3´ (sense) and 5´-ACATCATGTTTGAGCCCTGG-3´ (antisense); and for GAPDH, 5´-CAGAACATCATCCCTGCCTCT-3´ (sense) and 5´-GCTTGACAAAGTGGTCGTTGAG-3´ (antisense).

**Real-time PCR analysis**

Real-time PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM sense and antisense primers, 25 _l of SYBR Green Master Mix (Qiagen), and 0.5 units of uracil N-glycosylase (UNG; Epicentre Technologies). After incubation at 50°C for 2 minutes (UNG reaction), and at 95°C for 10 minutes (UNG inactivation and activation of the AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 seconds at 95°C for denaturation and 1 minute for annealing and extension at 60°C). Incorporation of SYBR Green dye into the PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems), allowing determination of the threshold cycle (Ct), at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with 1 peak, indicating the specificity of the amplification. A Ct value was obtained

from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative messenger RNA (mRNA) expression in chondrocytes was determined using the the ΔΔCt method, as detailed in the manufacturer's guidelines (Applied Biosystems). A ΔCt value was first calculated by subtracting the Ct value for the housekeeping gene GAPDH from the Ct value for each sample. A ΔΔCt value was then calculated by subtracting the ΔCt value of the control (unstimulated cells) from the ΔCt value of each treatment. Fold changes compared with the control were then determined by raising 2 to the –ΔΔCt power. Each PCR reaction generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCR reactions. Each PCR was performed in triplicate on 2 separate occasions for each independent experiment.

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP experiments were performed according to the ChIP protocol provided by Upstate/Millipore and previously published protocols (32,33). The primer sequences used were as follows: for the iNOS promoter, 5′-ATGAACTGCCACCTTGGACT-3′ (sense) and 5′-GTTTTCGACTCGCTACAAAGTT-3′ (antisense); for the COX-2 promoter, 5′-AAGACATCTGGCGGAAACC-3′ (sense) and 5′-ACAATTGGTCGCTAACCGAG-3′ (antisense); and for the MMP-13 promoter, 5′-ATTTTGCCAGATGGGTTTTG-3′(sense) and 5′-CTGGGGACTGTTGTCTTTCC-3_ (antisense).

**RNA interference**

Specific small interfering RNA (siRNA) for SET-1A, MLL-1, or scrambled control was obtained from Dharmacon. Chondrocytes were seeded in 6-well plates at 6 _ 105 cells/well and incubated for 24 hours. Cells were transfected with 100 nM siRNA using HiPerFect Transfection Reagent (Qiagen) following the manufacturer's recommendations. The medium was changed 24 hours later, and the cells were incubated for an additional 24 hours before stimulation with 100 pg/ml of IL-1 for 2 hours or 20 hours.

**Immunohistochemistry**

Cartilage specimens were processed for immunohistochemistry as previously described (30). The specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 _m) of paraffin-embedded specimens were deparaffinized in toluene and dehydrated in a graded series of ethanol. The specimens were then preincubated with chondroitinase ABC (0.25 units/ml in PBS, pH 8.0) for 60 minutes at 37°C, followed by a 30-minute incubation with 0.3% Triton X-100 at room temperature. Slides were then washed in PBS followed by 2% hydrogen peroxide/methanol for 15 minutes. They were further incubated for 60 minutes with 2% normal serum (Vector) and overlaid with primary antibody for 18 hours at 4°C in a humidified chamber. The antibody was a rabbit polyclonal anti-human SET-1A (Bethyl Laboratories), which was used at 10 μg/ml.

Each slide was washed 3 times in PBS, pH 7.4, and stained using the avidin–biotin complex method (Vectastain ABC kit; Vector). The color was developed with 3,3′-diaminobenzidine (Vector) containing hydrogen peroxide. The slides were counterstained with eosin. The specificity of staining was evaluated by using antibody that had been preadsorbed (1

hour at 37°C) with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200–1250 of human SET-1A (Bethyl), and by substituting nonimmune rabbit IgG (Chemicon) for the primary antibody at the same concentration. The evaluation of positive-staining chondrocytes was performed using our previously published method (30). For each specimen, 6 microscopic fields were examined under 40X magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated, and the results were expressed as the percentage of chondrocytes staining positive (cell score).

**Statistical analysis**

Results of the real-time PCR and ChIP analyses are expressed as the mean ± SD, and statistical significance was assessed by Student's 2-tailed t-test. Results of the immunohistochemical analyses are expressed as the median (range), and statistical analysis was performed using the nonparametric Mann-Whitney U test. P values less than 0.05 were considered statistically significant.

## RESULTS

### Induction of iNOS and COX-2 expression by IL-1 in cultured human chondrocytes

We first examined the effect of IL-1 on iNOS and COX-2 mRNA expression in human OA chondrocytes. Cells were stimulated with IL-1 for various time periods, and the levels of iNOS and COX-2 mRNA were determined by real-time RT-PCR. IL-1–induced changes in gene expression were expressed as the fold change over control (untreated cells) after normalization to the internal control GAPDH. Treatment with IL-1 (100 pg/ml) induced iNOS Mrna expression in a time-dependent manner. Levels of mRNA for iNOS started to gradually increase at 2 hours after stimulation to reach a peak at 6 hours. With the longer incubation times, we observed a gradual decline in the mRNA levels starting at 8 hours. Similarly, treatment with IL-1 led to a time-dependent increase in COX-2 mRNA (data available upon request from the author). COX-2 mRNA was rapidly and significantly induced at 1 hour following stimulation with IL-1, reached the maximum at 6 hours and started to decrease at 8 hours (data available upon request from the author).

Next, we performed Western blot analysis to determine whether changes in mRNA levels were paralleled by changes in iNOS and COX-2 protein levels. Consistent with its effects on iNOS and COX-2 mRNA, IL-1 induced the expression of iNOS and COX-2 protein in a time-dependent manner (data available upon request from the author). By 4 hours poststimulation, iNOS protein levels were significantly increased. These levels were further increased up to 8 hours and remained elevated until 24 hours. The induction of COX-2 protein expression occurred earlier (2 hours poststimulation) than iNOS protein expression, reached the maximum at 8 hours, and remained constant until 24 hours (data available upon request from the corresponding author).

These results confirmed that IL-1 is a potent inducer of iNOS and COX-2 expression in chondrocytes (2-4).

**IL-1 enhancement of H3K4 dimethylation and trimethylation, but not monomethylation, at the iNOS and COX-2 promoters**

Recent studies have provided abundant evidence indicating that histone methylation plays an important role in the regulation of gene expression and that H3K4 di- or trimethylation is strongly correlated with transcriptional activation when found at promoter sites (21-24). To determine whether H3K4 methylation might be involved in IL-1–induced COX-2 and iNOS transcription, we performed ChIP assays. Chondrocytes were stimulated with IL-1 for various time periods, and formaldehyde cross-linked DNA–proteins were immunoprecipitated using antibodies specific for mono-, di-, or trimethylated H3K4. Control Ig and no antibodies were used as controls. DNA isolated from the immunoprecipitates was analyzed by real-time PCR using specific primers spanning the transcription start site (+1), the TATA box, and the binding sites of several transcription factors in the proximal regions of the iNOS (bp -256 to +24), COX-2 (bp -270 to +7), and MMP-13 (bp -220 to +7) promoters.

As shown in Figures 1A–C, treatment with IL-1 enhanced the levels of di- and trimethylated H3K4 at the iNOS and COX-2 promoters in a time-dependent manner. In contrast, the levels of H3K4 methylation at the MMP-13 promoter remained unchanged, indicating that the observed modifications at the iNOS and COX-2 promoters are specific. The levels of di- and trimethylated H3K4 at the iNOS and COX-2 promoters were significantly increased at 0.5 hours after IL-1 stimulation, reached a maximum at 1–2 hours, and returned to a near basal level by 8 hours, whereas the level of monomethylated H3K4 did not appreciably change following IL-1

stimulation (Figure 1A). No immunoprecipitable COX-2 or iNOS promoter DNA was detected with the control Ig and with the no antibodies controls (data not shown). The induction of H3K4 di- and trimethylation by IL-1 at the iNOS and COX-2 promoter paralleled the increased transcription of iNOS and COX-2 (data available upon request from the author), suggesting that enhanced H3K4 di- and trimethylation may play a key role in IL-1–induced iNOS and COX-2 expression.

To determine whether the changes in H3K4 methylation seen at the iNOS and COX-2 promoters were not secondary to events causing global H3K4 methylation, we investigated the effect of IL-1 on global H3K4 methylation in chondrocytes. Cells were stimulated with IL-1 for various time periods, histones were extracted, and the levels of H3K4 methylation were measured by Western blot analysis using specific antibodies for mono-, di-, or trimethylated H3K4. As shown in Figure 1, the levels of mono-, di-, or trimethylated H3K4 were high in untreated chondrocytes, and treatment with IL-1 did not significantly change these levels. These results indicate that the alterations in H3K4 methylation seen in the ChIP assays were not due to nonspecific global histone modifications and are specific for the iNOS and COX-2 promoters.

**IL-1–enhanced recruitment of the H3K4 methyltransferase SET-1A to the iNOS and COX-2 promoters**

SET-1A and MLL-1 are H3K4-specific methyltransferases capable of di- and trimethylating H3K4 (25-28). Hence, we performed ChIP assays in IL-1–treated chondrocytes to examine the recruitment of SET-1A and MLL-1 to the iNOS and COX-2 promoters. As shown in Figure 2A, treatment with IL-1 resulted in sustained recruitment of SET-1A at the promoters of iNOS and COX-2. In contrast, IL-1 had no effect on the recruitment of MLL-1 to either promoter

(Figure 2B), suggesting that the H3K4 methyltransferase that is involved in H3K4 methylation at the iNOS and COX-2 promoters is SET-1A. No immunoprecipitable COX-2 or iNOS promoter DNA was detected with the control Ig and no antibodies controls (data not shown). Strikingly, SET-1A was recruited to the promoters of iNOS and COX-2 when the levels of di- and trimethylated H3K4 increased (Figures 1B and C), and this recruitment correlated well with the increased transcription of iNOS and COX-2 (data available upon request from the author). Immunoblotting of cell lysates did not show any changes in the levels of SET-1A protein (Figures 2C and D), suggesting that the enhanced recruitment of SET-1A to the iNOS and COX-2 promoters seen with the ChIP assays was not due to increased expression of SET-1A protein. Together, these data suggest an implication of SET-1A in IL-1–induced H3K4 methylation and iNOS and COX-2 expression.

**MTA reduction of IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 protein expression**

The previous data suggest that SET-1A is involved in H3K4 di- and trimethylation and may contribute to the induction of iNOS and COX-2 expression. To test this, we first investigated the effect of MTA, a histone methyltransferase inhibitor (34), on IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters. Chondrocytes were pretreated with increasing concentrations of MTA for 1 hour, before stimulation with IL-1 for an additional 1.5 hours. The status of H3K4 methylation at the iNOS and COX-2 promoters was evaluated using ChIP assays with antibodies against mono-, di-, and trimethylated H3K4. We found that MTA treatment dose-dependently decreased IL-1–induced di- and tri-methylation of H3K4 (Figures 3B and C), which had increased during transcriptional activation. However, MTA treatment did not change the level of H3K4

monomethylation, which was not affected during transcriptional activation of iNOS and COX-2 (Figure 3A).

Next, we investigated the effect of MTA on IL-1–induced iNOS and COX-2 protein expression. Chondrocytes were pretreated with increasing concentrations of MTA for 1 hour, before stimulation with IL-1 for 20 hours. As shown in Figure 3D, treatment with MTA dose-dependently suppressed the IL-1–induced iNOS and COX-2 expression. This reduction was coincident with the decline in H3K4 methylation following treatment with MTA. The inhibition observed was not a result of reduced cell viability, as confirmed by MTT assay (data not shown). These findings strongly suggest that the SET-1A methyltransferase activity contributes to IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters as well as iNOS and COX-2 expression.

**Prevention of IL-1–induced H3K4 methylation at the iNOS and COX-2 promoter as well as iNOS and COX-2 protein expression by siRNA-mediated depletion of SET-1A**

To confirm the role of SET-1A, we examined the impact of its silencing by siRNA on IL-1–induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters. Chondrocytes were transfected with the scrambled control siRNA, siRNA for SET-1A, or siRNA for MLL-1, and after 48 hours of transfection, the cells were stimulated or were not stimulated with IL-1 for 1.5 hours. SET-1A knockdown reduced IL-1– induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters (Figure 4A). In contrast, MLL-1 silencing had no effect (Figure 4B). These results support the notion that SET-1A mediates IL-1–induced H3K4 di- and trimethylation at the iNOS and COX-2 promoters.

Moreover, SET-1A silencing also markedly suppressed IL-1–induced iNOS and COX-2 expression (Figure 5A), whereas MLL-1 knockdown did not affect iNOS and COX-2 expression (Figure 5B). Taken together, these data strongly suggest that SET-1A contributes to IL-1–induced iNOS and COX-2 expression through up-regulation of H3K4 di- and trimethylation.

**Elevated SET-1A protein levels in OA cartilage**

To determine whether SET-1A levels were altered under conditions of OA, we performed immunohistochemical analysis on cartilage sections from OA patients and normal donors. As shown in Figures 6A and B, the immunostaining for SET-1A was located in the superficial and upper intermediate zones. Statistical evaluation of the cell score revealed a significant increase in the number of chondrocytes staining positive for SET-1A in OA cartilage (n = 14) as compared with normal cartilage (n = 14). The specificity of the staining was confirmed using an antibody that had been preadsorbed (1 hour at 37°C) with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200– 1250 of human SET-1A (Figure 6C) or nonimmune control IgG (data not shown).

**DISCUSSION**

The present study is the first to show that the induction of iNOS and COX-2 expression by IL-1 is accompanied by increased H3K4 di- and trimethylation at the iNOS and COX-2 promoters. These modifications correlated with the recruitment of SET-1A to the iNOS and COX-2 promoters. Blocking methyltransferase activity or reducing the expression level of SET-1A abrogated IL-1–induced H3K4 methylation, as well as iNOS and COX-2 expression. Taken together, these results indicate that H3K4 methylation by SET-1A participates in IL-1–induced iNOS and COX-2 expression and suggest that this pathway may represent a therapeutic target in OA.

Our finding that IL-1–induced transcriptional activation of iNOS and COX-2 is associated with H3K4 di- and trimethylation is consistent with recent studies showing that transcriptional activation of a number of inducible inflammatory genes correlates with increased methylation of H3K4 at target promoters. For instance, the induction of monocyte chemotactic protein 1 (MCP-1) and TNFα by the proinflammatory astrocytederived protein S100B or TNFα in THP-1 cells is strongly associated with H3K4 methylation (35). Similarly, H3K4 methylation was reported to be increased at the promoters of TNFα and iNOS upon stimulation of the murine macrophage cell line RAW 264.7 and Kupffer cells with lipopolysaccharide (36). Increased methylation of H3K4 was also observed at promoters of MMP-1 in phorbol 12-myristate 13-acetate–treated T98G cells (31), IL-6 and MCP-1 in TNFα-treated vascular smooth cells (37), class II major histocompatibility complex in IFNγ-treated colon 26 cells (38), and IL-17 in CD4+ T helper cells treated with a combination of transforming growth factor β1 and IL-6 (39).

Several histone methyltransferases have been identified, among which SET-1A and MLL play dominant roles in the di- and trimethylation of H3K4 (25-28). Therefore, we examined the

effect of IL-1 on the recruitment of SET-1A and MLL-1 to the iNOS and COX-2 promoters. ChIP results demonstrated that IL-1 enhanced the recruitment of SET-1A to the iNOS and COX-2 promoters, whereas the level of MLL-1 was not affected. Interestingly, the recruitment of SET-1A to the iNOS and COX-2 promoters was concomitant with the appearance of di- and trimethylated H3K4 at these sites, indicating that H3K4 methylation in response to IL-1 could be mediated by SET-1A. It is noteworthy that SET-1A appeared to be maintained at the iNOS and COX-2 promoters when the levels of di- and trimethylated H3K4 decreased. This suggests that specific H3K4 demethylases or inhibitors of SET-1A activity are recruited to the iNOS and COX-2 promoters and contribute to decreased H3K4 di- and tri-methylation.

The correlation between SET-1A recruitment and H3K4 di- and trimethylation suggests that SET-1A is implicated in these modifications and that H3K4 methylation by SET-1A contributes to IL-1–induced iNOS and COX-2 expression. Indeed, we found that MTA, a protein methyltransferase inhibitor (34), prevented IL-1–induced H3K4 methylation at the iNOS and COX-2 promoters and suppressed IL-1–induced iNOS and COX-2 protein expression. Moreover, the siRNAmediated knockdown of SET-1A diminished the IL-1–induced di- and trimethylation of H3K4 and blocked the expression of iNOS and COX-2. Collectively, these results suggest that SET-1A contributes to IL-1–induced iNOS and COX-2 expression by enhancing H3K4 methylation.

In addition to H3K4, methylation of H3K9, H3K27, H3K36, and H3K79 is also known to modulate gene transcription. Like H3K4, methylation of H3K36 and H3K79 is associated with transcriptional activation, whereas methylation of H3K9 and H3K27 is associated with transcriptional repression (21–24). Although the role of these modifications in the effects of IL-1

is still unknown, we cannot exclude the possibility that they may also be involved in iNOS and COX-2 transcription.

We also demonstrated that the levels of SET-1A were increased in OA cartilage as compared with normal cartilage. Interestingly, OA chondrocytes in these zones were shown to express elevated levels of iNOS and COX-2 (15, 40, 41). These data, together with the implication of SET-1A in the transcriptional activation of iNOS and COX-2 in cultured chondrocytes, suggest that increased expression of SET-1 may be among the mechanisms that mediate the up-regulation of iNOS and COX-2 OA cartilage.

There are a number of mechanisms by which H3K4 methylation could mediate the transcriptional activation of iNOS and COX-2. One possibility is that H3K4 methylation promotes transcriptional activation by enhancing the acetylation of neighboring histones by histone acetyltransferases and by preventing the binding of the NuRD deacetylase complex (42,4 3). Alternatively, methylated H3K4 may serve as a docking site for the recruitment of chromatin-remodeling complexes such as the nucleosome remodeling factor (44), and the chromo–ATPase/helicase–DNA binding domain 1 (45). Finally, H3K4 methylation can activate transcription by facilitating the assembly of active transcription complexes. Indeed, the basal transcription complex TFIID can directly bind to the trimethylated H3K4 via the plant homeodomain finger of its subunit TAF-3 (46), and the methyltransferase SET-1A was reported to associate with RNA polymerase II (47).

In addition to histones, nonhistone proteins, especially transcription factors, have been identified as targets for methylation (48). In this context, Yang et al (49) reported that methylation of the RelA subunit of NF-κB, which is critically involved in the induction of iNOS and COX-2 in chondrocytes, by the lysine methyltransferase SET-7/9 inhibits NF-κB activity by inducing the

degradation of RelA. On the other hand, Li et al (35) reported that SET-7/9 associates with the NF-κB p65 and up-regulates the expression of a subset of NF-κB target genes. Whether methylation of NF-κB contributes to the transcriptional activation of iNOS and COX-2 genes in chondrocytes remains to be determined.

In conclusion, the present study provides, to our knowledge, the first evidence that H3K4 methylation by SET-1A contributes to the induction of iNOS and COX-2 expression by IL-1. SET-1A may therefore be a novel therapeutic target for osteoarthritis and other human conditions associated with increased expression of iNOS and COX-2.

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**FIGURE LEGENDS**

**Figure 1. Effect of interleukin-1 (IL-1) on histone H3K4 methylation at the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) promoters.** Confluent chondrocytes were treated with 100 pg/ml of IL-1 for the indicated time periods. Chromatin immunoprecipitation (ChIP) assays coupled with real-time polymerase chain reaction were performed using antibodies specific for **A,** monomethylated H3K4 (H3K4me1), **B,** dimethylated H3K4 (H3K4me2), and **C,** trimethylated H3K4 (H3K4me3). The results (shown at the top) are expressed as the fold change in H3K4 mono-, di-, and trimethylation at the iNOS, the COX-2, or the matrix metalloprotease 13 (MMP-13) promoter relative to untreated cells. Values are the mean $\pm$ SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P<0.05$ versus unstimulated cells, by Student's 2-tailed *t*-test. In addition, confluent chondrocytes were treated as indicated, and histones were extracted and immunoblotted for mono-, di-, and trimethylated H3K4 as well as unmodified H3. Shown at the bottom are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results.

**Figure 2. Effect of IL-1 on the recruitment of SET-1A and mixed-lineage leukemia 1 (MLL-1) to the iNOS and COX-2 promoters.** A and B, Confluent chondrocytes were treated with 100 pg/ml of IL-1 for the indicated time periods, and ChIP assays were performed using specific anti–SET-1A **(A)** and anti–MLL-1 **(B)** antibodies. Results are expressed as the fold change in SET-1A and MLL-1 binding to the iNOS and COX-2 promoters relative to untreated cells. Values are the mean $\pm$ SD of 4 independent experiments. * = $P<0.05$ versus unstimulated cells, by Student's 2-tailed t-test. **C** and **D**, Confluent chondrocytes were treated as indicated, and cell lysates were prepared and analyzed for SET-1A **(C)** and MLL-1 **(D)** protein expression by Western blotting.

Blots were then stripped and reprobed with a specific anti–β-actin antibody. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

**Figure 3. Effect of 5'-deoxy-5'-(methylthio)adenosine (MTA) on IL-1–induced H3K4 methylation and COX-2 and iNOS protein expression.** Chondrocytes were pretreated for 1 hour with control vehicle (N,N-dimethylformamide; maximum concentration 0.05%) or with increasing concentrations of MTA prior to stimulation with 100 pg/ml of IL-1 for 1.5 hours **(A–C)** or 20 hours **(D)**. A–C, ChIP assays, coupled with real-time polymerase chain reaction analyses, were performed using antibodies specific for mono-, di-, and trimethylated H3K4. Results are expressed as the fold change in H3K4 mono-, di-, and trimethylation at the iNOS and COX-2 promoters relative to untreated cells. Values are the mean ± SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P<0.05$ versus IL-1–treated cells, by Student's 2-tailed t-test. D, Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. Blots were then stripped and reprobed with a specific anti–β-actin antibody. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

**Figure 4. Effect of SET-1A silencing on IL-1–induced H3K4 methylation at the COX-2 and iNOS promoters.** Chondrocytes were transfected with 100 nM SET-1A small interfering RNA (siRNA) **(A)**, mixed-lineage leukemia 1 (MLL-1) siRNA **(B)**, or control (CTL) scrambled siRNA. At 48 hours posttransfection, cells were left untreated or were treated for 1.5 hours with 100 pg/ml of IL-1. ChIP assays, coupled with real-time polymerase chain reaction analyses, were performed using antibodies specific for dimethylated (top) or trimethylated (middle) H3K4. Results are

expressed as the fold change in H3K4 di- and trimethylation at the iNOS and COX-2 promoters relative to untreated cells. Values are the mean $\pm$ SD of 4 independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on 2 separate occasions. * = $P<0.05$ versus nontransfected cells stimulated with IL-1, by Student's 2-tailed t-test. Knockdown of SET-1A and MLL-1 was confirmed by Western blotting using antibodies specific for SET-1A and MLL-1 (bottom). Blots were then stripped and reprobed with a specific anti–β-actin antibody. See Figure 1 for other definitions.

**Figure 5. Effect of SET-1A silencing on IL-1–induced COX-2 and iNOS protein expression.** Chondrocytes were transfected with 100 nM SET-1A small interfering RNA (siRNA) **(A)**, mixed-lineage leukemia 1 (MLL-1) siRNA **(B)**, or control (CTL) scrambled siRNA. At 48 hours posttransfection, cells were left untreated or were treated for 20 hours with 100 pg/ml of IL-1. Cell lysates were prepared and analyzed for iNOS and COX-2 protein expression by Western blotting. Blots were then stripped and reprobed with specific anti–β-actin or anti–COX-2 antibodies. SET-1A and MLL-1 silencing was confirmed by Western blotting using antibodies specific for SET-1A and MLL-1. Shown are representative blots from 1 experiment of 4 independent experiments performed, all of which yielded similar results. See Figure 1 for other definitions.

**Figure 6. Expression of SET-1A protein in normal and osteoarthritic (OA) cartilage. A** and **B**, Knee cartilage specimens from a normal donor (A) and a patient with OA (B) were immunostained for SET-1A protein. C, Knee cartilage specimen from a patient with OA was treated with anti–SET-1A antibody that had been preadsorbed with a 20-fold molar excess of the protein fragment corresponding to amino acids 1200–1250 of human SET-1A (control for staining specificity). Representative sections are shown. Original magnification X 100. D, The percentage

of chondrocytes expressing SET-1A in normal and OA cartilage samples was determined. Results are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. * = $P<0.05$ versus normal cartilage, by Mann-Whitney U test.

**Figure 1**

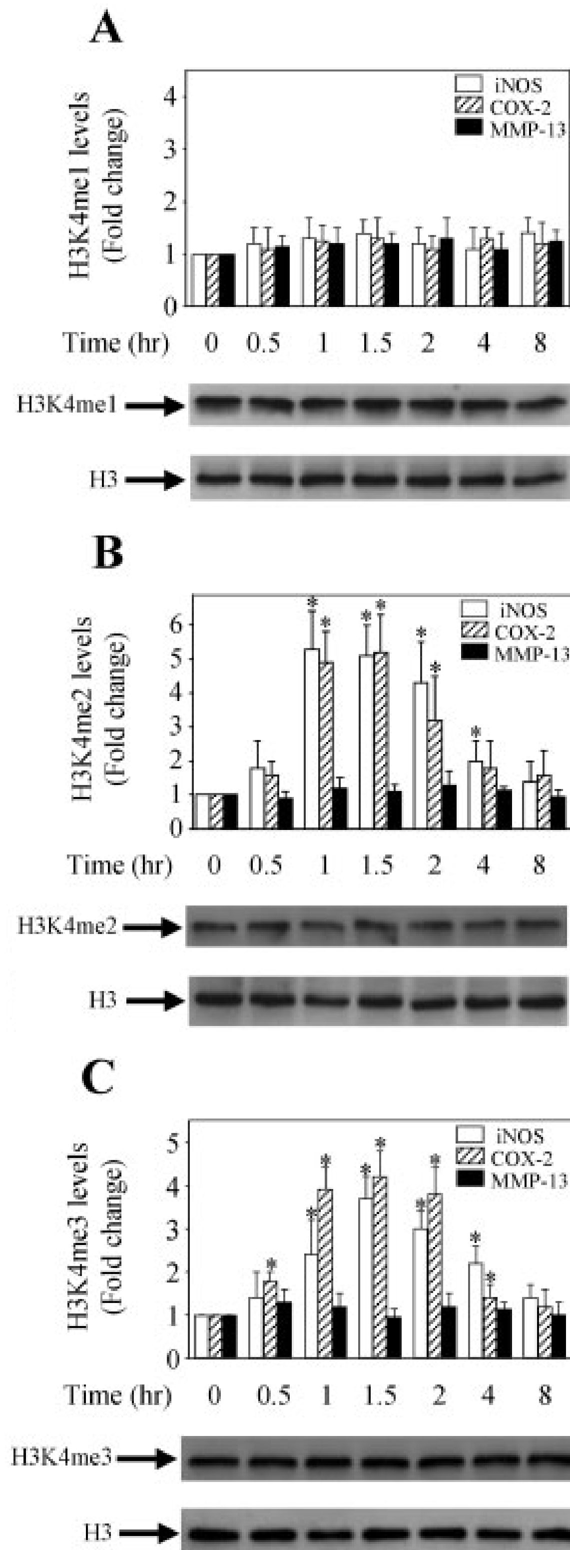


**Figure 2**

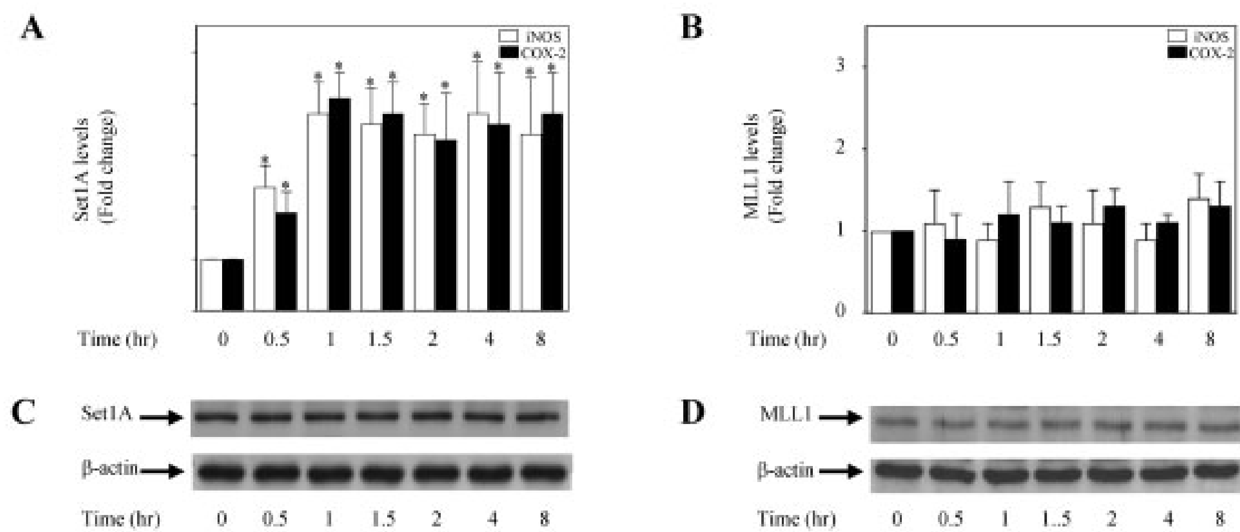


**Figure 3**

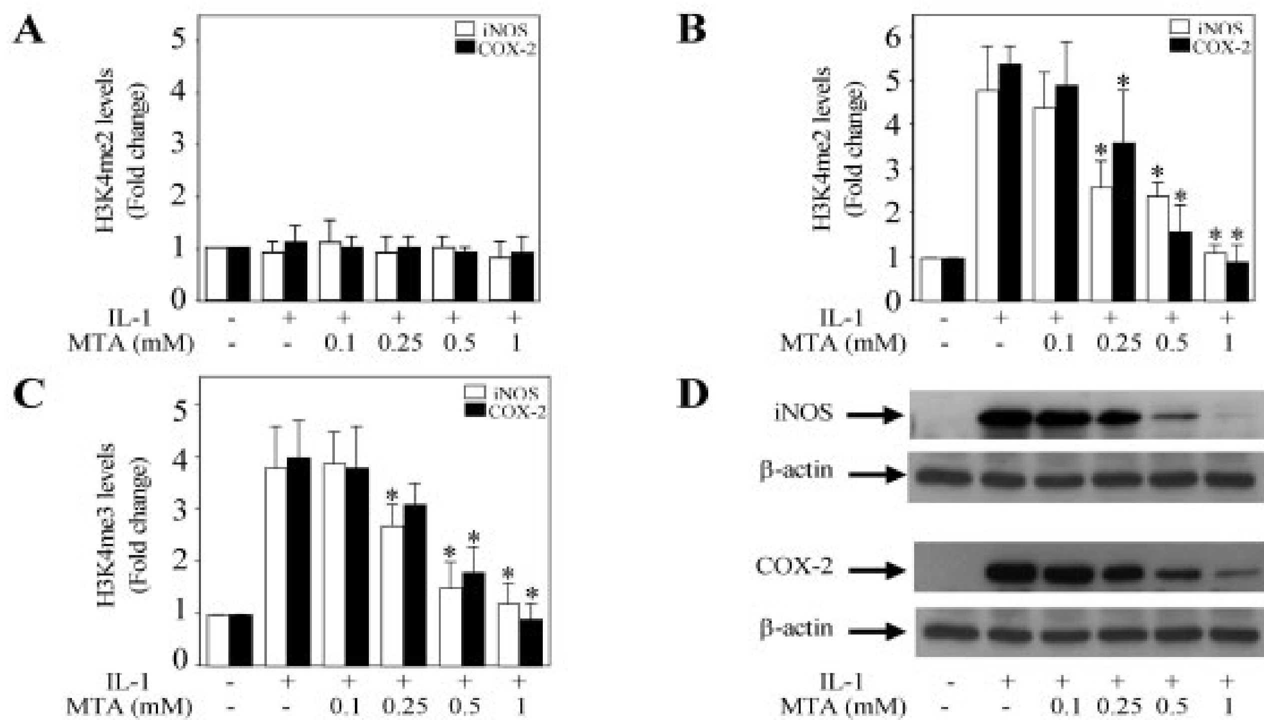
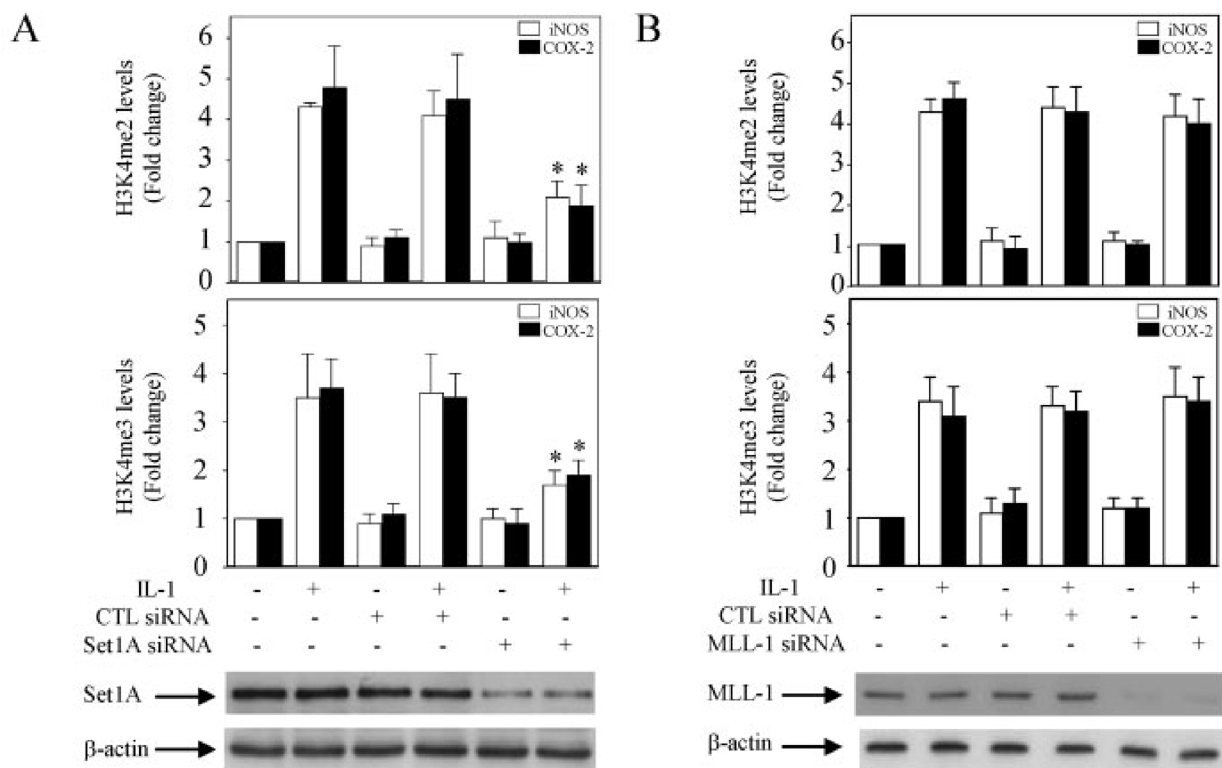


**Figure 4**



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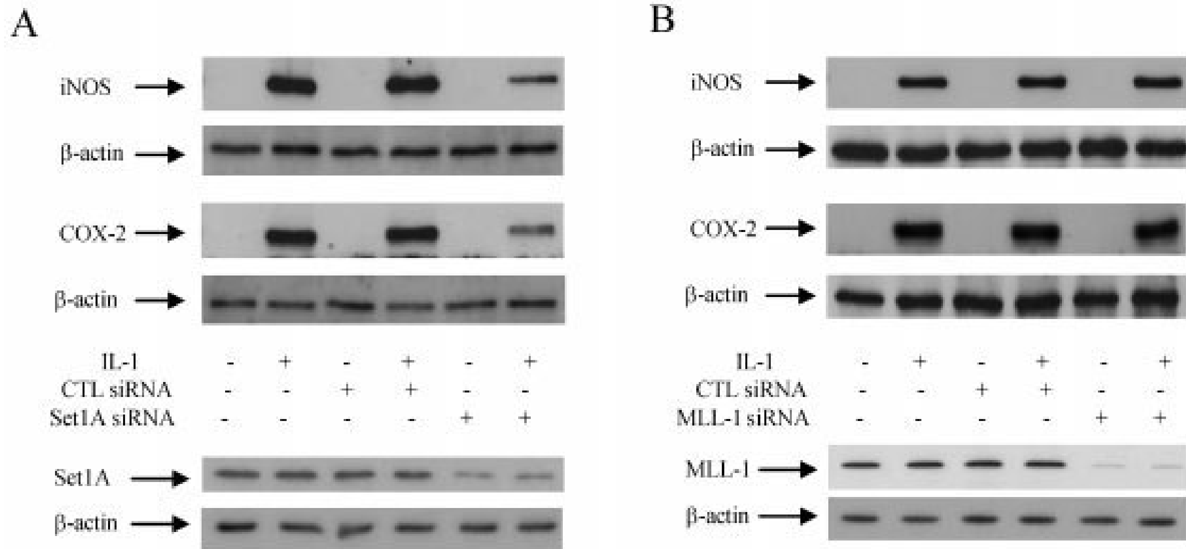
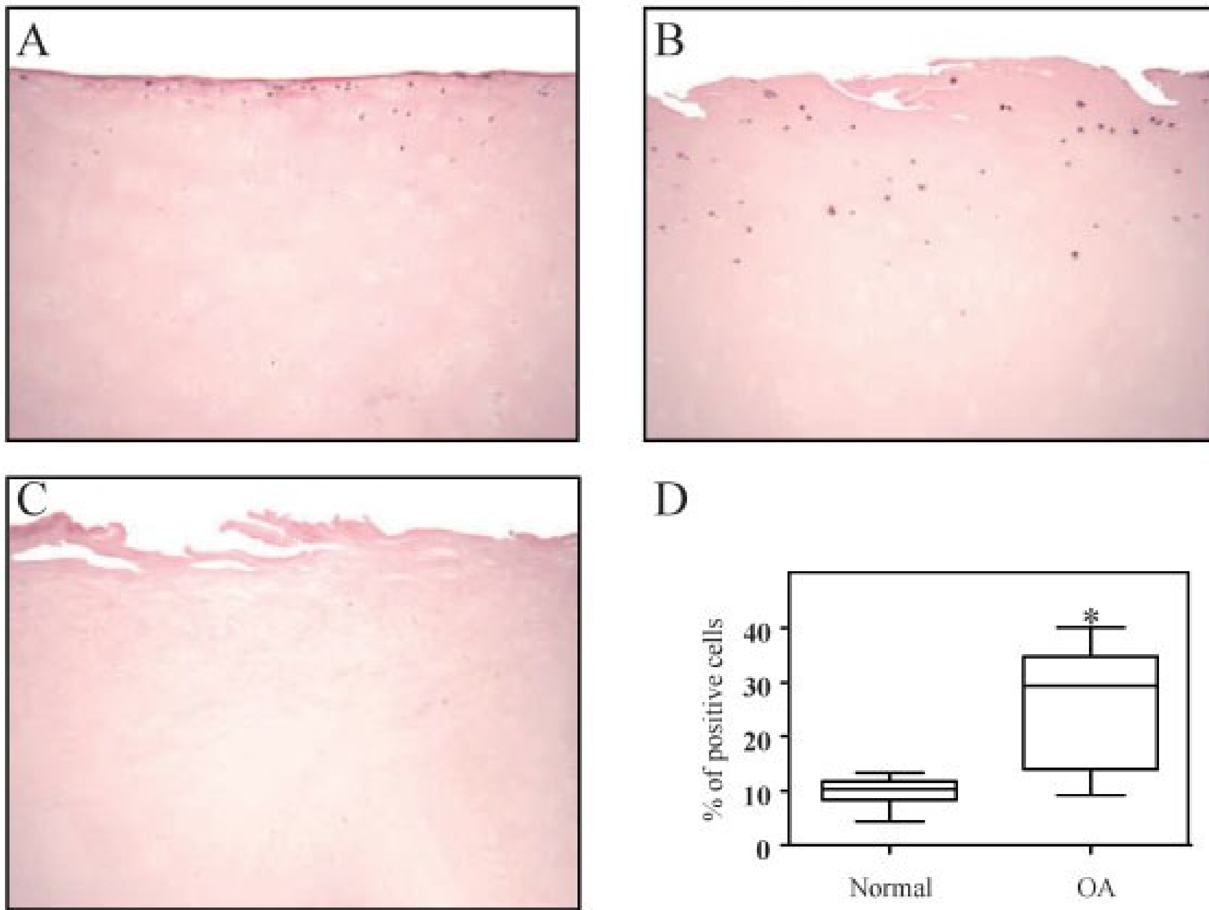


**Figure 6**



# Résumé: Article 2

**Objectif** : La prostaglandine E synthase microsomale-1 (mPGES-1) catalyse l´étape finale de la biosynthèse de la $PGE_2$, un médiateur principal dans la physiopathologie de l´OA. La méthylation des histones joue un rôle très important dans la régulation des gènes. Dans cette étude, on a examiné le rôle de la méthylation des histones H3K9 dans l´expression de la mPGES-1 induite par l´interleukine-1β (IL-1β) dans les chondrocytes humains.

**Méthodes** : Les chondrocytes ont été stimulées par l´IL-1β, et l'expression de l'ARN messager et des protéines de la mPGES-1 a été évaluée par la PCR en temps réel et transfert de Western, respectivement. La méthylation de H3K9 et le recrutement de l´histone deméthylase LSD1 au niveau du promoteur de mPGES-1 a été évalué par des analyses d'immunoprécipitation de la chromatine. Le rôle du LSD1 a été ensuite évalué en utilisant à la fois des inhibiteurs pharmacologiques, le tranylcypromine et le pargyline, et des siRNA pour une répression génique. Les niveaux de LSD1 ont été déterminés par la PCR en temps réel et l´immuhistochimie.

**Résultats** : l´induction de l´expression de la mPGES-1 par l´IL-1β est associée à une diminution des niveaux des histones H3K9 mono- et diméthylés au niveau du promoteur de la mPGES-1. Ces changements corrèlent temporairement avec le recrutement de l´histone deméthylase LSD-1. Les traitements avec le tranylcypromine et le pargyline, des inhibiteurs potentiels de LSD1, inhibent la deméthylation des histones H3K9 induite par l´IL-1β au niveau du promoteur de la mPGES-1 ainsi que l’expression de mPGES-1. De plus, la répression de LSD1 par Des siRNAs inhibe la deméthylation des histones H3K9 ainsi que l’expression de mPGES-1, ce qui suggère que l´expression de mPGES-1 induite par l´IL-1β est médiée par LSD1 via la deméthylation de H3K9.

Enfin, on a trouvé que l'expression de LSD1 est élevée dans le cartilage OA versus le cartilage normal.

**Conclusion** : Ces résultats indiquent que la deméthylation des histones H3K9 par LSD-1 contribue à l'expression de mPGES-1 induite par l'IL-1β. Ceci suggère que cette voie peut représenter une intervention thérapeutique potentielle pour le traitement de l'OA et autres maladies arthritiques.

# Paper 2

**LSD1-Mediated Demethylation of Histone H3 Lysine 9 Contributes to Interleukin 1-Induced Microsomal Prostaglandin E Synthase-1 Expression in Human Osteoarthritic Chondrocytes**

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I and Dr. Fahmi designed the study, carried out most of the experiments, analyzed data, and prepared the manuscript. H.A. contributed to the design study, the analysis and the interpretation of data. All authors contributed to the analysis and interpretation of data and read and approved the final manuscript.

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## ABSTRACT

**Objective:** Microsomal prostaglandin E synthase-1 (mPGES-1) catalyzes the terminal step in the biosynthesis of $PGE_2$, a critical mediator in the pathophysiology of osteoarthritis (OA). Histone methylation plays an important role in epigenetic gene regulation. In this study, we investigated the roles of histone H3 (H3K9) methylation in interleukin-1β (IL-1)-induced mPGES-1 expression in human chondrocytes.

**Methods**: Chondrocytes were stimulated with IL-1 and the expression of mPGES-1 mRNA was evaluated using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). H3K9 methylation and the recruitment of the histone demethylase lysine-specific demethylase 1 (LSD1) to the mPGES-1 promoter were evaluated using chromatin immunoprecipitation (ChIP) assays. The role of LSD1 was further evaluated using the pharmacological inhibitors, tranylcypromine and pargyline, and small interfering RNA (siRNA)-mediated gene silencing. The LSD1 level in cartilage was determined using RT-PCR and immunohistochemistry.

**Results:** The induction of mPGES-1 expression by IL-1β correlated with decreased levels of mono- and dimethylated H3K9 at the mPGES-1 promoter. These changes were concomitant with the recruitment of the histone demethylase LSD1. Treatment with tranylcypromine and pargyline, potent inhibitors of LSD1, prevented IL-1β-induced H3K9 demethylation at the mPGES-1 promoter and mPGES-1 expression. Consistently, LSD1 gene silencing with siRNA prevented IL-1β-induced H3K9 demethylation and mPGES-1 expression, suggesting that LSD1 mediates IL-1β-induced mPGES-1 expression via H3K9 demethylation. We show that the level of LSD1 was elevated in OA compared to normal cartilage.

**Conclusion:** These results indicate that H3K9 demethylation by LSD1 contributes to IL-1β-induced mPGES-1 expression and suggest that this pathway could be a potential target for pharmacological intervention in the treatment of OA and possibly other arthritic conditions.

## INTRODUCTION

Osteoarthritis (OA) is the most common joint disease and is a leading cause of disability in developed countries and throughout the world (1). Pathologically, OA is characterized by progressive degeneration of articular cartilage, synovial inflammation, and subchondral bone remodeling (2, 3). These processes are thought to be mediated largely through excess production of proinflammatory and catabolic mediators, among which prostaglandin $E_2$ ($PGE_2$) is considered a critical mediator in the pathophysiology of the disease (2, 3). The beneficial effects of nonsteroidal anti-inflammatory drugs (NSAIDs), the most widely prescribed drugs worldwide, are attributed to inhibition of $PGE_2$ production

$PGE_2$ is the most abundant prostaglandin in the skeletal system (4). Excessive levels of $PGE_2$ have been reported in serum and synovial fluid from patients with OA and rheumatoid arthritis (RA) (5). $PGE_2$ contributes to the pathogenesis of OA through several mechanisms, including induction of cartilage proteoglycan degradation (6), upregulation of matrix metalloproteinase (MMP) activity and production (7, 8), and promotion of chondrocyte apoptosis (9). $PGE_2$ is also a well-known mediator of pain and neoangiogenesis (10).

The biosynthesis of $PGE_2$ requires two enzymes acting sequentially. Cyclooxygenase (COX) enzymes convert arachidonic acid (AA) into $PGH_2$ which is in turn isomerized to $PGE_2$ by PGE synthase (PGES) enzymes. Two isoforms of the COX enzyme, COX-1 and COX-2, have been identified. COX-1 is expressed in most tissues and is responsible for physiological production of PGs. COX-2, in contrast, is almost undetectable under physiologic conditions, but is strongly induced in response to proinflammatory and mitogen stimuli (11).

At least three distinct PGES isoforms have been cloned and characterized, including cytosolic PGES (cPGES), microsomal prostaglandin E synthase 1 (mPGES-1), and mPGES-2 (12). cPGES, also called the heat shock protein-associated protein p23, is constitutively and ubiquitously expressed with, and is functionally coupled with COX-1, thus promoting immediate production of $PGE_2$ (13). In contrast, mPGES-1 which was originally named membrane-bound gluthatione S-transferase-1-like-1 (MGST-L-1), is markedly upregulated by inflammatory or mitogenic stimuli and is functionally coupled with COX-2, thus promoting delayed $PGE_2$ production (14). mPGES-2 is constitutively expressed in various cells and tissues and can be coupled with both COX-1 and COX-2 (15).

We and others have previously shown that expression of mPGES-1, but not cPGES, is elevated in articular tissues taken from patients with OA (16, 17) and patients with RA (18), as well as in the rat adjuvant induced arthritis model (19), suggesting that aberrant expression of this enzyme could contribute to the pathogenesis of arthritis. Importantly, mPGES-1 deficient mice exhibit reduced inflammatory and pain responses and were protected against experimental arthritis (20-22) and bone loss (23).

The pro-inflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been shown to induce mPGES-1 expression in several tissues and cell types including chondrocytes (16,17,24). However, little is known about the molecular mechanisms underlying the regulation of mPGES-1 expression.

Posttranslational modifications of nucleosomal histones, including acetylation, methylation, phosphorylation, and sumoylation, play important roles in the regulation of gene transcription through remodeling of chromatin structure (25, 26). To date, histone acetylation and

methylation are among the most intensively studied and best characterized modifications of nucleosomal histones. Methylation occurs on both lysine (K) and arginine residues. In histone H3, different lysine residues (K4, K9, K27, K36 and K79) can be methylated. Unlike acetylation, which is generally associated with transcriptional activation, histone lysine methylation is associated with either gene activation or repression, depending on the specific residue modified (27-29).

Methylation of the histone H3 lysine 4 (H3K4), H3K36 and H3K79 is generally associated with transcriptionally active genes, whereas methylation of H3K9, and H3K20 is associated with transcription silencing (27-29). Moreover, lysine methylation can exist in three different states (mono-, di-, and trimethylated), which may bring about additional regulatory complexity (27-29).

Lysine methylation is controlled by the opposing activities of lysine methyltransferases (KMTs) and lysine demethylases (KDMs) (27-29). There are two classes of lysine demethylases: the amine oxidase-related enzymes and the Jumonji (JMJ) C-terminal domain-containing enzymes. Lysine specific demethylase 1 (LSD1), also known as KDM1, p110b, BHC110, or NPAO, was the first histone demethylase discovered. It belongs to the superfamily of the flavin adenine dinucleotide (FAD)-dependent amine oxidases (30). Researchers in several studies demonstrated that LSD1 modulates gene expression through demethylation of either H3K4 (31-34) or H3K9 (30, 35-38).

In the present study, we demonstrated that the induction of mPGES-1 expression by IL-1β was associated with decreased levels of mono- and dimethylated H3K9 at the mPGES-1 promoter. These changes correlated with the recruitment of the histone demethylase LSD1. Both pharmacological inhibition of LSD1 and small interfering RNA (siRNA) knockdown prevented

IL-1β-induced H3K9 demethylation at the mPGES-1 promoter as well as concomitant mPGES-1 protein expression. Furthermore, we show that the level of LSD1 expression was elevated in OA cartilage. These data suggest that modulation of LSD1 in the joint may have therapeutic potential in the treatment of OA and possibly in other conditions associated with increased mPGES-1 expression and $PGE_2$ production.

## MATERIALS AND METHODS

### Ethical approval

The Clinical Research Ethics Committee of Notre Dame Hospital approved the study protocol and the use of human articular tissues. Informed consent was obtained from each donor or from a family member.

### Reagents and antibodies

Recombinant human (rh) IL-1β was obtained from Genzyme (Cambridge, MA, USA). Aprotinin, leupeptin, pepstatin, phenylmethylsulphonyl fluoride (PMSF), sodium orthovanadate ($Na_3VO_4$), pargyline and tranylcypromine were from Sigma-Aldrich Canada (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, foetal calf serum (FCS) and Trizol reagents were supplied by Life Technologies (Burlington, ON, Canada). Antibodies against mPGES-1 and cPGES-1 were purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against histone H3, mono-, di- and trimethylated H3K9, as well as mono-, di- and trimethylated H3K4, were purchased from EMD Millipore (Billerica, MA, USA). Antibodies against LSD1/KDM1, JMJD1A/JHDM2A/KDM3A, KIAA1718/JHDM1D/KDM7A, PHF8/JHDM1F/KDM7B and PHF2/JHDM1E/KDM7C were obtained from Abcam (Cambridge, MA, USA). Polyclonal rabbit anti-mouse immunoglobulin G (IgG) antibody, coupled with horseradish peroxidase (HRP) and polyclonal goat anti-rabbit IgG antibody with HRP, were obtained from Thermo Fisher Scientific (Rockford, IL, USA).

**Specimen selection and chondrocyte culture**

Human normal cartilage was obtained at necropsy, within 12 hours of death from donors with no history of arthritic disease (n = 13, mean ± SD age: 56 ± 14 years). To ensure that only normal tissue was used, cartilage specimens were thoroughly examined both macroscopically and microscopically, and only those with neither alteration were further processed. Human OA cartilage was obtained from patients undergoing total knee replacement (n = 47, mean ± SD age: 67 ± 20 years). All OA patients were diagnosed on the basis of criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (39). At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of NSAIDs or selective COX-2 inhibitors. Patients who had received intraarticular injection of steroids were excluded.

For Chondrocytes cultures, cartilage from tibial plateaus and femoral condyles was used. For immunohistochemical studies, only cartilage from femoral condyles was used. Chondrocytes were released from cartilage by sequential enzymatic digestion as previously described (40, 41). Cells were seeded at $3.5x10^5$ cells per well in 12-well culture plates (Costar, Corning, NY, USA) or at $6-7x10^5$ cells per well in 6-well culture plates in DMEM supplemented with 10% FCS, and cultivated at 37°C for 48 hours. Cells were washed and incubated for an additional 24 hours in DMEM containing 0.5% FCS, before stimulation with IL-1β alone or in the presence of pharmacological inhibitors of LSD1. Only first passaged chondrocytes were used.

**Western blot analysis**.

Chondrocytes were lysed in ice-cold lysis buffer (0.1% SDS, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1 mM PMSF, 10 µg/ml concentrations each of aprotinin, leupeptin and pepstatin, 1 mM $Na_3VO_4$, and 1 mM NaF). Lysates were sonicated on ice and boiled at 95 °C for 5 minutes and centrifuged at 12,000 rpm for 15 minutes. The protein concentration of the supernatant was determined using the bicinchoninic acid protein assay (Thermo Fisher Scientific). Twenty micrograms of total cell lysate was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitrocellulose membrane (Bio-Rad, Mississauga, ON, Canada). After blocking the cell lysate in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) non-fat dry milk, blots were incubated overnight at 4°C with the primary antibody and washed with a mixture of Tris-buffered saline, pH 7.5 and 0.1% Tween 20. The blots were then incubated with HRP-conjugated secondary antibody (Thermo Fisher Scientific), washed again, incubated with SuperSignal Ultra Chemiluminescent substrate (Thermo Fisher Scientific) and exposed to KODAK X-OMAT XAR autoradiography film (Eastman Kodak Ltd, Rochester, NY, USA).

**RNA extraction and reverse transcriptase-polymerase chain reaction**

Total RNA from stimulated chondrocytes was isolated using the TRIzol® reagent (Life Technologies) according to the manufacturer's instructions. To remove contaminating DNA, isolated RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA). The RNA was quantitated using the RiboGreen RNA assay kit (Molecular Probes, Eugene, OR, USA), dissolved

in diethylpyrocarbonate (DEPC)-treated-$H_2O$ and stored at -80°C until use. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, ON, Canada) as detailed in the manufacturer's guidelines. One fiftieth of the reverse transcriptase reaction was analyzed by real-time PCR as described below. The following primers were used: mPGES-1: sense 5'-GAAGAAGGCCTTTGCCAAC-3' and antisense 5'-GGAAGACCAGGAAGTGCATC-3'; MMP-13: sense 5'-TGAAGCAGTGAAGAAGGAC-3' and antisense 5'-CTGCTTTCTCTTGTAGAATC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-CAGAACATCATCCCTGCCTCT-3' and antisense 5'-GCTTGACAAAGTGGTCGTTGAG -3'.

**Real-time PCR**

Real-time PCR analysis was performed in a total volume of 50 µl containing template DNA, 200 nM of sense and antisense primers, 25 µl of SYBR® Green master mix (Qiagen, Mississauga, ON, Canada) and uracil-N-glycosylase (UNG, 0.5 Unit, Epicentre Technologies, Madison, WI, USA). After incubation at 50°C for 2 minutes (UNG reaction) and at 95°C for 10 minutes (UNG inactivation and activation of the AmpliTaq Gold enzyme (Life Technologies)), the mixtures were subjected to 40 amplification cycles (15 seconds at 95°C for denaturation and 1 minute for annealing and extension at 60°C). Incorporation of SYBR® Green dye into PCR products was monitored in real time using a GeneAmp 5700 Sequence detection system (Applied Biosystems, Foster City, CA, USA) to enable us to determine the threshold cycle ($C_T$) at which exponential amplification of PCR products began. After PCR, dissociation curves were generated

with one peak, which indicated the specificity of the amplification. We obtained a $C_T$ value from each amplification curve using the software provided by the manufacturer (Applied Biosystems).

Relative mRNA expression in chondrocytes was determined using the $\Delta\Delta C_T$ method, as detailed in the manufacturer's guidelines (Applied Biosystems). A $\Delta C_T$ value was first calculated by subtracting the $C_T$ value for the housekeeping gene GAPDH from the $C_T$ value for each sample. A $\Delta\Delta C_T$ value was then calculated by subtracting the $\Delta C_T$ value of the control (unstimulated cells) from the $\Delta C_T$ value of each treatment. Fold changes compared with the control were then determined by raising 2 to the $-\Delta\Delta C_T$ method. Each PCR generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis of test PCRs. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

**Chromatin immunoprecipitation (ChIP) assay**

The chromayin immuoprecipitation (ChIP) experiments were performed according to the ChIP protocol provided by EMD Millipore. The data are expressed as percentages of control (unstimulated cells) or fold changes relative to control conditions (unstimulated cells) calculated using the $\Delta\Delta$ $C_T$ method as detailed in the manufacturer' s guidelines and according to previously published methods (42,43). A $\Delta$ $C_T$ value was first calculated by subtracting the $C_T$ value for the input DNA from the $C_T$ value for the immunoprecipitated sample (ChIP analysis). A $\Delta\Delta$ $C_T$ value was then calculated by subtracting the $\Delta$ $C_T$ value of the control from the $\Delta$ $C_T$ value of each treatment. Fold changes compared with the control (unstimulated cells) were then calculated using the $2^{-\Delta\Delta C_T}$ method. The following primer sequences used were: mPGES-1 promoter sense 5'-

GTTTGAGGATTTGCCTGGAA -3’ and antisense 5’-CTGCTCATCACCAGGCTGT-3’; and MMP-13 promoter sense 5’-ATTTTGCCAGATGGGTTTTG-3’ and antisense 5’-CTGGGGACTGTTGTCTTTCC-3’. Primers were tested in a conventional PCR using genomic DNA as the template and checked on an agarose gel to ensure that the primer PCRs resulted in a single band of predicted size.

**RNA interference**

Specific siRNA for LSD1 and scrambled control siRNA were obtained from Dharmacon Inc (Lafayette, CO, USA). Chondrocytes were seeded in 6-well plates at $6.10^5$ cells/well and incubated for 24 hours. The cells were then transfected with 100 nM of siRNA using the HiPerFect Transfection Reagent (Qiagen) following the manufacturer’s recommendations. The medium was changed 24 hours later, and then the cells were incubated for an additional 24 hours before stimulation with 100 pg/ml IL-1β for 2 or 20 hours.

**Immunohistochemistry**

Cartilage specimens were processed for immunohistochemistry as previously described (40). The specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) of paraffin-embedded specimens were deparaffinized in toluene and dehydrated in a graded series of ethanol. The specimens were then preincubated with chondroitinase ABC (0.25 U/ml in phosphate-buffered saline (PBS) pH 8.0) for 60 minutes at 37˚C, followed by a 30-minute incubation with Triton X-100 (0.3%) at room temperature. Slides were then washed in PBS, followed by 2% hydrogen peroxide⁄methanol, for 15 minutes. They were further incubated for 60

minutes with 2% normal serum (Vector Laboratories, Burlingame, CA, USA) and overlaid with primary antibody for 18 hours at 4°C in a humidified chamber. The antibody used was a rabbit polyclonal anti-human Set1A Ab (Bethyl Laboratories, Montgomery, TX, USA), used at 10 µg/ml. Each slide was washed 3 times in PBS pH 7.4 and stained using the avidin-biotin complex method (VECTASTAIN ABC kit; Vector Laboratories). The color was developed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories) containing hydrogen peroxide. The slides were counterstained with eosin. The specificity of staining was evaluated using preadsorbed Ab (1 hour, 37°C) antibody with a 20-fold molar excess of protein fragment corresponding to amino acids 834-852 of human LSD1 (Abcam), and by substituting the primary antibody with non-immune rabbit IgG (Chemicon, Temecula, CA, USA), which was used at the same concentration as the primary antibody. The evaluation of positive-staining chondrocytes was performed using our previously published method (40). For each specimen, six microscopic fields were examined under 40X magnification. The total number of chondrocytes and the number of chondrocytes staining positive were evaluated, and results expressed as the percentage of chondrocytes staining positive (cell score).

**Flavin adenosine dinucleotide quantification**

Intracellular FAD was measured using the FAD Assay and Deproteinizing Sample Preparation Kit (BioVision Research Products, Mountain View, CA, USA).

**Statistical analysis**

Data are expressed as the mean ± SD. For chondrocyte culture studies, statistical significance was assessed by the one-way analysis of variance, followed by the Bonferroni

multiple-comparison post hoc test. The comparison of LSD1 expression in human and OA cartilage was analyzed using the two-tailed Student's t -test. P-values less than 0.05 were considered statistically significant. All statistics were generated using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

## RESULTS

### IL-1β decreased H3K9 mono- and di-, but not trimethylation, at the mPGES-1 promoter

First, we examined the effect of IL-1β on mPGES-1 mRNA expression in human OA chondrocytes. The cells were stimulated with IL-1β for various time periods, and the levels of mPGES-1 were determined by real-time RT-PCR. IL-1β-induced changes in mPGES-1 gene expression are expressed as fold changes over control (untreated cells) after normalizing to the internal control GAPDH. As shown in Fig. 1A, treatment with IL-1β (100 pg/ml) induced mPGES-1 mRNA expression in a time-dependent manner. mPGES-1 mRNA levels started to increase gradually at 2 hours after stimulation, were significantly increased by 4 hours poststimulation, increased further at 8 hours and peaked at 24 hours. With the longer incubation times, we observed a gradual decline in the mRNA levels starting at 36 hours poststimulation. These results confirmed previously published data showing that IL-1β is a potent inducer of mPGES-1 expression in human OA chondrocytes (16,17,24). The pattern of MMP-13 gene expression in response to IL-1β was similar to that of mPGES-1 and hence was used as a control comparator.

In numerous recent studies, researchers have demonstrated that transcriptional activation of a number of genes is associated with changes in the methylation state of H3K9, a critical epigenetic mark for gene silencing (30,35-38). To determine whether the induction of mPGES-1 by IL-1β was associated with changes in the levels of H3K9 methylation at the mPGES-1 promoter, we performed ChIP assays using specific antibodies for mono-, di- or trimethylated H3K9.

Chondrocytes were stimulated with IL-1β for different time periods, and ChIP enriched DNA was analyzed by real-time PCR using specific primers spanning the transcription start site (+1), the TATA box and several transcription factors' binding sites in the proximal regions of the mPGES-1 promoter (bp -259 to +10) and MMP-13 (bp –220 to + 7) promoters. Control Ig and no Ab were used as controls.

As shown in Fig. 1B and 1C, treatment with IL-1β decreased the levels of mono- and dimethylated H3K9 at the mPGES-1 promoter in a time-dependent manner. Their levels began to decrease at 2 hours after stimulation with IL-1β, persisted through 12 to 24 hours and then increased at 48 hours. In contrast, the levels of mono- and dimethylated H3K9 at the MMP-13 promoter did not appreciably change under the same conditions (during the treatment) (Fig. 1B and 1C), indicating that the observed modifications at the mPGES-1 promoter were specific. There were no significant changes in the levels of trimethylated H3K9 at the mPGES-1 or MMP-13 promoter at any time analyzed (Fig. 1D). No immunoprecipitable mPGES-1 promoter DNA was detected with the control Ig or the no-Ab controls (data not shown). The decrease in the levels of mono- and dimethylated H3K9 at the mPGES-1 promoter in response to IL-1□ paralleled transcriptional induction of mPGES-1 (Fig. 1A), suggesting that diminished levels of mono- and dimethylated H3K9 may play a key role in IL-1β-induced mPGES-1 expression.

Next we investigated the effect of IL-1β on global H3K9 methylation in chondrocytes. Cells were stimulated with IL-1β for various time periods, and the levels of H3K9 methylation were measured by Western blot analysis using specific antibodies for mono-, di- or trimethylated H3K9. Fig. 1B to 1D demonstrate that the levels of mono-, di- or trimethylated H3K9 were high in untreated chondrocytes, and treatment with IL-1β did not significantly change these levels.

These results indicate that the alterations in H3K9 methylation seen in ChIP assays were not due to nonspecific global histone modifications and are specific to the proximal region of the mPGES-1 promoter.

**IL-1β enhanced the recruitment of LSD1 to the mPGES-1 promoter**

Since the induction of mPGES-1 expression by IL-1β correlated with reduced H3K9 methylation, we hypothesized that IL-1β may mediate this effect by inducing the recruitment of H3K9 demethylases to the mPGES-1 promoter. To test this hypothesis, we first examined whether chondrocytes express LSD1/KDM1 (30), JMJD1A/JHDM2A/KDM3A (44), /KIAA1718 JHDM1D/KDM7A (45), PHF8/JHDM1F/KDM7B (46) and PHF2/JHDM1E/ /KDM7C (47). We focused on these proteins because they can demethylate H3K9me1 and H3K9me2, but not H3K9me3. As shown in Fig. 2A, Western blot analyses with nuclear extracts from four different chondrocyte populations indicated the presence of the five demethylases in all the cell populations tested. Hence, we performed ChIP assays to examine whether IL-1β would modulate the recruitment of these demethylases to the mPGES-1 promoter. The results demonstrated that LSD1 was present at the proximal region of the mPGES-1 promoter (Fig. 2B), and that treatment with IL-1β enhanced its level in a time-dependent manner. The level started to increase significantly at 2 hours after IL-1β stimulation, reached a maximum at 12 to 24 hours and then decreased by 48 hours. With regard to JMJD1A/JHDM2A/KDM3A, KIAA1718/JHDM1D/KDM7A, PHF8/JHDM1F/KDM7B and PHF2/JHDM1E/KDM7C, their binding signal at the mPGES-1 promoter was undetectable, the Ct values were equivalent to the non-template control (Ct $\geq$38) and IL-1β treatment had no significant effect on their recruitment at the mPGES-1 promoter. No

immunoprecipitable mPGES-1 promoter DNA was detected with the control Ig and no-Ab controls (data not shown).

Treatment with IL-1β did not affect the levels of LSD1 protein expression (Fig. 2C), suggesting that the recruitment of LSD1 to the mPGES-1 promoter seen with the ChIP assays was specific and was not due to increased expression of LSD1 protein.

The pattern of LSD1 levels at the mPGES-1 promoter correlated with decreased H3K9 methylation and is strikingly similar to transcriptional induction of mPGES-1 expression. This strongly suggests that IL-1β-induced mPGES-1 expression involves the recruitment of LSD1 and H3K9 methylation.

**Inhibition of LSD1 activity prevented IL-1β-induced H3K9 demethylation at the mPGES-1 promoter and mPGES-1 protein expression**

LSD1 demethylates lysine residue through a FAD-dependent reaction (30,48). This reaction is inhibited by monoamine oxidase inhibitors such as pargyline and tranylcypromine (35,49,50). Therefore, we investigated their effects on IL-1-induced H3K9 demethylation at the mPGES-1 promoter and on mPGES-1 protein expression. Chondrocytes were pretreated with increasing concentrations of pargyline or tranylcypromine for 1 hour before stimulation with IL-1β for an additional 8 or 24 hours. The levels of H3K9me1 and H3K9me2 and at the mPGES-1 promoter were analyzed using ChIP assays with Abs against mono-, and dimethylated H3K9.

We found that treatment with either pargyline (Fig. 3A and 3B) or tranylcypromine (Fig. 3D and 3E) dose-dependently prevented IL-1□-reduced H3K9me1 and H3K9me2 levels, which

decreased during transcriptional activation. However, pargyline and tranylcypromine treatment did not change the level of H3K9me3, which was not affected during IL-1β -induced mPGES-1 transcription (data not shown). Accordingly, pretreatment with either pargyline or tranylcypromine dose-dependently suppressed IL-1β-induced mPGES-1 protein expression (Fig. 3C and 3F). The inhibition observed was not a result of reduced cell viability, wich was confirmed in a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These findings strongly suggest that the LSD1 activity contributes to IL-1β-induced H3K9 demethylation at the mPGES-1 promoter and to mPGES-1 protein expression.

**LSD1 silencing with siRNA suppressed IL-1β-induced H3K9 demethylation at the mPGES-1 promoter and IL-1β-induced mPGES-1 protein expression**

To further define the role of LSD1, we determined the effect of its silencing by siRNA on IL-1β-induced H3K9 demethylation at the mPGES-1 promoter and on mPGES-1 protein expression. Chondrocytes were transfected with the scrambled control siRNA or siRNA for LSD1, and, after 48 hours of transfection, the cells were stimulated or not with IL-1β for 8 or 24 hours.

As shown in Figure 4, transfection with LSD1 siRNA prevented IL-1β-mediated diminished levels of H3K9me1 and H3K9me2 at the mPGES-1 promoter (Fig. 4A and 4B). Furthermore, LSD1 silencing markedly suppressed IL-1β-induced mPGES-1 expression (Fig. 4B). In contrast, transfection with scrambled control siRNA had no effect on either H3K9 demethylation or mPGES-1 expression. LSD1 protein levels were reduced by as much as 75-80%, confirming gene silencing (Fig. 4A and 4B). Together, these data strongly suggest that LSD1

contributes to IL-1β-induced mPGES-1 expression through downregulation of H3K9 mono- and dimethylation.

**Effect of IL-1β on H3K9 methylation, LSD1 recruitment and flavin adenosine dinucleotide levels in normal and osteoarthritis chondrocytes**

OA chondrocytes (n = 3 donors) and normal chondrocytes (n = 3 donors) from age-matched donors were treated with IL-1β for different time periods, and the levels of H3K9 methylation at the mPGES-1 promoter were analyzed by performing ChIP assays using specific Abs for mono-, di- or trimethylated H3K9. We observed a time-dependent decrease in the level of H3K9me2 and H3K9me1 at the mPGES-1 promoter in OA and normal chondrocytes, whereas the level of H3K4me3 remained unchanged (Figures 5A and 5B).

Next, we investigated the effect of IL-1β on LSD1 recruitment at the mPGES-1 promoter in normal and OA chondrocytes. As shown in Figure 5C, treatment of normal chondrocytes with IL-1β resulted in LSD1 recruitment at the mPGES-1 promoter, suggesting that, as observed in OA chondrocytes (Figure 5D), the H3K9 demethylase involved in H3K9me1 and H3K9me2 demethylation at the mPGES-1 promoter in normal chondrocytes is LSD1.

LSD1 utilizes FAD as an essential cofactor in catalyzing demethylation of mono- and di-methylated H3K9 [30]. We therefore examined whether IL-1β -induced H3K9 demethylation and LSD1 recruitment to the mPGES-1 promoter were associated with changes in FAD levels. As shown in Figures 5E and 5F, treatment of chondrocytes with IL-1β did not affect the content levels of FAD at any time point. These data indicate that IL-1-induced H3K9 demethylation and LSD1 recruitment in human chondrocytes were not associated with significant changes in FAD

levels.

**Effect of IL-1β on histone H3K4 methylation at mPGES-1 promoter**

H3K4 methylation is a critical epigenetic marker of transcriptional activation (27-29). We therefore examined the effect of IL-1β on H3K4 methylation at the mPGES-1 promoter. As shown in Figure 6, treatment with IL-1β enhanced the levels of H3K4 methylation at the mPGES-1 promoter in a time-dependent manner. The levels of di- and trimethylated H3K4 were significantly enhanced at 4 hours after IL-1β stimulation, reached a maximum at 12 hours, persisted through 24 hours and decreased at 48 hours. In contrast, the level of monomethylated H3K4 remained almost unchanged following IL-1β stimulation. The increase in H3K4 di- and trimethylation by IL-1β at the mPGES-1 promoter paralleled the increased transcription of mPGES-1 (Fig. 1A), suggesting that, in addition to H3K9 demethylation, H3K4 methylation also contributed to IL-1β-induced mPGES-1 expression.

**LSD1 levels are elevated in OA cartilage**

To investigate the expression of LSD1 in vivo, we analyzed its mRNA levels in total cartilage from healthy donors (n = 10) and OA donors (n = 10) using real-time quantitative RT-PCR. As shown in Fig. 7A, the level of LSD1 mRNA was about 1.7-fold higher in OA cartilage compared with normal cartilage.

mPGES-1 catalyzes the terminal step in the biosynthesis of $PGE_2$ , a critical mediator in the pathophysiology of osteoarthritis (OA). Histone methylation plays an important role in

epigenetic gene regulation. In this study, we investigated the roles of histone H3 lysine 9 (H3K9).

Next, we used immunohistochemistry to analyze the expression level of LSD1 protein. Typical normal and OA sections immunostained for LSD1 and the corresponding negative control are shown in Fig. 7B to 7E. LSD1 expression was seen in normal and OA cartilage in all superficial, middle and deep layers of the articular cartilage, and we observed that the expression levels were relatively high in the superficial and middle zones.

Statistical calculation of the cell score revealed that the percentage of cells expressing LSD1 was approximately 1.8-fold higher in OA cartilage (n = 10) than in normal cartilage (n = 10) (Fig. 7G). The specificity of the staining was confirmed using an Ab that had been preadsorbed (1 hour at 37°C) with a 20-fold molar excess of the peptide antigen (Fig. 5E) or non-immune control IgG (data not shown). Together, these data indicate that the expression level of LSD1 is increased in OA cartilage.

## DISCUSSION

Histone methylation and demethylation play important roles in transcriptional control (27-29). Histone methylation may positively or negatively regulate gene expression, depending on which residue is modified and how many methyl groups are added. H3K9 methylation usually suppresses transcription, whereas H3K4 methylation generally activates transcription (27-29).

In the present study, we showed that IL-1β-induced mPGES-1 expression in human OA chondrocytes correlated with reduced levels of H3K9me1 and H3K9me2 at the mPGES-1 promoter. We identified LSD1 as the responsible demethylase, since inhibition of LSD1 activity or its knockdown prevented IL-1β-induced H3K9 demethylation and mPGES-1 expression. We also demonstrated that LSD1 levels were elevated in the superficial and midlle zones of OA cartilage. These data indicate that H3K9 demethylation by LSD1 contributes to IL-1β-induced mPGES-1 expression and suggest that this pathway could be a potential target to modulate $PGE_2$ levels.

Our finding that the induction of mPGES-1 expression by IL-1β is associated with demethylation of H3K9 is consistent with the results of several recent studies in which researchers showed that transcriptional activation of a number of inducible inflammatory genes correlates with decreased methylation of H3K9 at target promoters. For instance, the induction of the p40 subunit of interleukin 12 (IL12p40), the macrophage-derived chemokine (MDC), as well as EBV-induced molecule 1 ligand chemokine (ELC) by lipopolysaccharides (LPS) in dendritic cells was observed to be accompanied by loss of H3K9 methylation at the three gene promoters (51). Reduced H3K9 methylation was also observed at the IL-1β and TNF-α promoters in LPS-treated THP-1 cells (52, 3), at the MMP-9 promoter in phorbol 12-myristate 13-acetate– treated HeLa

cells (54) and at the NF-κB-p65 promoter in a model of transient hyperglycemia in bovine aortic endothelial cells (55). Similarily, H3K9 methylation was reduced at the promoters of IL-1β, macrophage colony stimulating factor-1 (MCSF), and monocyte chemoattractant protein-1 (MCP-1) (56). In line with this finding, and in the context of cancer, transcriptional activation of several genes was associated with decreased H3K9 methylation, including androgen receptor-induced, prostate specific antigen (PSA) expression in LNCaP cells (35), and estrogen receptor-induced GREB1 expression in MCF7 cells (37). Loss of H3K9 methylation during varicella zoster virus reactivation from latency has also been reported (38).

Several H3K9me1/2 demethylases have been identified, including LSD1/KDM1 (57), JMJD1A /JHDM2A/KDM3A (44), KIAA1718/JHDM1D/KDM7A (45), PHF8/JHDM1F/KDM7B (46) and PHF2/JHDM1E/KDM7C (47). We therefore sought to identify which of these demethylases might be involved in the reduction of H3K9me1 and H3K9me2 levels at the mPGES-1 promoter.

Treatment with IL-1β increased the level of LSD1 at the mPGES-1 promoter, but had no effect on the recruitment of the other demethylases, suggesting that the H3K9 demethylase that is involved in H3K9me1 and H3K9me2 demethylation at the mPGES-1 promoter is LSD1. It is noteworthy that the recruitment of LSD1 at the mPGES-1 promoter coincides with decreased H3K9 mono- and dimethylation and correlates well with the increased transcription of mPGES-1. Taken together, these results strongly suggest that LSD1 recruitment to the mPGES-1 promoter and H3K9 demethylation contribute to IL-1β-induced mPGES-1 expression.

Having established that LSD1 is recruited to the mPGES-1 promoter, we next examined the effect of its pharmacological inhibition or silencing on IL-1β-induced H3K9 demethylation

and mPGES-1 expression. The amino oxidase inhibitors tranylcypromine and pragyline, known as potent inhibitors of LSD1 activity, prevented both IL-1β−induced H3K9 demethylation at the mPGES-1 promoter and IL-1β-induced mPGES-1 protein expression. Furthermore, siRNA-mediated LSD1 knockdown suppressed IL-1β-induced H3K9 demethylation and concomitant mPGES-1 protein expression. These results further support the model in which LSD1 contributes to IL-1β-induced mPGES-1 expression through H3K9 demethylation.

Our finding that H3K9 demethylation by LSD1 activates mPGES-1 expression extends similar findings showing transcriptional activation of a number of genes by LSD1. For instance genome-wide ChIP assay based on DNA selection and ligation (DSL) analysis in MCF7 cells treated with 17 β-estradiol revealed the presence of LSD1 at 42% of all Polymerase II-positive promoters and 74% of LSD1-positive genes were expressed (37). Moreover, LSD1 was reported to demethylate H3K9 and to mediate ligand-dependent transcription of both androgen receptor- and estrogen receptor-dependent genes (35,37). LSD1 was also shown to act as transcriptional activator during lytic replication of the herpes simplex virus (38), the expression of MMP-9 in retinal endothelia cells (58) and the expression of vascular endothelial growth factor in prostate cancer cells (59).

As stated above, H3K9me1 and H3K9me2 can also be demethylated by JMJD1A /HDM2A/KDM3A (44), KIAA1718/JHDM1D/KDM7A (45), PHF8/JHDM1F/KDM7B (46) and PHF2/JHDM1E/ KDM7C (47). Although we failed to detect the recruitment of these enzymes at the mPGES-1 promoter in our ChIP analysis, we cannot exclude their involvement through binding to other regions of the mPGES-1 promoter, which we did not analyzed in the present study. Moreover, our results, which are consistent with key roles of H3K9 demethylation in IL-

1β-induced mPGES-1 expression, do not rule out the possibility that changes in the methylation status of other residues might also participate in IL-1β-induced mPGES-1 expression. Indeed, methylation of H3K4, H3K27, H3K36 and H3K79 is also known to modulate gene transcription.

Our ChIP findings demonstrated the occupancy of LSD1 at the mPGES-1 promoter in IL-1β-treated cells. However, it is unclear how LSD1 is recruited to the mPGES-1 promoter. One possibility is that LSD1 is recruited to the mPGES-1 promoter by transcriptional regulatory co-factors. Such a mechanism has been reported by Liang *et al*., who demonstrated that the expression of viral immediate early genes in herpes simplex virus and Varicella zoster virus involves recruitment of LSD1 by the cellular transcriptional co-activator, host cell factor-1 (HCF-1), to viral immediate early promoters (38).

Another possibility is that LSD1 is recruited to the mPGES-1 promoter by key transcription factors that play key roles in its transcriptional activation, such as hypoxia-inducible factor 1α (HIF1α) (60) and Krüppel-like factor 5 (KLF5) transcription factor (61). Indeed, LSD1 has been shown to physically associate with HIF1α in melanoma inhibitory activity human pancreatic carcinoma MIA PaCa-2 cells (62) and with KLF5 in embryonic stem cells (63). Therefore, it is possible that these transcription factors direct LSD1 to the mPGES-1 promoter. In this context, LSD1 has been shown to be recruited by the androgen receptor and to stimulate transcription through H3K9 demethylation (35). The transcription factor TLX, an essential neural stem cell regulator, has also been reported to mediate LSD1 recruitment to the promoters of TLX target genes in neural stem cells (64) and the Y79 retinoblastoma cells (65).

We also demonstrate that the induction of mPGES-1 by IL-1β was associated with H3K4 methylation. This extends similar previous findings showing H3K4 methylation at the promoters

of several inflammatory genes, including inducible nitric oxide synthase and COX-2, in human chondrocytes (40). The increased level of H3K4 methylation at the mPGES-1 promoter might rely on the ability of LSD1 to anchor other factors at the mPGES-1 promoter rather than on its own enzymatic activity. Indeed, LSD1 is usually found as part of a multiprotein complex with several distinct enzymatic activities, including transcription factors, other demethylases and histone methyltransferases. For instance, Liang et al. reported that the activation of α-herpesvirus lytic replication and its reactivation from latency involve H3K9 demethylation and H3K4 trimethylation through recruitment of (1) a multiprotein complex containing LSD1 and (2) the H3K4 methyltransferases mixed lineage leukemia 1 (MLL1) and Set1A (38). Similarly, Le Douce et al. showed that the recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic markers through corecruitment of LSD1 and the histone methyltransferase hSET1 at the integrated provirus (66). Wang et al. demonstrated that LSD1 associates with the MLL1 complex, which mediates H3K4 trimethylation at the growth hormone promoter during developmental activation (67). Therefore, it is likely that multiprotein complexes such as these, which contain LSD1 and histone methylases, coordinate H3K4/H3K9 methylation and cooperate to mediate IL-1β -induced mPGES-1 expression.

H3K9 demethylation may mediate IL-1β-induced mPGES-1 expression through several non-exclusive mechanisms. H3K9 demethylation may promote transcriptional activation by enhancing lysine acetylation and allowing better access to DNA for transcription factors and RNA polymerase. Such a mechanism was reported by Escoubet-Lozach et al, who showed that LSD1 participates in pomalidomide-induced $p21^{WAF}$ expression in Burkitt's lymphoma cells by favoring H3K9 acetylation (68). Similarily, Zhong et al reported that LSD1-mediated MMP-9 expression during diabetes involves increased H3K9 acetylation (58). H3K9 demethylation can also

contribute to IL-1β-induced mPGES-1 expression by preventing DNA methyl transferase (DNMT) recruitment and local DNA methylation, which is often associated with transcriptional silencing. Indeed, H3K9 methylation is required for DNA methylation (69,70). In addition, H3K9 demethylation can participate in IL-1β-induced mPGES-1 expression by modifying the binding of chromatin factors/regulators. In this context, El Gazzar et al. demonstrated in a THP-1 model of endotoxin tolerance, that the loss of H3K9 methylation at the TNF-□ promoter induces the gene expression by decreasing the level of the heterochromatin protein 1 α (HP1α), which is known for its role in gene silencing (53). In the present study, we found no evidence of either of these mechanisms. Additional biochemical analyses are clearly warranted to resolve this issue.

We show here that LSD1 expression was higher in OA cartilage than in normal tissue. Interestingly, we and others have previously reported elevated levels of mPGES-1 in OA tissues (16,17,24), suggesting that high expression of LSD1 may be responsible for increased levels of mPGES-1. These data, together with our findings that LSD1 mediates IL-induced mPGES-1 expression in cultured chondrocytes, suggest that elevated levels of LSD1 may be part of the mechanisms responsible for increased mPGES-1 expression in OA cartilage.

**CONCLUSIONS**

The results of the present study demonstrate that the histone demethylase LSD1 contributes to IL-1β-induced mPGES-1 expression in human chondrocytes through H3K9 demethylation. Our findings thus provide insight into the regulatory mechanism underlying mPGES-1 expression and may have implications for the design of new anti-OA and anti-inflammatory drugs.

## LIST OF ABBREVIATIONS

AA: Arachidonic acid; ChIP: Chromatin immunoprecipitation; COX: Cyclooxygenase; cPGES: Cytosolic prostaglandin E synthase; CT: Threshold cycle; DMEM: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; H3K9: Histone H3 lysine 9; HRP: Horseradish peroxidase; Ig: Immunoglobulin; IL: Interleukin; KDM: Lysine demethylase; KMT: Lysine methyltransferase; LPS: Lipopolysaccharide; LSD1: Lysine-specific demethylase; MMP: Matrix metalloproteinase; mPGES-1: Microsomal prostaglandin E synthase 1; mPGES-2: Microsomal prostaglandin E synthase 2; NSAID: Nonsteroidal anti-inflammatory drug; OA: Osteoarthritis; $PGE_2$: Prostaglandin E2; PMSF: Phenylmethylsulfonyl fluoride; RA: Rheumatoid arthritis; siRNA: Small interfering RNA; TNF-α: tumor necrosis factor α; UNG: Uracil N-glycosylase.

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## FIGURE LEGENDS

**Figure 1. Effect of interleukin 1β on histone H3 lysine 9 methylation at the microsomal prostaglandin E synthase 1 promoter. (A)** Osteoarthritis (OA) chondrocytes were treated with 100 pg/ml interleukin 1β (IL-1β) for the indicated time periods. Total RNA was isolated, reverse-transcribed into cDNA, and microsomal prostaglandin E synthase 1 (mPGES-1), matrix metalloproteinase 13 (MMP-13) and glyceraldehyde3-phosphate dehydrogenase mRNAs were quantified using real-time PCR. All experiments were performed in triplicate, and negative controls without template RNA were included in each experiment. The results are expressed as fold changes, assuming 1 as the value of untreated cells, and represent the mean ± SD of four independent experiments using cells from four different OA donors. $^{*}P < 0.05$ compared with unstimulated cells. **(B)**- through **(D)** Confluent OA chondrocytes were treated with 100 pg/ml IL-1β for the indicated time periods. Chromatin immunoprecipitation (ChIP) assays, coupled with real-time PCR, were performed using antibodies specific to mono- **(B)**, di- **(C)** and trimethylated **(D)** histone H3 lysine 9 (H3K9). me1, Monomethylation; me2, Dimethylation; me3, Trimethylation. The results are expressed as percentages of control values (that is, untreated cells) and are represent the mean ± SD of four independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on two separate occasions. $^{*}P < 0.05$ compared with unstimulated cells. The lower panels show chondrocytes that were treated as indicated. The levels of mono-, di- and trimethylated H3K9 and unmodified H3 were evaluated by immunoblotting. The blots are representative of similar results obtained in four independent experiments in which we used cells from four different OA donors. **(E)** and **(F)** Schematic diagrams of the mPGES-1 and MMP-13 promoters showing the locations of the PCR primers (arrows) used in the ChIP analyses.

**Figure 2. Effect of interleukin 1β on the recruitment of lysine-specific demethylase 1 to the microsomal prostaglandin E synthase 1. (A)** nuclear extracts (20 μg) from four different osteoarthritis (OA) chondrocyte populations obtained from four different donors were studied by Western blot analysis and hybridized to antibodies specific to LSD1/KDM1, JMJD1A/JHDM2A/KDM3A, KIAA1718/JHDM1D/KDM7A, PHF8/JHDM1F/KDM7B and PHF2/JHDM1E/KDM7C. **(B)** Confluent OA chondrocytes were treated with 100 pg/ml interleukin 1β (IL-1β) for the indicated time periods, and chromatin immunoprecipitation (ChIP) assays were performed using a specific antibody against lysine-specific demethylase 1 (LSD1). The results are expressed as fold changes of LSD1 binding to the microsomal prostaglandin E synthase 1 (mPGES-1) or matrix metalloproteinase-13 (MMP-13) promoter relative to untreated cells and represent the mean ± SD of four independent experiments. *P < 0.05 compared with unstimulated cells. **(C)** Confluent OA chondrocytes were treated as described in part **(B)**, and cell lysates were prepared and analyzed for LSD1 protein expression by Western blotting. In the lower panels, the blots were stripped and reprobed with a specific anti-β-actin antibody. The blots are representative of similar results obtained from four independent experiments using cells from four separate donors.

**Figure 3. Effect of pargyline and tranylcypromine on interleukin 1β-induced histone H3 lysine 9 demethylation and microsomal prostaglandin E synthase 1 protein expression.** Osteoarthritis (OA) chondrocytes were pretreated with control vehicle (dimethyl sulfoxide) or increasing concentrations of pargyline **(A)** through **(C)** and tranylcypromine (TCP) **(D)** through **(F)** for 1 hour prior to stimulation with 100 pg/ml interleukin 1β (IL-1β) for 8 hours **(A, B, D and E)** or 24 hours **(C)** and **(F). (A), (B), (D)** and **(E)** Chromatin immunoprecipitation (ChIP) assays, coupled with real-time PCR, were performed using antibodies specific to mono- and dimethylated

histone H3 lysine 9 (H3K9). The results are expressed as the percentage of control values (that is, untreated cells) and represent the mean ± SD of four independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on two separate occasions. *$P < 0.05$ compared with IL-1β-treated cells. TCP, tranylcypromine. **(C)** and **(F)** Cell lysates were prepared and analyzed for microsomal prostaglandin E synthase 1 (mPGES-1) protein expression by Western blotting. In the lower panels, the blots were stripped and reprobed with specific anti-β-actin antibody. The blots are representative of similar results obtained in four independent experiments using cells from four separate donors. cPGES, Cytosolic prostaglandin E synthase; me1, Monomethylation; me2, Dimethylation; me3, Trimethylation.

**Figure 4. Effect of lysine-specific demethylase 1 silencing on interleukin 1β–induced histone H3 lysine 9 demethylation at microsomal prostaglandin E synthase 1 promoter.** Osteoarthritis (OA) chondrocytes were transfected with 100 nM control scrambled small interfering RNA (siRNA) or lysine-specific demethylase 1 (LSD1). At 48 hours posttransfection, cells were left untreated or treated with 100 pg/ml interleukin 1β (IL-1β) for 8 hours **(A)** or 24 hours **(B)**. CTL, Control. **(A)** Chromatin immunoprecipitation (ChIP) assays, coupled with real-time PCR, were performed using antibodies specific to mono- and dimethylated histone H3 lysine 9 (H3K9). The results are expressed as percentages of control values (that is, untreated cells), and the data are the mean ± SD of four independent experiments. For each ChIP assay, the immunoprecipitated DNA was quantitated in triplicate on two separate occasions. *$P < 0.05$ compared with nontransfected cells stimulated with IL-1β. **(B)** Cell lysates were prepared and analyzed for microsomal prostaglandin E synthase 1 (mPGES-1) protein expression by Western blotting. The blots were stripped and reprobed with specific anti-β-actin antibody. The blots are representative of similar results obtained from four independent experiments using cells from four separate donors.

Knockdown of LSD1 was confirmed by Western blotting using a specific anti-LSD1 antibody (lower panels).

**Figure 5. Effect of interleukin 1 on histone H3 lysine 9 methylation, lysine-specific demethylase 1 recruitment and flavin adenine dinucleotide levels in normal and osteoarthritis chondrocytes.** Normal **(A)** and **(C)** and osteoarthritis (OA) **(B)** and **(D)** chondrocytes were treated with 100 pg/ml interleukin 1β (IL-1β) for the indicated time periods. Chromatin immunoprecipitation (ChIP) assays, coupled with real-time PCR, were performed using antibodies specific to mono-, di- and trimethylated histone H3 lysine 9 (H3K9) **(A)** and **(B)** and lysine-specific demethylase 1 (LSD1) **(C)** and **(D)**. The results are expressed as percentages of control values (that is, untreated cells) or fold changes, and the data are the mean ± SD of three independent experiments using cells from three different donors. *$P < 0.05$ compared with unstimulated cells. Normal **(E)** and OA **(F)** chondrocytes were treated as indicated, and the levels of flavin adenine dinucleotide (FAD) were determined using a FAD assay kit. The results are expressed in picomolar units per 106 cells, and the data are the mean ± SD of three independent experiments using cells from three different donors. me1, Monomethylation; me2, Dimethylation; me3, Trimethylation.

**Figure 6. Effect of interleukin 1β on histone H3 lysine 4 methylation at microsomal prostaglandin E synthase 1 promoter.** Osteoarthritis (OA) chondrocytes were treated with 100 pg/ml interleukin 1β (IL-1β) for the indicated time periods, and chromatin immunoprecipitation (ChIP) assays were performed using antibodies specific to mono-, di- and trimethylated histone H3 lysine 4 (H3K4). The results are expressed as fold changes relative to control (that is unstimulated cells), and the data are the mean ± SD of three independent experiments using cells from four different donors. For each ChIP assay, the immunoprecipitated DNA was quantitated in

triplicate on two separate occasions. *P < 0.05 compared with unstimulated cells. me1, Monomethylation; me2, Dimethylation; me3, Trimethylation.

**Figure 7. Expression of lysine-specific demethylase 1 protein in human normal and osteoarthritis cartilage. (A)** RNA was extracted from normal cartilage (n = 10) and osteoarthritis (OA) cartilage (n = 10), reverse-transcribed into cDNA and processed for real-time PCR. The threshold cycle values were converted to the number of molecules. The data are expressed as copies of the gene's mRNA detected per 10,000 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) copies. *P < 0.05 versus normal samples. Representative immunostained images of human normal cartilage **(B)** and OA cartilage **(C)** for lysine-specific demethylase 1 (LSD1) protein are shown. **(D)** and **(E)** Higher-magnification views of the areas within the rectangles in **(B)** and **(C)**, respectively. The arrow shows postitive expression of LSD1. **(F)** Cartilage specimens treated with the anti-LSD1 antibody that was preadsorbed with a 20-fold molar excess of the protein fragment corresponding to amino acids 834 to 852 of human LSD1 protein (control for staining specificity). **(G)** Percentage of chondrocytes expressing LSD1 in normal and OA cartilage. The data are the mean ± SD of 10 normal and 10 OA specimens. *P < 0.05 versus normal cartilage.

**Figure 1**

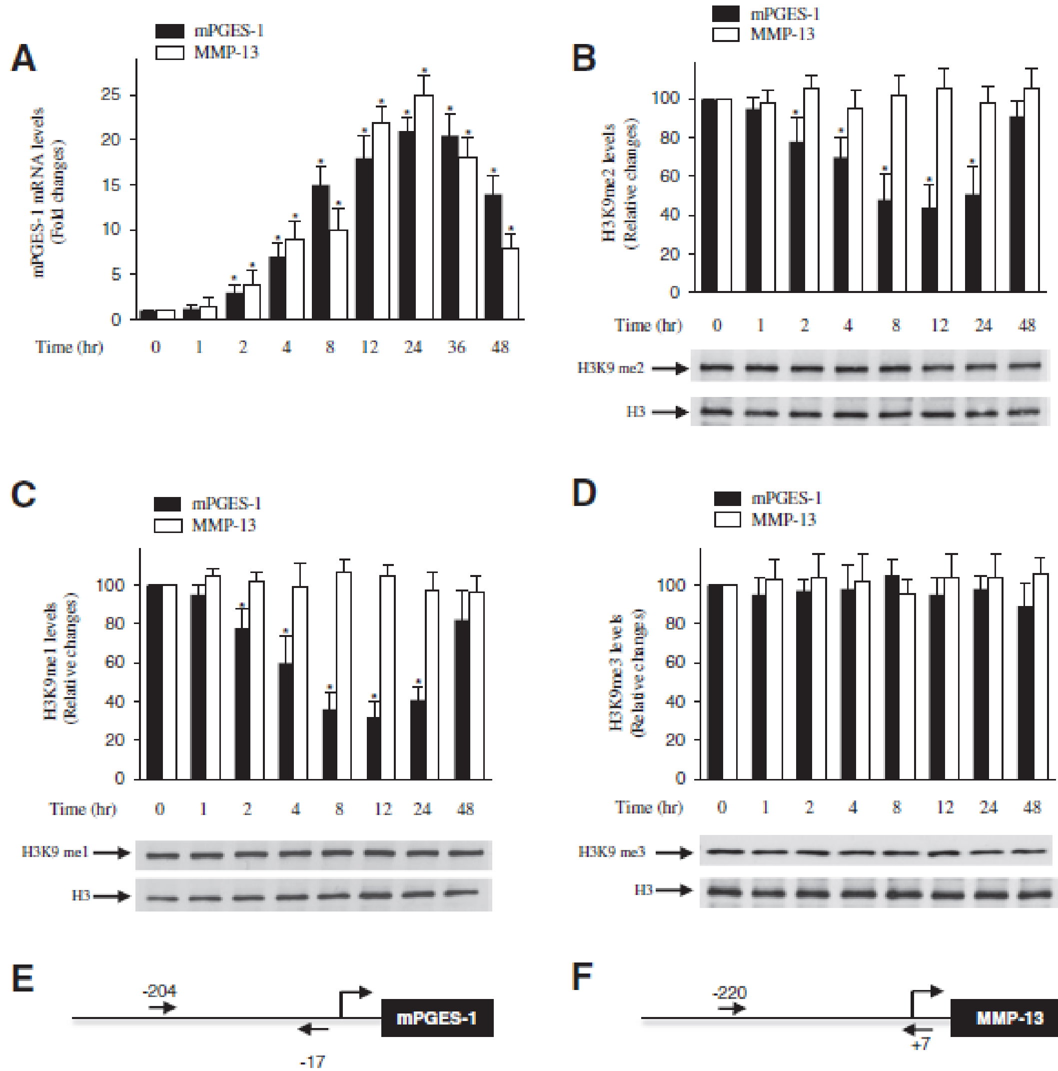


**Figure 2**

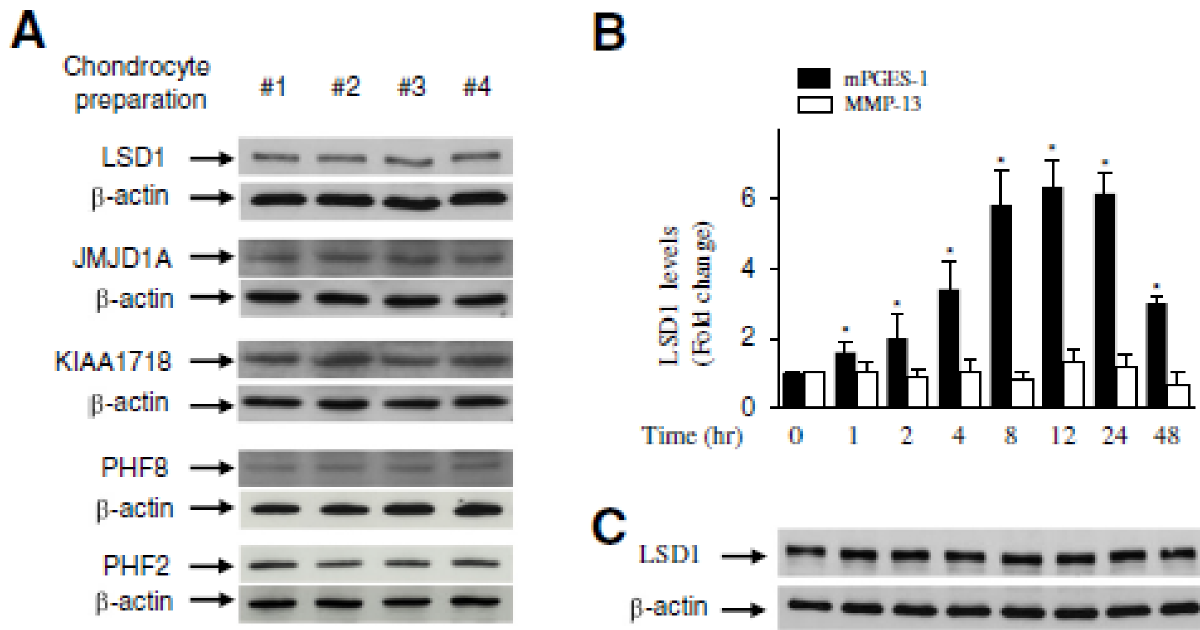


**Figure 3**

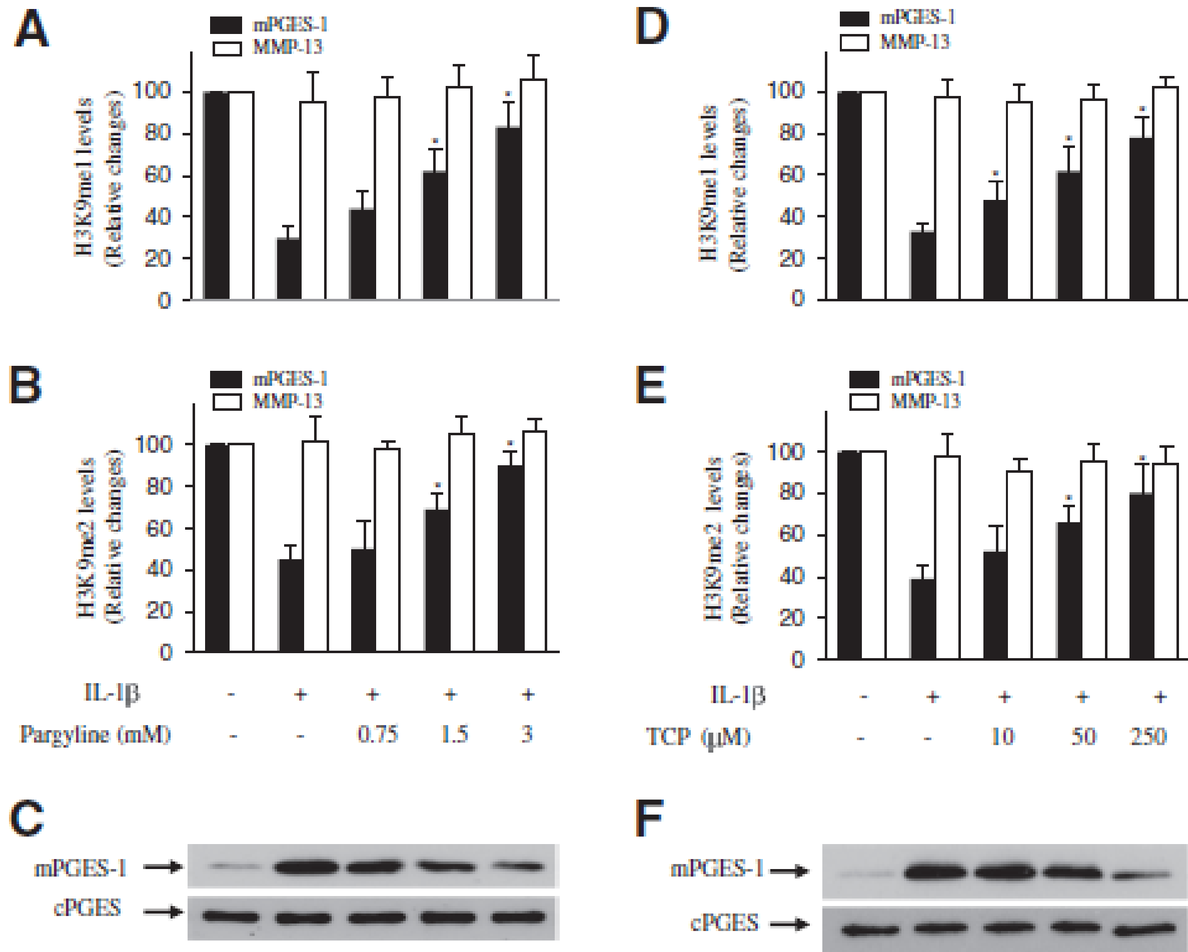


**Figure 4**

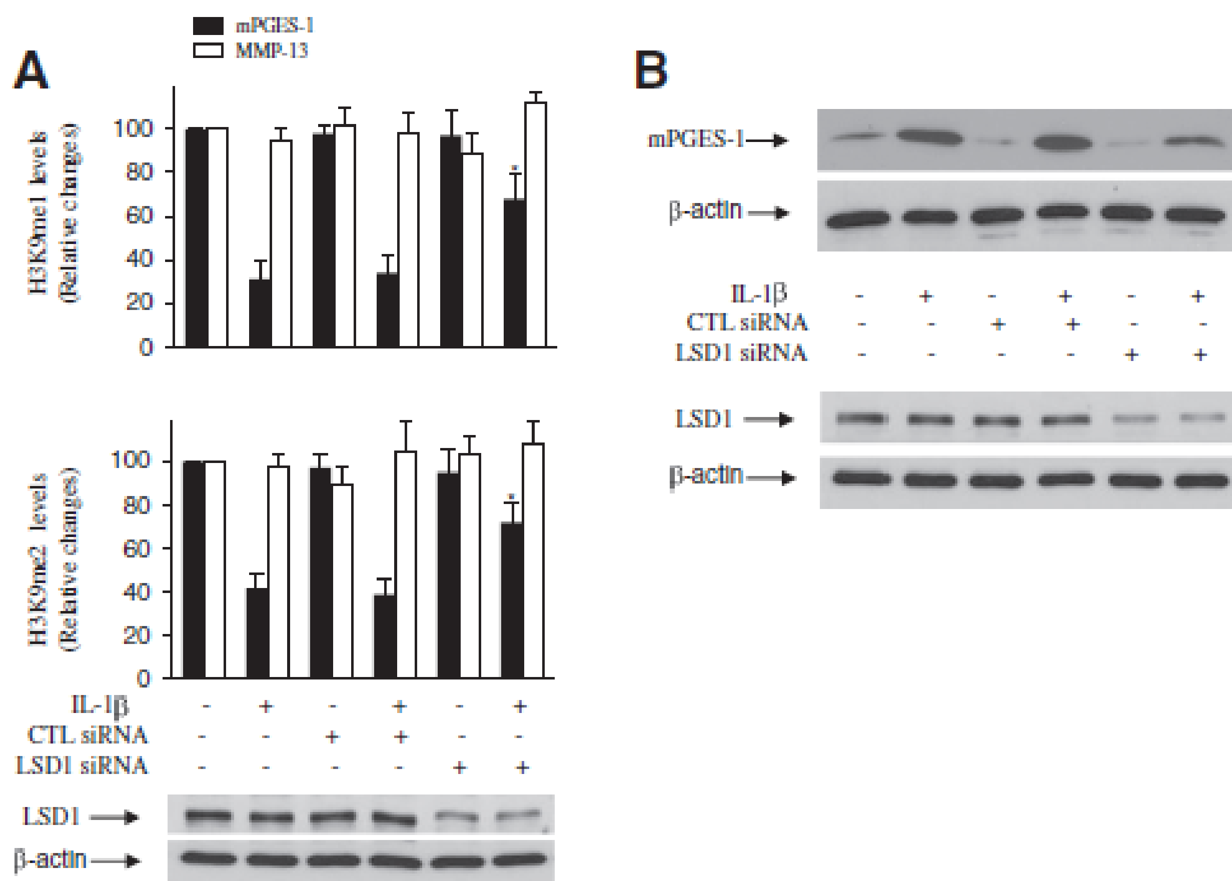


**Figure 5**

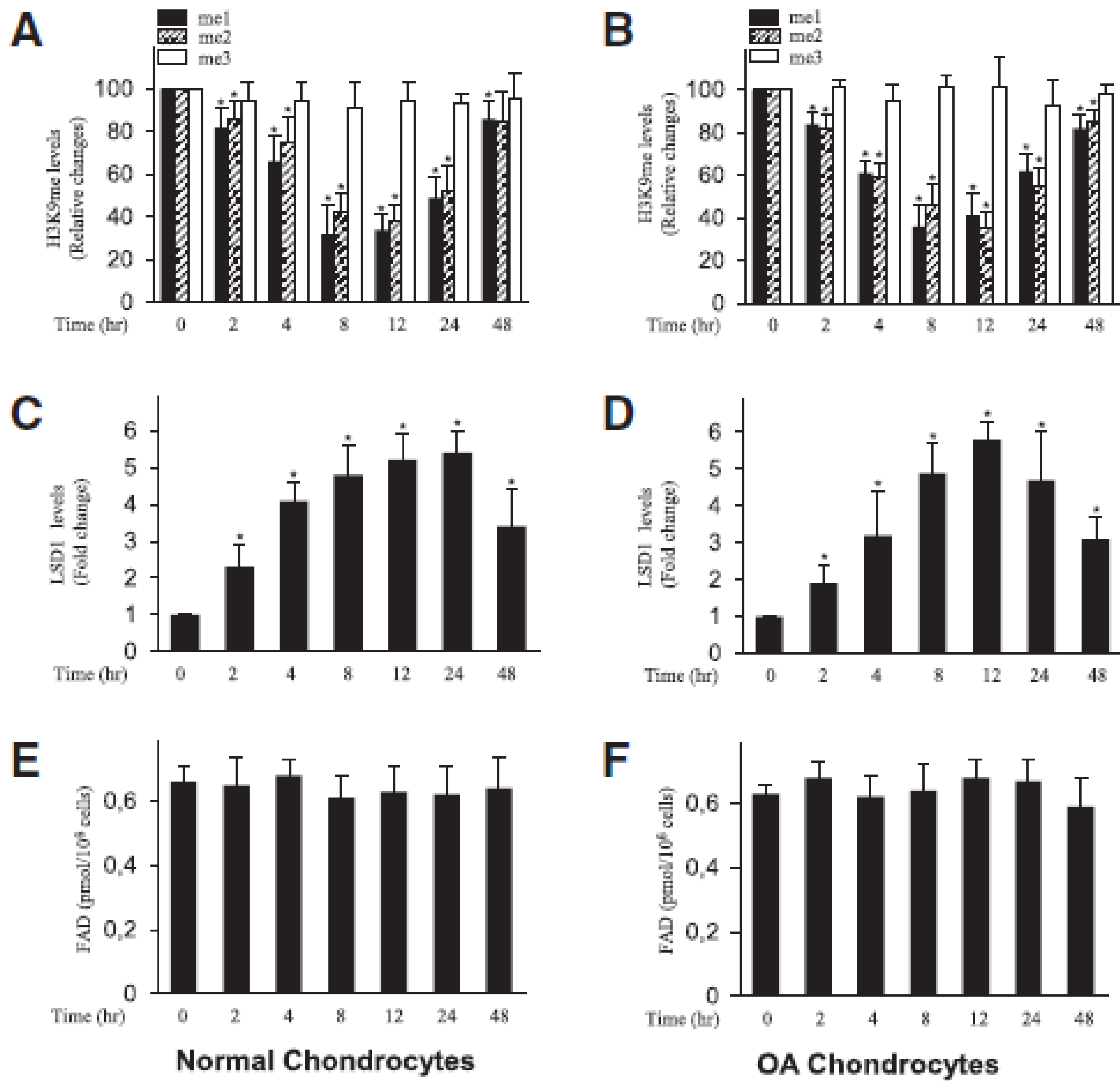


**Figure 6**

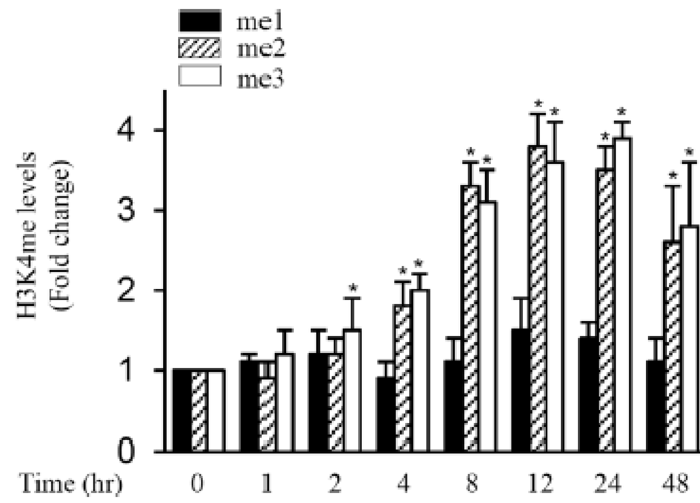
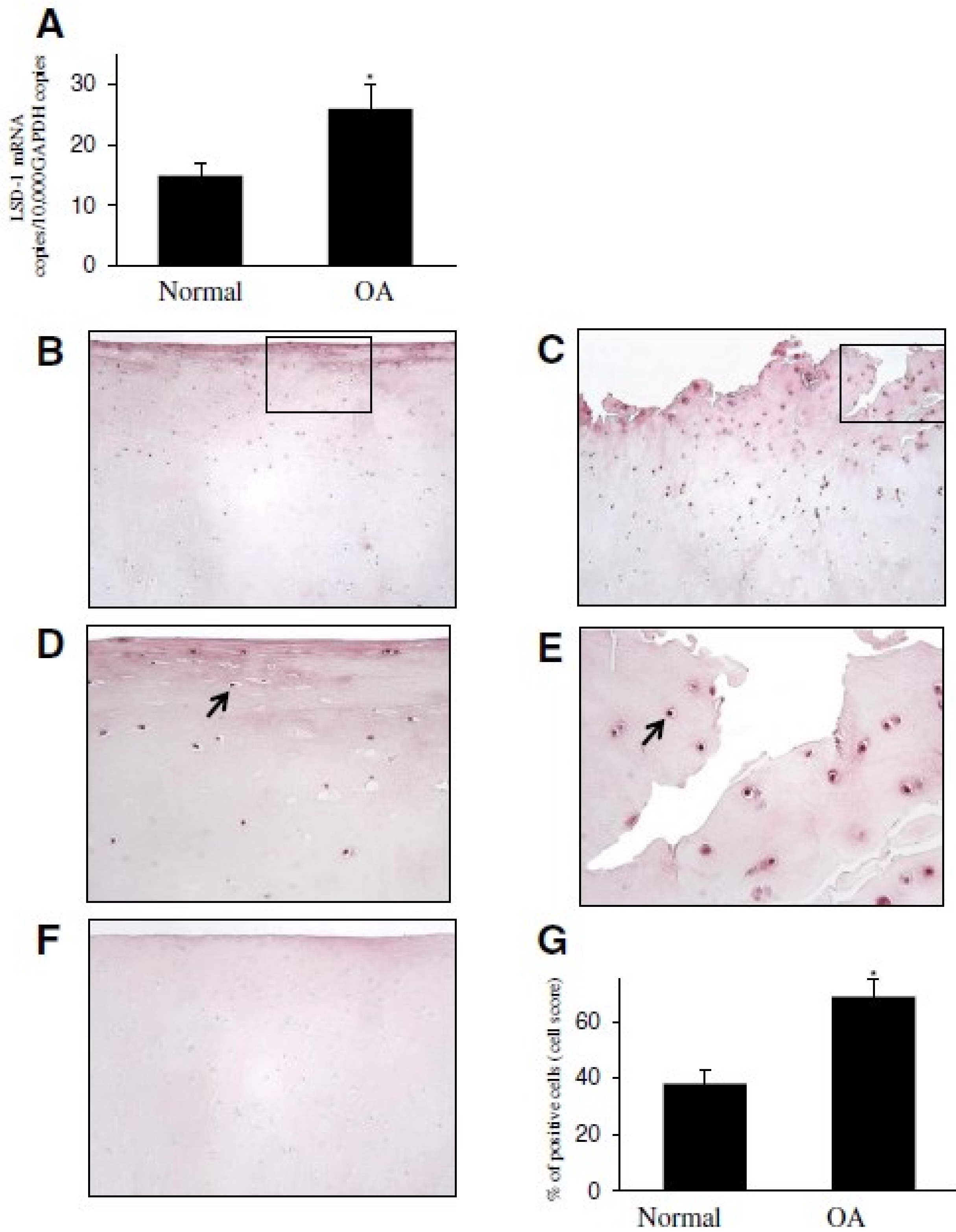


**Figure 7**



# Discussion

OA is a degenerative joint disease characterized by an imbalance of physiologic processes that trigger a network of inflammatory cascades. Although OA implicates all tissues of the joint, cartilage represents the most important component of this pathophysiology because of the major damage and the multitude of biochemical processes that are activated within this tissue. Prominent pro-inflammatory mediators, including cytokines, prostaglandins, and ROSs are pivotal players in this pathogenesis (9, 15, 34).

IL1β is a key cytokine in the inflammatory response. It is a major inducer of cartilage catabolism in OA. This pro-inflammatory cytokine has the ability to modify chondrocyte metabolism, thus affecting cartilage ECM homeostasis (60).

$PGE_2$ is another strong player in the inflammatory response; it mediates a variety of bioactivities in inflammatory and arthritis diseases (61). Arthritic joint tissues were reported to produce large quantities of $PGE_2$ (125). Chondrocytes are the major source of $PGE_2$ in the joint. Direct evidence has been provided for the role of $PGE_2$ in arthritic diseases and particularly in the course of OA (372). Gene expression analyses of both intact and damaged cartilage, obtained from OA patients, has shown an increase in the expression of COX-2 as well as mPGES-1 with consequent increase in $PGE_2$ production in diseased compared to normal cartilage. Further studies have provided a clear evidence for an overexpression of both COX-2 and mPGES-1 in OA human cartilage (373). These two synthases, COX-2 and mPGES-1, has attracted much attention regarding the major role of these enzymes in inflammation and pain (153, 156).

The production of this prostaglandin was demonstrated to be highly induced in the presence of specific pro-inflammatory cytokines. High levels of $PGE_2$ production within OA tissues are found in response to IL-1β stimulation (79). Similar to IL-1β, pro-inflammatory

cytokines like TNF-α and IL-17 have been shown to induce both COX-2 and mPGES-1-mediated-$PGE_2$ production in OA (124, 132, 373). $PGE_2$ can also contribute to joint cartilage damage by inhibiting collagen and proteoglycan synthesis (152). Thus, modulation of $PGE_2$ synthesis in patients with OA may represent a major target for therapeutic strategies.

Further to the role of $PGE_2$ in the pathophysiology of OA, NO is another potent inflammatory mediator produced by and acting on various cells. Several studies have reported the major implication of NO in OA (101, 187). It is a pivotal catabolic factor that contributes to OA through mediating a variety of processes, including apoptosis, and perpetuating the expression of pro-inflammatory cytokines. It has been shown that NO is spontaneously released from human OA cartilage in sufficient quantities to further increase cartilage damage. Increased levels of iNOS expression have been detected in OA cartilage (374). This up-regulation of iNOS expression results in elevated levels of NO. Numerous studies have reported the catabolic effects of NO on cultured chondrocytes and cartilage. One of these major effects is the up-regulation of the synthesis of targeted MMPs (375) resulting in the ECM destruction. NO mediates also the synthesis of the IL-1-converting enzyme, the caspase required for the maturation of targeted cytokines (IL-1β and IL-18) (376); thereby, further promoting the inflammatory response.

Inflammation triggers also the production of catabolic agents mainly MMPs owing to increased cartilage breakdown and impaired repair. MMPs play a critical role in the physiological and pathological conditions of ECM (202, 377). Studies have shown that MMP-13 levels are increased in OA cartilage contributing to cartilage catabolism. In vivo studies have further revealed the important role of MMP-13 in arthritic damage (377).

All of these mediators are under critical genetic control regulated by epigenetic mechanisms. Epigenetic regulation plays a key role in the expression of genes contributing to

gene transcriptional regulation (378). Of particular, histone lysine methylation has a critical role in the generation of specific molecular marks in chromatin. Histone lysine methylation has attracted much attention due to its implication in both activation and repression of gene expression (261, 379). Methylation of histone lysine residues is catalyzed by KMTs, which can add one or more methyl groups to regulate transcription. Whereas, HDMs can catalyze the removal of these methyl groups on lysine residues of histone tails (261, 276). Both KMTs and HDMs have tremendous specificity and their output can be either activation or repression of transcription (249, 298, 380).

Studies from our lab have previously reported the implication of histone deacetylation in the regulation of these pro-inflammatory mediators in OA. HDAC inhibitors, TSA and BA, suppressed IL-1-induced $PGE_2$ and NO synthesis, COX-2 and iNOS expression, as well as proteoglycan degradation (119). Further studies of our team have demonstrated that HDACi, TSA, BA, and VA inhibited IL-1-induced mPGES-1 protein expression in human synovial fibroblasts. Interestingly, overexpression of HDAC4 enhanced IL-1-induced mPGES-1 promoter activation, indicating that HDAC4 contributes to mPGES-1 gene expression. However, HDAC4 silencing reduced IL-1-induced mPGES-1 promoter activation. Additional analyses have shown that HDAC4 overexpression enhances Egr-1-mediated activation of the mPGES-1 promoter (380).

Published data have provided clear evidence for the contribution of histone modifications (deacetylation and phosphorylation) in COX-2 expression. It has been shown that HDAC1 decreases LPS-induced COX-2 gene expression (380, 381). Conversely, treatment with HDACis increased the expression of COX-2 (382). NaB and TSA accentuated LPS-induced COX-2 gene expression through mitogen-activated protein kinase-dependent increase of phosphorylation and acetylation of histone H3 at the COX-2 promoter (382, 383, 384).

These data support the implication of histone modifications in the regulation of these key inflammatory genes. Other previous studies have also claimed the involvement of histone modifications in COX-2, iNOS and mPGES-1 expression (385, 386, 387, 388, 389). However, up to now, the implication of histone lysine methylation in the expression of these pro-inflammatory genes in OA is still unknown. Thus, it is of great importance to study the role of histone lysine methylation in the regulation of inflammatory genes associated with OA.

The objective of my thesis was to investigate the role of histone lysine methylation and demethylation in the inflammatory process in human OA chondrocytes. In this project, we investigated the implication of histone lysine methylation H3K4 and demethylation of H3K9 in the regulation of COX-2, iNOS, and mPGES-1 genes. Here, I discuss the role of histone lysine methylations particularly histone H3K4 and H3K9 methylations, and the associated histone methyltransferases and demethylases, in controlling the expression of these genes as master regulator of inflammation in OA.

**H3K4 methylation regulates the inflammatory response**

We evaluated first the role of histone lysine methylation in the expression of COX-2 and iNOS genes in human OA cartilage. With the ultimate goal of clarifying this role, we demonstrated, for the first time, that IL-1β induced histone H3K4 di- and tri-methylation at COX-2 and iNOS promoters in human OA chondrocytes. Since we found that H3K4 at COX-2 and iNOS promoters can be di and tri-methylated but not monomethylated, we targeted KMTs that induce only di- and tri-methylation of H3K4: SET1A and MMLs (390). Therefore, we examined the effect of IL-1β, specifically, on the recruitment of SET-1A and MLL-1 to the COX-2 and iNOS promoters. ChiP analyses revealed that IL-1β induced the recruitment of SET-1A and not

MLL-1 to the COX-2 and iNOS promoters and this was concomitant with di- and trimethylated H3K4 at these sites. Of note, SET-1A levels seemed to be maintained at the COX-2 and iNOS promoters while the levels of di- and trimethylated H3K4 decreased suggesting that specific H3K4 demethylases or inhibitors of SET-1A activity are recruited to both COX-2 and iNOS promoters and contribute to decreased H3K4 di- and tri-methylation. The association of SET-1A recruitment with H3K4 di- and trimethylation suggests that SET-1A is involved in these modifications and that SET-1A-methylated-H3K4 participates to IL-1-induced COX-2 and iNOS expression.

Methylthioadenosine (MTA) is powerful inhibitor of histone methylation (391). We found that treatment of chondrocytes with this inhibitor prevented IL-1-induced H3K4 di- and trimethylation at COX-2 and iNOS promoters and inhibited IL-1-induced COX-2 and iNOS protein expression. Thus, the increase of these pro-inflammatory genes in OA pathology is associated with chromatin remodeling and mediated by histone methylation since the inhibitory effect of MTA treatment results in a significant down-regulation of COX-2 and iNOS genes. Our findings are consistent with several studies showing that MTA inhibits LPS-induced TNFα expression in RAW (murine macrophage cell line) and Kupffer cells (392). We further silenced SET-1A expression by SiRNAs. We found that SET-1A knockdown inhibited the IL-1-induced H3K4me2/3 and prevented COX-2 and iNOS expression. We were then interested to examine the expression of SET-1A in OA cartilage. In that aim, we showed that OA cartilage expresses elevated levels of SET-1A compared with normal cartilage. Interestingly, the expression of SET-1A correlated with increased levels of COX-2 and iNOS expressed by OA chondrocytes in these zones (74, 79, 393). In conclusion, SET-1A may represent a novel therapeutic target for OA and other pathological conditions associated with increased expression of COX-2 and iNOS.

Both the dimethyl- and trimethyl-H3-K4 modifications are enriched at actively transcribed genes (283, 284, 394). Our data are in concordance to previous studies indicating, that activation of numerous inducible inflammatory genes, correlates with elevated methylation of H3K4 at specific promoters. For instance, H3K4 methylation contributes to the induction of monocyte chemotactic protein 1 (MCP-1) and TNFα by the pro-inflammatory astrocyte derived protein S100B or TNFα in THP-1 cells (395). In addition, high levels of methylated H3K4 have been detected at the promoters of TNFα and iNOS in LPS-stimulated-murine macrophage cell line RAW 264.7 and Kupffer cells (396). Similarly, methylation of H3K4 was also reported to be increased at promoters of MMP-1 in phorbol 12-myristate 13-acetate–treated T98G cells (396), class II major histocompatibility complex in IFNγ-treated colon 26 cells (397), IL-6 and MCP-1 in TNFα-treated vascular smooth cells (398), and IL-17 in transforming growth factor β1 and IL-6-treated-CD4 T helper cells (399). Further studies have reported the association of elevated p19 expression in aged cells with di- and tri-methylation of H3K4 and binding of specifically c-Rel at p19 promoter (400).

**H3K4 methylation and transcriptional activation**

Transcription starts with recruitment and assembly of specific transcription factors at gene promoters and enhancers in order to facilitate the binding of RNA polymerase. To ensure that genes are only expressed at the right place and time, this process is highly regulated. Histone lysine methylation contributes to these mechanisms of regulation (250).

The exact mechanism of how H3K4 methylation mediates transcription activation is presently unknown. However, there are several scenarios that may explain how H3K4 methylation could mediate the activation of genes like COX-2 and iNOS transcription. First, H3K4

methylation may activate transcription through enhancing acetylation of histones, located near to H3K4, by HATs indicating that methylated H3K4 are subjected to acetylation by p300 in the presence of H-acetyl CoA (401). Previous data have also noted that methylated H3K4 disrupts binding of nucleosome remodeling and deacetylase NuRD repressor complex; thus, methylated H3K4 may promote activation of transcription through blocking the NuRD deacetylase complex binding (402). Methylated H3K4 may serve as marks to recruit chromatin-remodeling complexes like the nucleosome remodeling factor (NURF) showing that NURF-mediated ATP dependent chromatin remodeling is directly coupled to H3K4me3 in order to maintain Hox gene expression patterns during development (403, 404). Another alternative is that methylated H3K4 can induce transcriptional activation through facilitating the assembly of active transcription complexes. The TFIID transcription complex can directly bind to the H3K4me3 via the plant homeodomain finger of its subunit TAF-3 (405). Methylated H3K4 may also contribute to iNOS and COX-2 activation by displacing factors that mediates transcriptional silencing like HDACs (396, 400, 406, 407).

H3K4me3 accounts for 75% of all human gene promoters in several cell types, playing a critical role in mammalian gene expression (408, 409, 410) such as developmental genes in animals and embryonic development (408, 411). H3K4me3 levels are correlated with increased gene expression (412, 413). Most H3K4me3-containing promoters contains also acetylated H3K9 and H3K14 (414). Additionally, H3K36me3 and H3K79me2 are significantly enriched downstream of H3K4me3-containing promoters in transcriptionally active genes. Such combinatorial arrangement of H3K4me3 and other histone marks support somehow the "histone code" hypothesis (237, 408). H3K4me3 may also modulate transcription by mediating interactions with RNA polymerase associated proteins. It enhances active transcription by the removal of repressive lysine methylation. H3K4me3 can either facilitate the recruitment of reader proteins or

prevent the recruitment of others. Furtheremore, it has been demonstrated that methylation of H3R2 inhibits the methylation of H3K4 and vice versa (415, 416). Asymmetric dimethylation of H3R2me2a mark prevents the methylation of H3K4 by the Set1 lysine methyltransferase (415, 417) and was shown to associate genome-wide with silenced chromatin. However, symmetric dimethylation of H3R2me2s facilitates H3K4me.

Additionally, evidence has reported that H3K4me3 prevents DNA methylation, a mechanism, which is associated with transcription repression (250). An example for the interplay between DNA methyation and histone modifications is DNMT3L and H3K4. DNMT3L interacts with H3 and induces de novo DNA methylation by recruiting DNMT3A. However, when H3K4 is methylated, this interaction is inhibited (418). This mechanism could explain how methylated H3K4 may mediate transcriptional activation of COX-2 and iNOS gene.

**H3K9 methylation regulates the inflammatory response**

As mentioned above, histone lysine methylation can either activate or repress gene transcription depending on the residue, which is modified, and the degree of modification (number of methyl groups that are added). H3K4 methylation generally activates transcription while methylation of H3K9 represses transcription (419, 420). We were interested to investigate the implication of H3K9 methylation in the regulation of inflammation in OA. Microsomal PGES-1 has been widely reported to mediate $PGE_2$ production in inflammatory related arthritic conditions (153, 156). Numerous studies have focused on the critical role of mPGES-1 in OA indicating that mPGES-1 could represent a novel therapeutic target in OA with less side effects.

We have previously shown, in a study to which I participated, that valproic acid (VA) suppresses the expression of mPGES-1 as well as the production of $PGE_2$ in OA chondrocytes.

The expression of mPGES-1 is dependent on the synthesis of de novo protein suggesting that other proteins are implicated in the suppression of mPGES-1 by VA. We found that the physiological co-repressor of Egr-1 transcription factor called NAB-1 (NGF1-Abinding protein 1) contributes to mPGES-1 suppression since VA induces its expression. The binding of NAB-1 to Egr-1 inhibits the transcriptional activity of Egr-1 (348). However, the implication of histone lysine methylation in mPGES-1 regulation remains unknown.

In the second part of my thesis, we extended our investigations to evaluate the role of histone lysine methylation/demethylation in the regulation of mPGES-1 expression in OA cartilage. We demonstrated that IL-1β-induced mPGES-1 expression coincides with reduced levels of mono- and dimethylated H3K9 at the mPGES-1 promoter in OA chondrocytes. This is the first evidence that supports that H3K9 demethylation contributes to IL-1β-induced mPGES-1 expression and suggesting that this pathway might be a potential target for modulation of $PGE_2$ levels.

Our findings, that the induction of mPGES-1 expression by IL-1β was associated with demethylation of H3K9 (421), are in concordance with data of recent studies showing that the transcriptional activation of numerous inducible inflammatory genes is concomitant with decreased methylation of H3K9 at target promoters. Emerging evidences reported that the transcriptional activation of IL-12p40, the macrophage-derived chemokine, as well as Epstein-Barr virus–induced molecule 1 ligand chemokine in LPS-induced-dendritic cells is accompanied by loss of H3K9 methylation at the these gene promoters (422). Further studies have reported reduced levels of methylated H3K9 at the MMP-9 promoter in phorbol 12-myristate 13- acetate–treated HeLa cells (423), at the IL-1β and TNF-α promoters in LPS-treated THP-1 cells (424, 425), and at the NF-κB-p65 promoter in a model of transient hyperglycemia in bovine aortic

endothelial cells (426). Additionally, reduced levels of H3K9 methylation at the promoters of IL-1β, macrophage colony-stimulating factor 1 and monocyte chemoattractant protein 1 have been detected in TNF-α-stimulated-murine vascular smooth muscle cells (427).

There are several H3K9 demethylases that target specifically mono- and dimethyl groups of H3K9 including LSD1, JMJD1A/JHDM2A (329), KIAA1718/JHDM1D (428), PHF8/JHDM1F (312) and PHF2/JHDM1E (429). Thus, we were interested to determine which of these demethylases might be implicated in the decreased levels of H3K9me1/2 at the mPGES-1 promoter. Our findings demonstrated that IL-1β induced particularly the recruitment of LSD1 at the mPGES-1 promoter among all the tested demethylases. This data suggest that LSD1 is the H3K9 demethylase, which is implicated in H3K9me1/2 demethylation at the mPGES-1 promoter. Of interest, the recruitment of LSD1 at the mPGES-1 promoter correlates with reduced levels of mono- and dimethylated H3K9 and coincides with increased mPGES-1 transcription. Together, our data indicates that H3K9 demethylation and LSD1 recruitment to the promoter of mPGES-1 contributes to IL-1β-induced expression of mPGES-1.

We next examined the effect of inhibiting LSD1 on IL-1β-induced H3K9 demethylation and mPGES-1 expression. Treatment with tranylcypromine (TCP) and pargyline, amino oxidase inhibitors known as potent inhibitors of LSD1 activity, inhibited IL-1β-induced H3K9 demethylation at the mPGES-1 promoter as well as mPGES-1 protein expression. Similarly, the MAO inhibitors TCP and biguanide inhibit LSD1 in acute leukemias (430). TCP acts by forming a covalent adduct with the FAD co-factor that resides at the base of the active site (430, 431). Silencing of LSD1 with siRNAs repressed IL-1β-induced H3K9 demethylation and mPGES-1 protein expression. Thus, both inhibition of LSD1 and knockdown suppressed IL-1β-induced H3K9 demethylation and mPGES-1 expression. Our results are in concordance with findings

showing transcriptional activation of several genes by LSD1. It was reported that LSD1 demethylate H3K9 and mediate ligand-dependent transcription of estrogen receptor and androgen receptor-dependent genes (320). In addition, LSD1 activates the expression of MMP-9 in retinal endothelial cells (432) and the expression of vascular endothelial growth factor in prostate cancer cells (433). Further analyses of LSD1 Knockdown have showed increased levels of H3K9me2 on C/EBPα promoter (434).

We found finally that LSD1 expression was higher in OA cartilage than in normal tissue. Immunohistochemical analyses showed that the expression of LSD1 was elevated in the superficial and middle zones of human OA cartilage. These results correlate with elevated levels of mPGES-1 in OA tissue previously found (124, 373), suggesting that high expression of LSD1 may be responsible for increased levels of mPGES-1. Thus, LSD1 contributes to IL-1β-induced mPGES-1 expression in human chondrocytes through H3K9 demethylation. These findings provide insight into the regulatory mechanisms underlying mPGES-1 expression and may represent a novel therapeutic target for OA.

**H3K9 methylation and transcriptional repression**

The precise mechanism behind LSD1 recruitment and contribution to mPGES-1 expression is unclear; however, several hypothetic mechanisms might be implicated. Studies have reported that the expression of viral immediate early genes in herpes simplex virus and varicella zoster virus implicates LSD1 recruitment by the cellular transcriptional co-activator, host cell factor 1, to viral immediate early promoters (435). Alternatively, transcription factors, like HIF-1α (436) and Krüppel-like factor 5 (KLF5) (437) might be involved in LSD1 recruitment to the mPGES-1 promoter. LSD1 have been documented to associate with HIF-1α in melanoma

inhibitory activity human pancreatic carcinoma MIA PaCa-2 cells (438) and with KLF5 in embryonic stem cells (439). Moreover, LSD1 can be recruited by the TLX transcription factor, an essential neural stem cell regulator, to the promoters of TLX target genes in neural stem cells (440) and Y79 retinoblastoma cells (441).

LSD1 demethylates mono- and dimethylated H3K4 but not trimethylated H3K4 (408, 442) through an amine oxidase reaction (277, 394). When LSD1 is alone, it demethylates H3K4me1/2 on histones; but, when it is associated with Co-REST, it demethylates nucleosomal H3K4 (408, 442, 443, 444). The enzymatic activity of LSD1 may be regulated by its associated proteins like CoREST (394, 443).

Furthermore, we cannot exclude the contribution of methylation of other histone residues in IL-1β-induced mPGES-1 expression. Similar to COX-2 and iNOS promoters, we found that induction of mPGES-1 by IL-1β was associated with H3K4 methylation. LSD1 can be also found as a part of multiprotein complexes like transcription factors, other demethylases and histone methyltransferases. Both H3K9 demethylation and H3K4 trimethylation are implicated in the activation of α-herpesvirus lytic replication by recruitment of a multiprotein complex containing LSD1 and the H3K4 methyltransferases MLL1 and Set1A (435). Moreover, the recruitment of LSD1 at the HIV-1 proximal promoter is concomitant with both H3K4me3 and H3K9me3 methylation by corecruitment of LSD1 and hSET1 at the integrated provirus (445). Studies have also reported that LSD1 associates with the MLL1 complex and mediates H3K4 trimethylation at the growth hormone promoter during developmental activation (446). Thus, multiprotein complexes containing LSD1 and histone methylases may coordinate H3K4/H3K9 methylation and contribute to mediate IL-1β-induced mPGES-1 expression.

Nfat1, one of the Nuclear Factor of Activated T-cells (NFAT) transcription factors, plays

an essential role as transcriptional regulator of chondrocyte homeostasis in adult articular cartilage. The absence of Nfat1 can cause OA-like damage in adult mice. It has been noted that an increase in Nfat1 expression in articular chondrocytes correlates with increased H3K4me2 (transcriptional activation), while a decrease in Nfat1 expression is associated with increased H3K9me2 (transcriptional repression) (447). A decrease of LSD1 and JHDMJ2A expression coincides with increased H3K4me2 and H3K9me2 levels respectively at the transcriptional starting site of the Nfat1 promoter and up-regulation of Nfat1 expression in chondrocytes.

Other findings have reported that iNOS promoter is basically rich in H3K9 methylation in endothelial cells (424, 448). This study has showed that treatment with LSD1 inhibitors delayed Hp-induced activation of iNOS suggesting the implication of epigenetic mechanisms in the control of human iNOS gene expression upon hp exposure. Furthermore, increased H3K4me2 and decreased H3K9me levels are the main drivers of iNOS activation upon Hp exposure (448). All of these data supports our findings and helps to better understand the mechanisms of regulation of these genes during OA (**Figure 20**).

Additionally, there is “cross-talk” among the histone modifications and DNA methylation. DNMTs have been reported to interact with histone methyltransferases, such as SUV39h1, Setdb1 and G9a (449). DNMT3A and DNMT3L can recognize unmethylated H3K4 and trimethylated H3K9 (378). Thus, one modification may influence the interpretation of another modification on a neighbor site, which may explain how mPGES-1 is regulated (450).

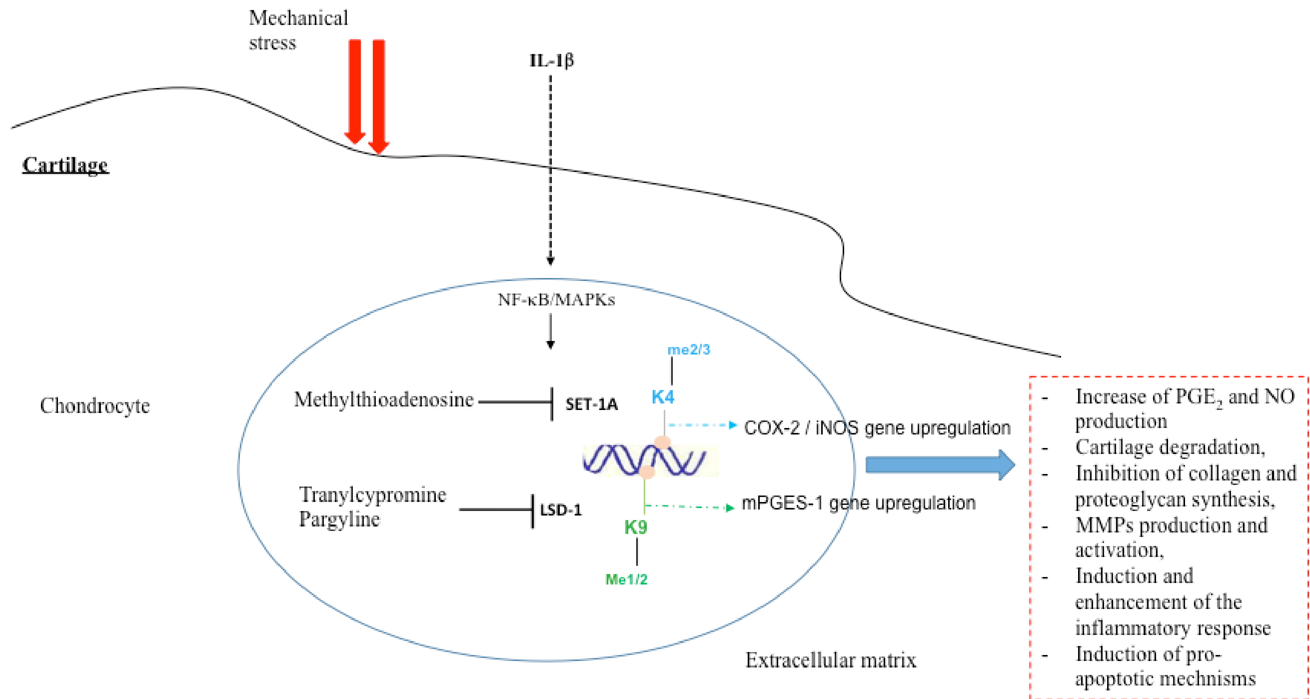



**Figure 20:** Histone lysine methylation/demethylation in OA. Histone lysine methylation/ demethylation of COX-2, iNOS, and mPGES-1 promoters enhances the inflammatory response. SET-1mediated-H3K4 methylation of COX-2and iNOS promters and LSD1-mediated-H3K9 demethyation enhance the catabolic and inflammatory response during OA.

Several studies have provided a clear evidence for an overexpression of both COX-2, mPGES-1, and iNOS in OA cartilage (373). These genes have attracted much attention regarding their major role in inflammation and pain (153, 156).

In here, we showed that SET-1A-methylated-H3K4 contributes to IL-1-induced COX-2 and iNOS expression. We also demonstrated that LSD1-demethylated- H3K9 contributes to IL-1β-induced mPGES-1 expression. Both of these pathways mediate the inflammatory response resulting in increased $PGE_2$ and NO production. Accumulating these data, we suggest that histone lysine methylation regulates the expression of COX-2, mPGES-1 and iNOS and their products $PGE_2$ and NO during the inflammatory process in OA.

Interestingly, COX-2 and mPGES-1 colocolize in the endoplasmic reticulum and the perinuclear membrane (127). They are both induced by the same stimuli including cytokines and growth factors. They are copexpressed in a variety of pathological conditions. Thus, there is a functional coupling of COX-2 and mPGES-1. Previous studies have reported that silencing mPGES-1 by siRNA reduced COX-2-mediated-$PGE_2$ production (451). Similarly, iNOS/NO mediate the pro-inflammatory process during OA. This mediator is induced by the same factors as COX-2/mPGES-1/$PGE_2$ including IL-1β and TNF-α which enhances the inflammatory response.

Our data suggest that these pathways might be potential target for modulation of $PGE_2$ and NO levels in OA.

**Expression of COX-2/mPGES-1/$PGE_2$ and iNOS/NO in animal models of OA**

Numerous factors have been reported to be implicated in OA like cytokines, growth factors, and MMPs; However, $PGE_2$ and NO remains the main contributers to OA. Both of these factors are produced in abundance in OA cartilage; they enhance the inflammatory and catabolic

response. They both induce pain, increase cartilage degradation and pro-inflammatory mediators like IL-1β and NF-κB, increase MMPs production and activation, enhance pro-appototic mechanisms by increasing chondrocytes death, promote angiogenesis, and inhibit collagen and aggrecan synthesis.

Among all COX and PGES synthases (**Figure 9**; **Figure 12**), mPGES-1/COX-2 and iNOS play the most critical role in the production and release of $PGE_2$ and NO in pathophysiologic conditions and particularly OA. These enzymes are highly produced to mediate the inflammatory response.

The role of mPGES-1 in experimental arthritis was analyzed using mPGES-1 knockout mice. Native chicken col-II was subcutaneously administrated to mPGES-1 knockout mice versus wild-type. The results demonstrated significant reduction in the severity of inflammation in knockout animals compared with wild-type. This reduction correlated with the reduction of several histopathological parameters in mPGES-1 -/- mice, such as grade of hyperplasia, loss of proteoglycan, bone erosion, and destruction of cartilage surface (153). Additionally, previous studies have also reported the chondroneutral effect of COX-2 inhibtors specifically, celecoxib, on OA animal models (452).

Studies have been undertaken to evaluate the effect of iNOS inhibitor S-methylisothiourea (SMT) in monosodium iodoacetate (MIA)-induced osteoathritic pain and disease progression in rats. Animals were orally gavazed with different doses of SMT. SMT showed an analgesic effect in a dose dependent manner. It reduced NO production in synovial fluid. Histopathological results indicated also a complete cartilage formation formation in rats treated with SMT as evidence for reduced disease progression (453).

Thus, further studies for understanding the mechanism of regulation of COX-2, mPGES-1, and iNOS regarding their main implication in the development and progression of OA, are needed. To understand the molecular mechanism of the regulation of these genes, we investigated the role of histone lysine methylation in the regulation of mPGES-1/COX-2 and iNOS expression and their products $PGE_2$ and NO.

**Epigenetic based therapeutic interventions for OA and arthritis**

Understanding the role of epigenetics in arthritis diseases like OA and RA has provided another view from a different perspective. This could lead to novel and efficient therapeutic interventions.

To date, most studies have focused on histone deacetylation targeted treatments. Rat adjuvant arthritis animals treated with phenylbutyrate or TSA reduced joint infiltration and swelling, which correlates with reduced expression of TNF in the synovium. Further studies on collagen-induced-arthritis (CIA) model have shown that treatment with entinostat prevented and reversed arthritis and protected from inflammation and bone erosion (454). Furthermore, the high efficacy of MS-275 over other HDACis in CIA models has been noted, indicating that such efficacy might be due to its specificity for class I HDACs, specifically HDAC1 (455, 456). Recently, the efficiency of givinostat in several models of arthritis including CIA and rat adjuvant arthritis has been reported. Clinically, givinostat was administrated orally at 1.5 mg $kg^{-1}$ $day^{-1}$ to patients with systemic juvenile inflammatory arthritis (454).

In RA animal models, HDACi have been reported as effective therapeutic agents. A single intravenous infusion of FK228, an inhibitor of HDAC1 and 2, inhibits synovial swelling and bone and cartilage degradation, reduces TNF-α and IL-1β production in autoantibody-mediated arthritis (456, 457). Administration of HDACi to both animal models of RA and individuals with juvenile

arthritis has shown efficacy in attenuating inflammation and tissue damage (458).

To date, there is no reported data as for the *in vivo* implication of histone lysine methylation in OA animal models and much work is needed. Our studies have created advances in the field by elucidating the role of histone lysine methylation in the regulation of inflammatory genes, like COX-2, iNOS, and mPGES-1 in the pathophysiology of OA; thus, allowing to understand more the mechanisms of regulation and suggest new approaches to modulate these promising therapeutic targets.

# Conclusion & perspectives

In closing, our results demonstrate, for the first time, that histone lysine methylation contribute to the regulation of inflammatory genes associated with OA. We showed, in the first study, that H3K4 methylation by SET-1A contributes to IL-1-induced iNOS and COX-2 expression in human OA chondrocytes. In the second part of my project, we demonstrated that LSD1 contribute to IL-1-induced mPGES-1 expression. These results provide, to our knowledge, the first evidence that histone lysine methylation regulates the expression of inflammatory genes associated with in human OA. Our findings suggest that this pathway could be a potential target for pharmacologic intervention in the treatment of OA and possibly other arthritic diseases. Our findings showed that epigenetic modifications contribute to the activation of inflammatory genes. The suppressive effect of HMT and HDM inhibitors shed the light on a new area of clinical investigation of these drugs. However, many questions remain regarding the implication of histone lysine methylation in the precise molecular mechanisms in the regulation of these genes. Further studies are needed to investigate the implication of histone lysine methylation in synovial tissue. Answering to these questions will help to better understand the role of histone lysine methylation in the pathophysiologic process of OA.

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