The Effects of Link N on Human Osteoarthritic Cartilage in the Presence of Interleukin-1

By

Motaz AlAqeel

Department of Surgical Research

McGill University

Montreal, Canada

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Abstract

Osteoarthritis (OA) is a disease of diarthrodial (synovial) joints associated with pain and disability caused by proteolytic degradation of the cartilage extracellular matrix (ECM) under inflammatory conditions. Currently, there is no therapy to prevent, reverse or modulate the course of the disease. We have recently shown that Link N (LN) could prevent disease progression in a rabbit model of OA. The aim of the present study was to evaluate the regenerative potential of LN in human OA cartilage in an inflammatory milieu and if LN could affect pain-related behavior in a mouse injury model of knee OA.

OA cartilage-bone explants and OA chondrocytes isolated from various donors were treated with LN in the presence of IL-1β to simulate an OA environment. Quantitative PCR was performed on OA chondrocytes isolated from different donors to determine the effect of LN on matrix protein synthesis, catabolic enzymes, cytokines and neurotrophic factor NGF expression. The ECM proteins and proteoglycans were extracted from the explants. The sulfated glycosaminoglycans (GAG) were analyzed by the dimethyl methylene blue (DMMB) dye-binding assay while the expression of type II collagen and nuclear factor-kappa B (NF-κB) signaling was analyzed by western blot. Partial Medial Meniscectomy (PMM) was performed on 8-10 week-old C57BL/6 mice. Twelve weeks post-surgery, mice were given a 5 µg intra-articular (IA) injection of LN or PBS and a Von Frey test was conducted over 24 hours to measure the degree of mechanical allodynia in the hind paw.

Although IL-1β decreased the expression of aggrecan and significantly increased the expression of cartilage degrading enzymes and inflammatory molecules, including ADAMTS-4, MMP-3, IL-1β, IL-6, and NGF, when in combination with LN, these catabolic factors were supressed. The proteoglycan content was significantly decreased when OA explants were treated

IL-1β, however, this effect was inhibited in the presence of LN. The quantity of extractable type II collagen was also increased when explants from OA cartilage were treated with LN in the presence of IL-1β. Upon investigation of the canonical signaling pathways IL-1β, NF-□B, LN significantly inhibited its activation in a dose dependent manner. Surprisingly, IA injection of LN into mouse knee joints significantly reduced PMM-induced OA pain.

LN can modulate proteoglycan and collagen synthesis in human OA cartilage through inhibition of IL-1β-induced biological effects. In addition, LN suppressed mechanical allodynia in an OA PMM-mouse model. These results support the concept that biological repair by LN administration could provide therapeutic potential in OA.

Résumé

L'arthrose ou l'ostéo-arthrite est une maladie dégénérative touchant les articulations diarthrodiales (synoviale). Elle est causée par la dégradation protéolytique de la matrice extra-cellulaire (MEC) du cartilage dans des conditions inflammatoires et se manifeste par une douleur articulaire et par une amplitude de mouvement réduite. Elle est Il n'y a actuellement aucun traitement pour prévenir, inverser ou moduler l'évolution de la maladie. Nous avons montré récemment que Link N (LN) pourrait contrer la progression de la maladie dans un modèle d'arthrose chez le lapin. Le but de la présente étude était d'évaluer le potentiel régénératif de LN dans le cartilage arthrosique humaine en milieu inflammatoire et de déterminer si LN pouvait affecter les comportements associés à la douleur dans un modèle d'arthrose du genou chez la souris.

Des explants d'os-cartilage et des chondrocytes ont été isolés à partir du cartilage arthrosique de donneurs et ont été incubés avec LN en présence d'IL-1β pour simuler le milieu inflammatoire de l'arthrose. Des analyses par PCR quantitative ont été effectuées afin de déterminer l'effet de LN sur la synthèse des protéines matricielles, des enzymes protéolytiques, des cytokines pro-inflammatoires et sur l'expression génique du facteur neurotrophique NGF.

Les protéines matricielles et les protéoglycanes ont été extraits des explants. Le dosage quantitatif des GAG a été déterminée par la méthode au bleu de diméthylméthylène (DMB), tandis que la synthèse du collagène de type II et la signalisation du facteur nucléaire-kappa B (NF-KB) ont été évalué par Western blot. Une méniscectomie médiale partielle (MPM) a été appliqué chez les souris C57BL/6 âgées de 8 à 10 semaines. Douze semaines après la chirurgie, les souris ont reçu une injection intra-articulaire (IA) de 5 µg de LN ou de PBS et l'allodynie mécanique au niveau de la patte postérieure a été mesuré par le test de Von Frey pour 24 heures.

Pendant que l'incubation avec IL-1β ait diminué l'expression génique de l'agrécane en augmentant de manière significative l'expression d'enzymes protéolytiques et de molécules inflammatoires qui dégradent le cartilage, incluant ADAMTS-4, MMP-3, IL-1β, IL-6 et NGF, l'expression de ces facteurs cataboliques a été inhibé lors de la co-incubation avec LN.

La concentration en protéoglycanes était significativement réduite lorsque les explants de cartilage arthrosique étaient traités avec IL-1β. Cependant, cet effet était inhibé en présence de LN. La quantité de collagène de type II a était également augmentée lorsque les explants étaient traités avec LN en présence du IL-1β. Lors de l'étude des voies de la signalisation canoniques IL-1β, LN a inhibé de manière dose-dépendante activation du NF-κB. Surprenant, l'injection intra-articulaire de LN dans les genoux de souris a réduit de manière significative la douleur reliée à l'arthrose induite par le PMM.

LN can modulate proteoglycan and collagen synthesis in human OA cartilage through inhibition of IL-1β-induced biological effects. In addition, LN suppressed mechanical allodynia in an OA PMM-mouse model. These results support the concept that biological repair by LN administration could provide therapeutic potential in OA.

En conclusion, LN peut moduler la synthèse de protéoglycanes et de collagène dans le cartilage humain arthrosique en inhibant les effets biologiques induits par IL-1β. De plus, LN a supprimé l'allodynie mécanique dans un modèle d'arthrose induite par PMM chez la souris. Ces résultats confirment le rôle potentiel du LN pour le traitement de l'arthrose.

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Motaz AlAqeel

SECTION 1: INTRODUCTION

A) Rationale and Objectives

Due to an imbalance of the anabolic and catabolic activities of chondrocytes, the extracellular matrix of the articular cartilage exhibits an increase in degradation leading to joint osteoarthritis (OA). Interleukin-1 beta (IL-1β) is a major catabolic cytokine implicated in OA. It downregulates the synthesis of matrix proteins and enhances their degradation through upregulation of catabolic enzymes such as Matrix Metalloproteinases (MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS). Therefore, cartilage repair requires both, the stimulation of matrix production and the inhibition of the detrimental effect of IL-1β on matrix degradation. Link N (DHLSDNYTLDHDRAIH) is a naturally occurring peptide generated in vivo by the N-terminal proteolytic fragmentation of the link protein during tissue turnover. It is present in the extracellular matrix of many tissues including articular cartilage and intervertebral disc. Our laboratory has previously shown that Link N can stimulate the synthesis of extracellular matrix proteins like aggrecan (Agg) and collagen type II (Col II) while down-regulating the expression of proteolytic enzymes in the disc, and can stimulate matrix production in vitro, in vivo in a rabbit model of disc degeneration and in ex vivo human intervertebral disc (IVD) organ culture. Our goal is to determine whether Link N can stimulate matrix production and suppress protease production in human OA cartilage explants and chondrocytes in the presence of IL-1β, and, if so, to determine the molecular mechanism for Link N. We hypothesize that Link N may be capable of preserving matrix protein synthesis and weather in part, be due to its ability in suppressing IL-1β-induced activation of NF-κB.

B) Review of the Literature

1. Articular Cartilage

Articular cartilage (AC) is the smooth thin layer of specialized connective tissue that lines the epiphyses of joint ends of bones (Figure 1)(1). It is a unique form of tissue, formed by only one cell type and has a rich extracellular matrix (ECM) that is highly specific. It is perfectly adapted to provide a lubricated, low-friction load-bearing surface for the articulating bones(1).

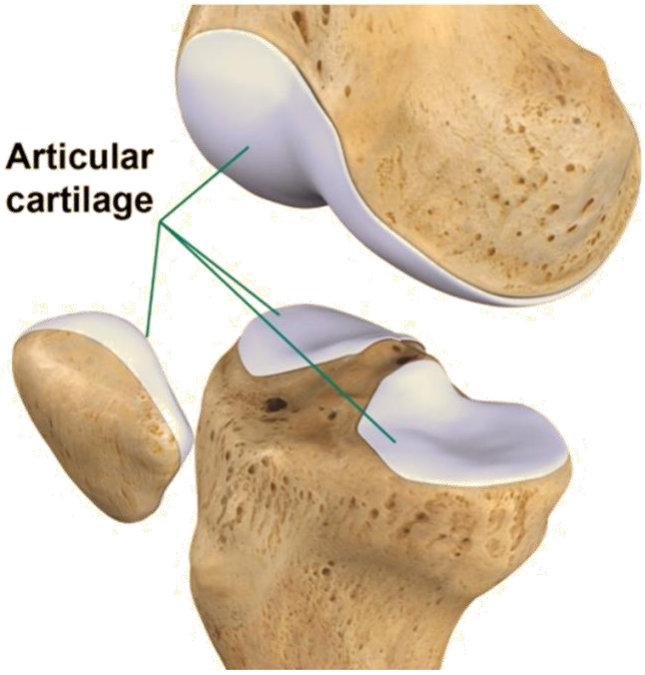

Figure 1: Articular cartilage in the knee joint.

AC is exposed to different loads and compressive forces depending on its location in the body. For instance, the forces exhibited in the hip joint have been measured to be 3.3 times a person's

bodyweight, while the contact pressure (compressive stresses) routinely reaches 2-11 MPa, increasing up to18 MPa during more stressful exercises like standing up (2). The knee joint encounters a load that is estimated to be 3.5-times bodyweight, while in the ankle joint the load is around 2.5 times bodyweight (3). Mature healthy cartilage can withstand these forces for decades without expressing signs of deterioration or failure.

AC function is intimately linked to its unique biomechanical properties:

- provides a shock absorptive capability that can adjust to the different biomechanical forces during joint movement, distributes the compressive forces and endures the shear stress (4);
- has a high deformation capability, decreasing the contact stress, by increasing the area of contact between surfaces (4);
- provides lubrication with minimal wear, during joint movements over decades of constant use (4).
- the articular chondrocytes produce an ECM which can withstand and absorb the compressive forces applied to the joint during movement.

These unique tensile and viscoelastic properties originate from the cartilage ECM composition, architecture, and organization.

1.1. Extracellular Matrix of the Articular Cartilage

The three-dimensional matrix of the AC is mainly composed of collagen, proteoglycans, and water (5). It also contains other components such as noncollagenous proteins and glycoproteins, which are present in much smaller concentrations. This mixture of fluid and ECM contributes to the biomechanical advantage of low friction characteristics of AC.

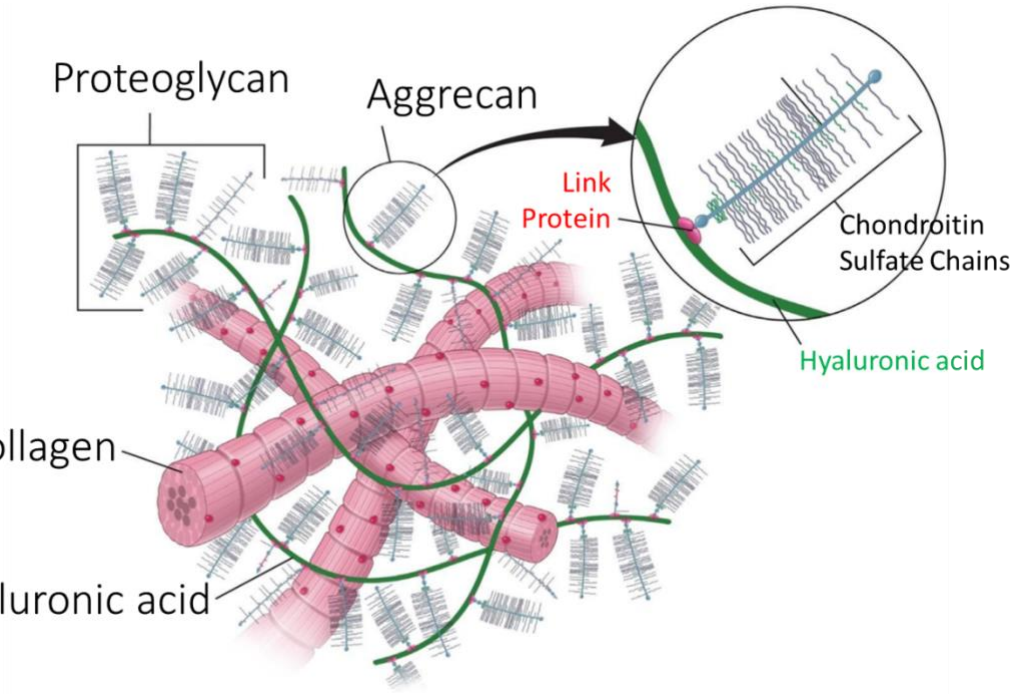

Figure 2: The major extracellular macromolecules of the articular cartilage

Collagen (10%-25%) serves as the main skeleton of AC, forming a comprehensive network throughout the matrix. The major fibrous component is type II collagen, which provides the tensile strength of cartilage. The size of collagen fibrils varies between different zones of the cartilage, from 20nm in the superficial zone up to 120 nm in the deep zone (6).

Charged proteoglycan (PG) aggregates are embedded in the fibrous collagen network (7) and play a crucial role in the organization and structure of cartilage ECM (Figure 2). PGs constitute the second largest proportion (5%-15%) in AC after collagen and play a significant role in cell regulation mechanisms, due to their strong interaction with growth factors and cytokines through glycosaminoglycan (GAG) chains. The major cartilage PG is the aggrecan molecule, which interacting with hyaluronic acid (HA) forms large aggregates (size 1–4 μm). The negatively charged aggrecan-HA complexes are highly hydrated exhibiting gel-like properties. The osmotic pressure exerted by the charged groups of the hydrophilic GAG bristles of the aggrecan molecules leads to swelling of the PG assemblies. The swelling is constrained by the collagen network, placing it under tension. The mechanical properties of cartilage are governed by the balance of the swelling pressure of the aggrecan-HA complexes and the elastic pre-stress developed in the collagen network (6-8).

Despite the broad representation of collagen and PG in AC, water is the most abundant component. Up to 65%-80% of AC wet weight is due to water molecules (3). The collagen-PG matrix contains most of the water within the interstitial intrafibrillar space, where the PG aggrecan provides the negative charge.

The composition of AC varies between different joints (3); the ankle joint has a higher concentration of PG in comparison to the knee joint. However, there is a similar collagen content between ankle and knee joint. Different zone of AC has a different concentration of PG; the superficial zone has the lowest level of PG followed by the middle and deep zones with the highest concentration of PG (2).

1.2. Chondrocytes

Cartilage chondrocytes are the only AC resident cells responsible for both synthesis and turnover of the abundant ECM (7). They are surrounded and protected by the ECM. They lack access to vascular supply and depend on diffusion of nutritional supply, glucose transporter proteins, and active membrane transport (9, 10). They exist at low oxygen tension within ECM ranging from 10% at the superficial zone to less than 1% in deep zones. By upregulating hypoxia-inducible factor-1-alpha (HIF-1α), chondrocytes can adapt to low oxygen tension, stimulate the expression of many factors such as vascular endothelial growth factor (VEGF) (11) and glucose transporters (GLUTs) (9) as well as various genes related to cartilage anabolism and chondrocyte differentiation (12).

The volume of chondrocytes in the AC varies between 1%- 6% of the ECM. These cells ensure also that cartilage functions properly by facilitating fluid exchange within the matrix, which is essential to absorb nutrients and to remove waste (7).

In the embryonic stage, cartilage starts to develop and continues to grow reaching a final stage of maturity around adulthood. At this stage, the chondrocytes function changes from cartilage building to cartilage maintenance (13). Additionally, the number of chondrocytes is much higher during the neonatal and infancy period. The chondrocytes in developing cartilage are called adult cartilage chondrocytes and exhibit low anabolic and proliferative activities (13).

Chondrocytes are unevenly distributed in AC, being surrounded by a pericellular matrix composed mainly of PG and noncollagenous proteins (4). The pericellular matrix has distinguished functions in AC; it provides a biomechanical barrier to protect chondrocytes from mechanical

stress and plays a major role in conduction of signaling events between chondrocytes and surrounding environments (14).

Giving the avascular nature of AC, chondrocyte metabolism depends on diffusion of nutrients, and other regulatory factors from synovial fluid and subchondral bone. This diffusion is facilitated by joint motion and load, which help in creating a flow of the extracellular fluid from in or out AC. Chondrocytes are heterogeneous in shape and function, and their phenotype is influenced by their depth within the cartilage, cell surface receptors and joint specific demands. Thus, each zone of AC has distinct chondrocyte metabolism activity, producing different matrix composition that corresponds to the mechanical, physicochemical and electrical signals that the cell receives (14).

1.3. The Structure of the Articular Cartilage

AC matrix is highly organized from the surface to the deepest layers into four architectural zones, with striking variations in both chondrocytes and their surrounding ECM: the superficial zone, the transitional zone, the deep (or radial) zone, and the calcified cartilage zone (Figure 3). Each zone has its unique feature that is linked intimately to function, matrix composition and organization, collagen fiber orientation, and cell phenotype and morphology (1, 6). Although a progressive transition is observed between these zones, there is no clear boundary (1).

The superficial zone is the thinnest zone of AC (Figure 3) that encounters considerable friction forces. This zone contains the highest proportion of fibrillar collagen but a small amount of PGs (7). The collagen fibers in this zone are intersecting between each other and are oriented parallel to the AC surface, which provides the high tensile force and resistance to shear stress at the joint surface. Toward the middle of this zone, the fibers exhibit an oblique orientation (5). This zone

has the highest water content and a thin film of synovial fluid called lubricin covers the cartilage and contributes to the gliding surface (15). This layer also acts as a filter for large macromolecules, thereby protecting the cartilage.

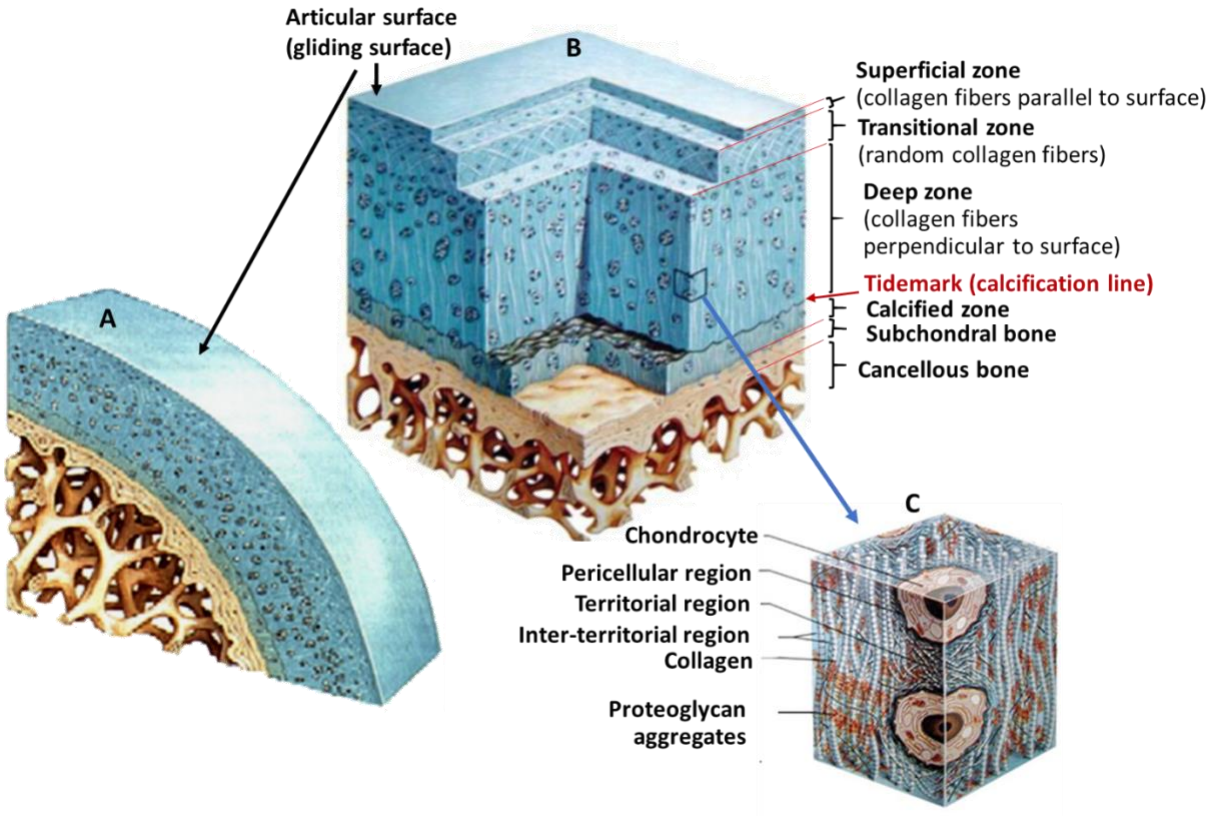

Figure 3: Schematic representation of human articular cartilage and pericellular matrix of the chondrocytes. A) Architectural layout of the articular cartilage according to its various layers; B) Articular cartilage has four distinctive zones: superficial zone, transitional zone, deep zone and calcified zone; C) Representation of a fragment of articular cartilage (Figure obtained with permission from OrthoLab, modified from Sopena-Juncosa et al (16).

The chondrocytes in this zone have a flattened ellipsoid shape, lining parallel to the joint surface (Figure 4). These chondrocytes express the Proteoglycan 4 (PRG4) gene, which encodes for superficial zone protein (17, 18).They synthesize a high concentration of collagen and a low concentration of PGs. They also secrete abundantly the superficial zone protein known as lubricin (5) Lubricin will attach covalently to the glycoprotein and serve to decrease the coefficient of friction and facilitate gliding motion. Regulation of Lubricin production is critical to the homeostasis and maintenance of AC. A mutation in the PRG4 gene inhibits lubricin expression that may result in an alteration in AC surface due to non-inflammatory early-onset cartilage degradation and joint failure (5). The loss of cellular lubricin followed by the alteration to the superficial zone of AC was also demonstrated in the early onset of OA in a meniscectomized sheep model for early OA (19).

The unique chondrocyte morphology in this zone, characterized by less pericellular matrix around the chondrocytes, flatten the surface. These chondrocytes express a series of particular cells surface markers and other proteins. Also, they contain the highest concentration of progenitor cell that express cell surface markers such as Stro-1 or Notch-1, which display the potential of differentiation to different lineages such as adipocytes or osteoblasts (7).

The transitional zone represents more than half of AC height and is composed of rounded chondrocytes surrounded by abundant ECM (Figure 4). This zone is rich in large-diameter type II collagen fibers, PG aggrecan, and noncollagenous proteins (8). Collagen fibers express an oblique pattern crisscrossing at the right angle to each other in a leaf-like manner, serving as the first line of resistance against compressive forces (Figure 4). In the transition to the deep zone, the collagen fiber shows a vertical orientation (5). During the presence of a compressive force, large volumes

of water exit the transitional zone toward the joint and once the mechanical stress removed, new fluid returns to the cartilage.

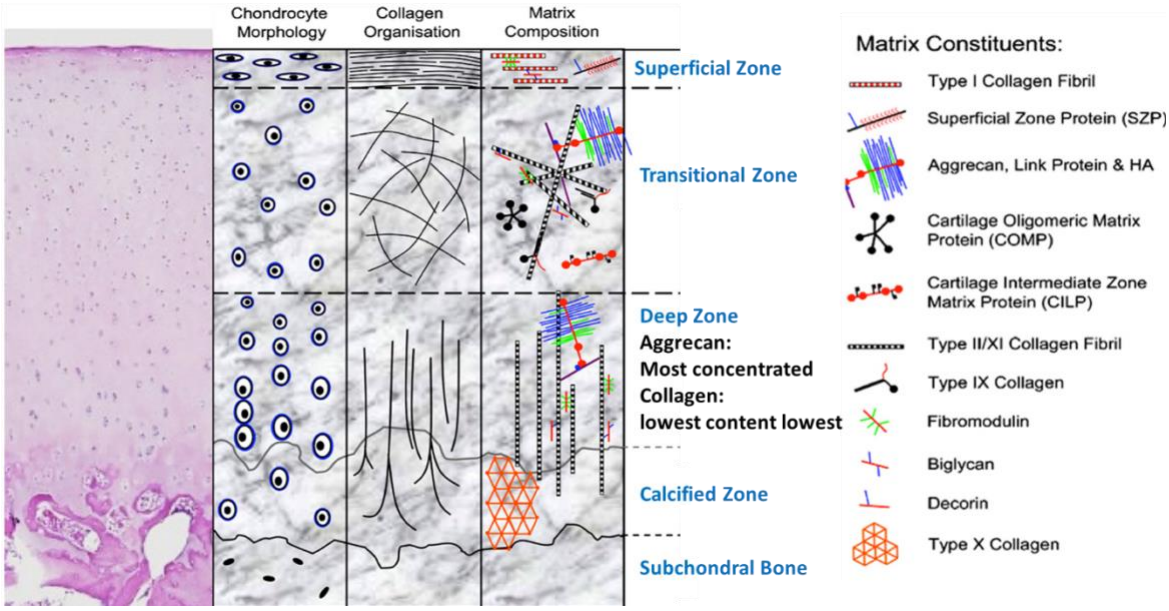

Figure 4: Chondrocytes morphology, collagen organization and matrix composition along the cartilage structural zones. (Figure obtained with permission from OrthoLab)

The number of chondrocytes is less in the transition zone. They have a rounded shape (Figure 4) and a higher number of synthetic organelles such as Golgi bodies, mitochondria, and endoplasmic reticulum (3). They produce fewer but larger randomly-oriented collagen fibrils, compared to those fibrils in the superficial zone.

The deep zone represents a third of the AC volume (Figure4) and is characterized by the highest consistency of PGs, the lowest concentration of water (65%) (20) and the lowest number of cells (21, 22). This zone comprises the longest collagen fibrils in a radial arrangement, being responsible for providing the highest resistance against compressive forces. Chondrocytes are oval, aligned parallel to collagen fibers and vertical to the joint line.

Chondrocytes in this zone are subjected to lower oxygen tension, driving them to adapt to the environment by upregulation of HIF-1α(23). Another important marker expressed by the deep zone chondrocytes is collagen type X, a marker for chondrocyte hypertrophy and an indicator of endochondral ossification during development (15).

Closer to the calcified cartilage zone, AC gets mineralized to form a distinct histological feature known as *the tidemark* (Figure 3). The tidemark separates the deep zone from the calcified zone and is composed of a thick, irregular surface of type I collagen organized in a horizontal fashion. It forms a firm foundation for the attachment of the type II collagen extending from the deep zone (24).

The calcified zone plays a major role in anchoring the cartilage to the subchondral bone. The vertically oriented collagen fiber in the deep zone infiltrates the calcified cartilage zone forming a robust and tight transition (1). The calcified zone is characterized by a small number of rounded, hypertrophic chondrocytes surrounded by a chondroid matrix with calcified salt. The metabolic activity of these chondrocites is limited only to type X collagen synthesis (25). This zone is also marked by the absence of PGs and the presence of large collagen fibers that are arranged perpendicular to the articular surface and anchored in the underlying subchondral bone surfaces (Figure 4) (25). This unique feature of the calcified zone helps in transforming shear stresses into compressive and tensile forces during normal joint motion (26).

Historically, the calcified cartilage zone was viewed as an impermeable barrier. However, there are several evidences suggesting that the calcified cartilage and the subchondral bone can communicate through a dense subchondral vasculature (27, 28).

1.4. Collagen

Collagen is a generic term used to name a protein with a triple helix configuration or three polypeptide chains (Figure 5). Currently, up to 26 types of collagen are genetically identified (29, 30). All members of the collagen family reside in the ECM, having variable size, shape, function and tissue distribution. Their tensile strength and torsional stability lead to the integrity of these tissues. Although the collagen family has a broad range of structural diversity, they all share one characteristic feature, a right-handed triple helix composed of three alpha chains (31). A structural requirement to assemble the collagen peptide into a triple helix is a glycine residue (Gly). Being the smallest amino acid, it allows a rotational configuration of the triple helix. Collagen itself is highly enriched in glycine, with glycine residues making up 20 to 25 percent of the amino acids in a typical collagen molecule (31). It is found in every third position of the polypeptide chain, in (Gly-X-Y)n repeats, with Hydroxyproline and Proline commonly occupying the X and Y positions. Almost 90% of the total collagen are fibril-forming.

Collagen is a major cartilage matrix component as it provides the endoskeletal structure to withstand the mechanical forces. It is the most abundant macromolecule in AC, and it makes up about 60% of the dry weight of cartilage.

Type II collagen: is the predominant contributor to the AC fibrillar matrix. It represents 90%-95% of the collagen in ECM and forms fibrils and fibers intertwined with PG aggregates. It is constituted of three identical Alpha-1 chains in a helical form; each has 1050 amino acids, with a long continuous triple helical region and a non-triple helical region called telopeptides at the terminal ends. These telopeptides do not have the characteristic (Gly-X-Y) repeats, which are usually found in the triple helical region. Initially, type II collagen forms as a precursor form called procollagen, and it has the needed peptide for proper assembly. Once it is released from the

cell, these propeptides are removed, and fibril formation occurs. In contrast to other types of collagen, type II collagen has a higher content of hydroxylysine, glucosyl and galactosyl residues, which mediate the interaction with PG forming a fiber-reinforced composite solid matrix (32).

Other collagens: Collagen types I, IV, V, VI, IX, and XI are also present in a minor proportion, helping to form and stabilize the type II collagen fibril network.

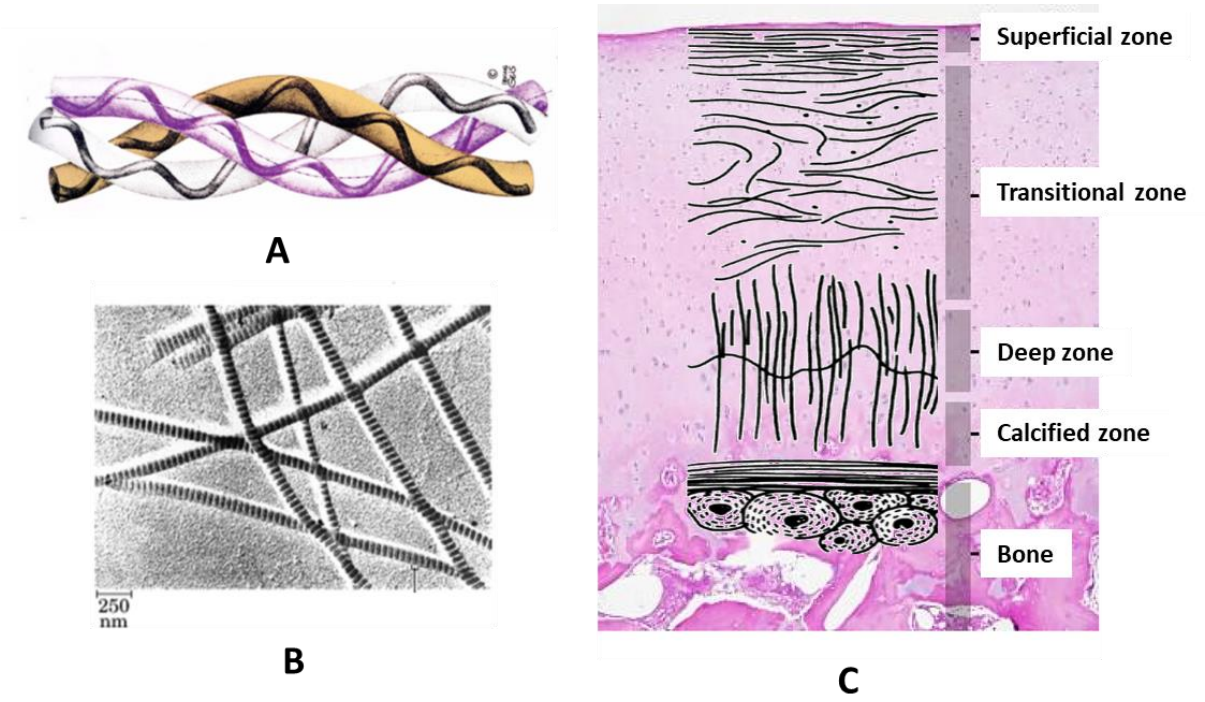

Figure 5: **Structure and distribution of collagen within the ECM of articular cartilage.** A) Schematic representation of the collagen triple helix; B) Scanning electron microscopy of collagen fibers; C) Schematic representation of the collagen distribution within articular cartilage (Figure obtained with permission from OrthoLab).

Type X collagen: is considered a special component of hypertrophic chondrocyte in the fetal and juvenile growth plate(31) (33). It is present in the calcified zone of the cartilage and plays a role in the endochondral ossification and matrix calcification. It is speculated to be involved in the calcification process in the deeper hypertrophic zone (31)(33). However, the function of type X collagen is not understood entirely.

The tensile strength of the collagen type II is strongly correlated with the crosslinks between the collagen fibrils (33)(38), which influence the stability of the collagen molecules within the tissue (34) (37). Therefore, the collagen cross-linking plays a key role in the mechanical performance of the AC (35, 36) (39, 40).

1.5. Proteoglycan

Proteoglycans (PGs) are a large family of a macromolecules that exist in ECM and cell surface (37). These include aggrecan, fibromodulin, decorin, and lumican. A unique feature in common is the presence of multiple GAG side chains, which are linked covalently to the core protein. These GAG chains have multiple regulatory mechanisms, from cell signaling, morphogenesis, cell migration and matrix homeostasis (30). Each member of the PG family serves a particular function, determined directly by the core protein and number of GAG chains. AC is distinguished by a high level of PG, which is responsible for the cushioned nature of cartilage providing it with shock absorbance property and resistance to compressive loads (37).

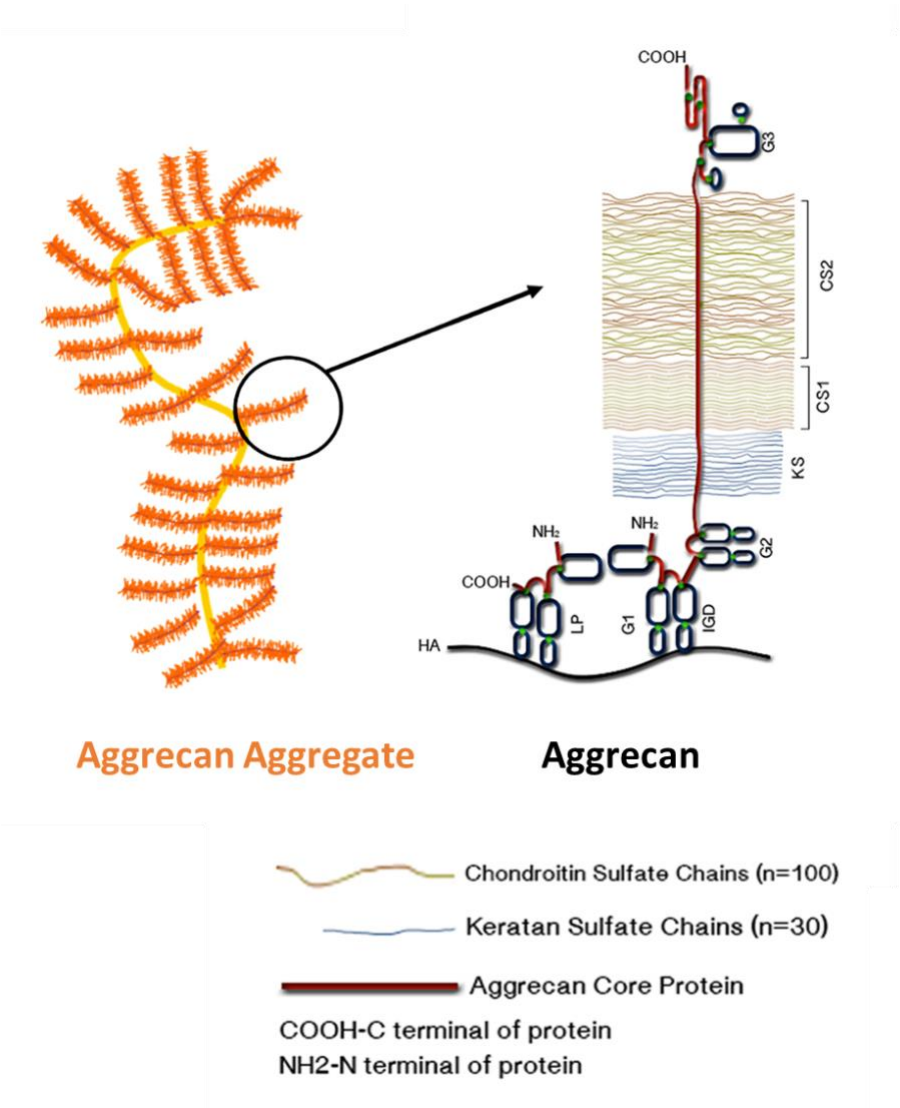

Figure 6: The structure of aggrecan. Diagram showing aggrecan, the most common proteoglycan in articular cartilage, bound to hyaluronic acid (HA) through the amino-terminal G1 globular domain. The stability of this binding is reinforced by Link Protein (LP), a molecule that resembles structurally and functionally to G1 domain. The keratan sulphate (KS) chains and chondroitin sulfate chains (CS1 and CS2 domains) are directly bound to the aggrecan core protein. Aggrecan-hyaluronan aggregates can be 5 μm long. (Figure obtained with permission from OrthoLab).

PGs play a significant role in cell regulation mechanisms, due to their strong interaction with growth factors and cytokines through the GAG chains. Aggrecan is the major PG in AC. It constitutes 35% of the dry weight of protein found in cartilage and presents in the form of highly condensed aggregates (38, 39). They are highly negatively charged due to the large number of GAG chains, attracting water and giving the cartilage the high-water content (40). Each aggrecan aggregate contains a long hyaluronan (HA) filament, with up to 100 aggrecan molecules branching from it (Figure 2 and 6). The binding sites for aggrecan are stabilized by link protein (LP), which interacts with both aggrecan and HA (41).

The main function of PGs is to provide resiliency to AC. Sudden loading is initially absorbed by the fluid excursion to the synovial space, serving to absorb the energy of the impact (42). When the load is removed, the water is attracted back into AC by the highly negative charged PG. Aggrecan aggregates play also an important role in nutrient and solute transport in cartilage due to their negative charge.

Aggrecan has a unique structure consisted of three globular regions: G1, G2, and G3 (Figure 6). G1 region is located at the amino terminal of the core protein where it interacts with HA. It forms three domains bonded by a di-sulphate bond (A, B, B′). The A domain is responsible for interacting with LP while the B domains interact with HA (43, 44). Therefore, the stability of the aggregates depends on the interplay between these structures. In contrast to the G1 region, the G2 region has two domains, similar to the B domains in G1. However, they do not have the capability of binding to HA, due to the presence of additional N- linked oligosaccharide that prevents the folding of this region and binding to HA (45, 46). The exact function of G2 is not clear. However, some authors suggest that it has a role in inhibiting aggrecan secretion before GAG modification in Golgi (47). The region in between G1and G2 is called the Interglobular domain (IGD) and is

composed of 127 protein residues. This region has significant value and has been the focus of many researchers due to its susceptibility to proteolytic cleavage. The region between G2-G3 represents more than 80% of the molecular mass of Aggrecan and is filled with long GAG attachments. This region is divided into three domains. The Keratin Sulfate (KS) rich domain is the closest domain to G2, where as many as 30 KS chains are attached to serine residues via O-linked oligosaccharide (48). This domain is followed by Chondroitin Sulfate (CS) domain, which is divided into two subdomains, CS1 and CS2. This region is crucial for aggrecan core functions, cartilage hydration and resistance to compressive loading. The CS1 domain contains repeats of nineteen amino acids which contain serine-glycine replicates, each serine residue serving as possible attachment for CS. The CS2 domain has a different amino acid sequence than CS1, which alter the susceptibility to proteolysis.

Chondroitin sulfate domains are followed by The G3 region at the carboxy terminus of the core protein. The G3 region is composed of two epidermal growth factor-like domains, one C-type lectin-like domain and one complement regulatory protein-like domain. The exact function of G3 and its domains are not clearly understood, but it was found that the G3 region is missing from mature aggrecan, and the number of aggrecan that contain G3 is decreasing with age (49). This suggests that the G3 region has a function in the early stages of aggrecan synthesis and secretion, but a lesser role subsequently. Since GAG chains position, length, and sulfation of vary throughout life, aggrecan does not have a constant structure.

Aggrecan strongly interacts with hyaluronic acid and link protein (LP) through G1 domain, forming stable ternary complexes in the extracellular matrix (Figure 7). LP plays a major role in the stability of aggrecan aggregates and a protective function preventing the degradation of HA by hyaluronidases or free radicals (50).

The assembly of aggrecan aggregates requires a meticulous organization by chondrocytes. It starts with HA synthesis at the cell surface and pericellular space. HA has more than 10,000 disaccharide units that might serve as a possible attachment site to the G1 domain (51). However, due to the large size of the aggrecan molecule and the GAG chains, each aggrecan molecule is spaced by a 50-disaccharide unit. However, it is not understood where and when the interaction between the aggrecan and the HA takes place.

In a load-free environment, aggrecan swells as the GAG chains get hydrated. However, this swelling is being balanced by the tensile force of collagen fibers. When AC is subjected to a compressive force, the water is displaced from the matrix, the size of aggrecan shrinks and the aggrecan molecules are brought into closer proximity balancing the applied load. When the compressive force dissipates, the aggrecan swells again, restoring the original equilibrium. Therefore, proper functioning of the AC requires: a high concentration of aggrecan, the formation of large aggregates, and a high degree of aggrecan sulfation (52).

The aggrecan content and structure vary throughout life and its functional ability declines with age (53). Aggrecan encounters two major deleterious changes; the first is a decrease in aggrecan molecule numbers and aggregate, the second is core protein truncation due to proteolysis (53). When cleavage of aggrecan occurs at the IGD or glycosaminoglycan-attachment region, this results in a significant loss of the anionic charge of aggrecan molecules. The two major families that are responsible for proteolytic cleavage of aggrecan are MMPs, mainly stromelysin (MMP3) and MMP13, and ADAMTS, in particular ADAMTS4 and ADAMTS5, the two major aggrecan-degrading enzymes (54).

1.6. Link Protein (LP)

Cartilage-Link Protein (LP, 40–48 kDa) is a small globular glycoprotein that stabilizes the interaction between aggrecan and hyaluronan in large PG aggregates. It has a structural resemblance to the G1 region of aggrecan (55, 56) and has three main domains (A, B, B') (Figure 7). The A domain of LP interacts with the A domain of the aggrecan G1 region while the B domains interact with HA. In the absence of LP, the binding between the HA and aggrecan is fragile and dissociates at pH 5 or lower.

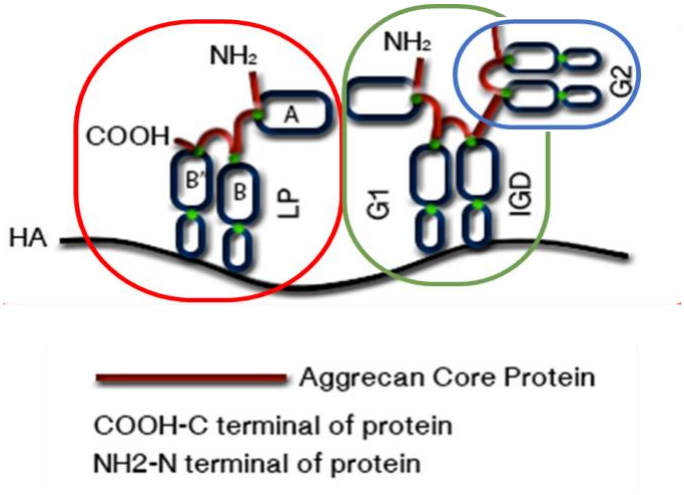

Figure 7. Interactions between Hyaluronic Acid (HA) and Link Protein (LP). Schematic diagram showing relative positions of G1 domain of core protein (green) and loop A of LP (red) and their site of interaction. Also showing the interaction of loop B and B' of LP. (Figure obtained with permission from OrthoLab).

In addition to stabilizing the PG aggregates, LP also serves two additional functions. First, it forms a coat along the HA that helps protect the HA from degradation by hyaluronidases or free radicals (50). Second, it participates in the process of delayed aggregation (57). During the synthesis of new aggrecan molecules, the G1 domain is not able to interact with HA until it goes

through folding and conformational changes, known as delayed aggregation (58). LP plays a role in inducing the conformational changes (57).

In the cartilage, LP exists in three isoforms that originate from the same core protein structure: LP1 (48 kDa), LP2 (44 kDa), and LP3 (41kDa) (46, 59). The difference between LP1 and LP2 is the degree of glycosylation and an additional N-linked oligosaccharide present only in LP1 isoform. However, there is no evidence for any functional difference between the two LP forms.

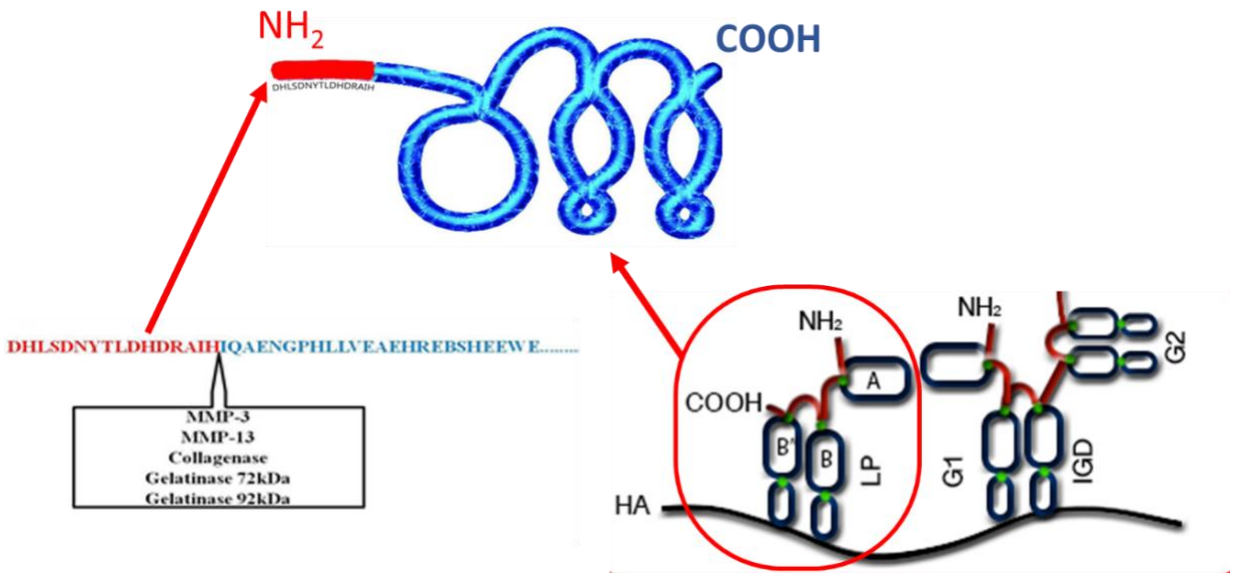

Figure 8: Generation of Link-N peptide. Cleavage site between His16-Ile17 residues on Link Protein (LP) generating the 16 amino acid Link N peptide. (Figure obtained with permission from OrthoLab)

LP3 is a proteolytic cleavage product of either LP1 or LP2 generated by MMPs during tissue turnover with the release of the N-terminal 16 amino acid fragment called Link N peptide (DHLSDNYTLDHDRAIH) (Figure 8) (60-62). The amount of LP3 accumulates with age as a consequence of this cleavage. Despite the loss of the N-terminal peptide, LP3 maintains functional properties similar to those of the intact molecule, and a powerful ability to stabilize aggrecan to HA (46, 47, 63).

1.7. Articular Cartilage Homeostasis

AC is a metabolically active tissue that performs a constant turnover (64). In normal conditions, AC matrix homeostasis is dependent upon the anabolic and catabolic pathways controlled by the resident chondrocytes (Figure 9) (33). They maintain and remodel the cartilage matrix framework in response to a variety of factors within joint mechanical and chemical environment(65). The cells produce the main cartilage structural macromolecules involved in tissue formation, such as collagen and PG, but also a variety of metalloproteinases, such as collagenase, gelatinase, stromelysin, and aggrecanase which are responsible for tissue turnover.

Since chondrocytes are responsible for synthesis and maintenance of AC, the active area of degradation and synthesis in AC is focused directly in the chondrocyte surrounding zone rather than in the territorial and interterritorial regions of the tissue (66). Additionally, not all the molecular components are reformed at the same rate. For instance, collagen has a very slow turnover rate as it takes around 117 years (67), while aggrecan has a relatively accelerated turnover rate with a half-life of 3.4 years days in human cartilage (68). Therefore, differences in the turnover exist based on the type of protein and its location within the tissue(69).

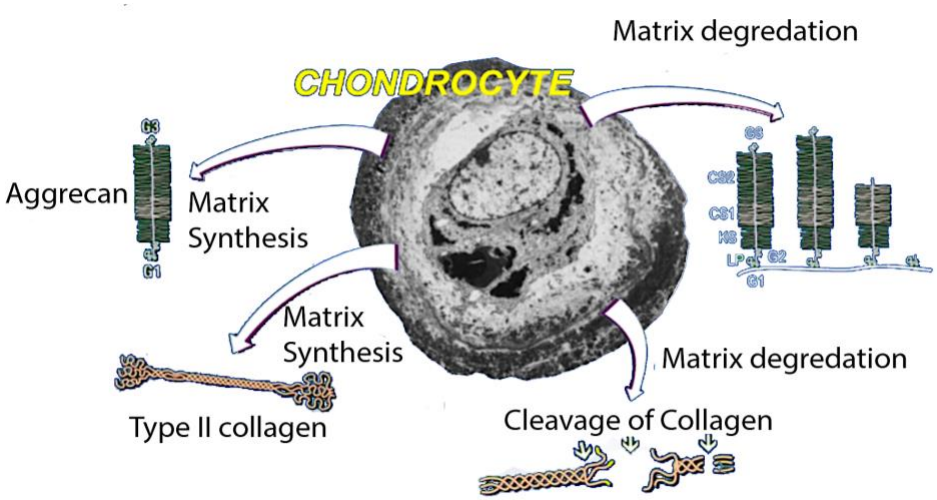

Figure 9: Extracellular matrix balance. In a healthy articular cartilage, there is an equilibrium between the catabolic and anabolic factors which the keeps balance in collagen and aggrecan synthesis and degradation. (Figure obtained with permission from OrthoLab)

Cartilage turnover in development and tissue repair involves a precise regulation of matrix protein degradation, anabolic processes exceeding the catabolic activities. However, the capability of chondrocytes to synthesize various ECM proteins, as well as their response to anabolic stimuli and their proliferative capacity diminishes with age. When chondrocytes fail to maintain the equilibrium and catabolic processes overtake anabolic activities, cartilage degeneration occurs with the onset of OA (7).

2. Osteoarthritis

2.1. Introduction to Osteoarthritis

Osteoarthritis (OA) is one of the most prevalent types of arthritis and a leading cause of pain and disability affecting around 15% of the adult population across the developed and developing world, and over 49% of the population aged 65 and over (70). Recent estimates from US data indicate that the prevalence of OA increased from 21 million adults aged 25 years or older in 1995 to 27 million in just over a decade (71), and in expected reach 130 million people worldwide by 2050 (72). Based on this increasing prevalence, OA has been identified as one of the priorities for the "Bone and Joint Decade" (70).

OA is more than a simple process of "cartilage wear and tear". It is a whole-joint disease affecting all anatomical structures of the joint, such as subchondral bone, synovial capsule, and membrane. However, AC has the dominant role in the onset and progression of OA. Major clinical symptoms include chronic pain, joint instability, stiffness and radiographic joint space narrowing (71).

OA can be broadly classified by etiology into primary (idiopathic) or secondary. Primary OA is the more commonly diagnosed, being usually associated with natural aging of the joint. Damage from mechanical stress (i.e. repetitive joint overloading during sports activities) with insufficient self-repair by joints is also recognized as a cause for primary OA (73). Secondary OA is usually monoarticular and is caused by another disorder or condition, such as obesity, joint injury from trauma (post-traumatic arthritis), joint anatomic abnormality (i.e. joint morphology, limb alignment, leg length discrepancy and poor muscle strength), or inherited metabolic disorders (74).

Regardless of the underlying mechanism, OA implies AC damage, bony osteophyte formation, and sclerosis of the subchondral bone, and in advanced cases, subchondral cyst formation.

2.2. Pathogenesis of OA

Although OA is a multifactorial disease that affects the entire joint structure and function, cartilage destruction is the central feature(75). The pathophysiology of OA can be subdivided into three overlapping stages:

1. matrix alteration at a molecular level;
2. alteration of cartilage homeostasis and response to tissue damage;
3. failure to restore AC and progressive loss of ECM (Figure 10).

In early stages, only microscopic changes could be observed, such as superficial matrix disruption and localized fibrillation of the AC. As degeneration continues, the fibrillation extends deeper and through the cartilage zone until it reaches the subchondral bone (75). When cartilage breaks down, ECM degradation products, such as fibronectin, small leucine-rich PGs and collagen type II fragments, reach the synovium, eliciting an inflammatory response by the synoviocyte resulting in the upregulation of catabolic and proinflammatory mediators (76). These mediators will start a vicious "feedback amplified matrix destruction" cycle by activating the chondrocytes to intensify the synthesis of matrix metalloproteinases (MMP-4 and MMP-13) and cytokines (e.g., IL-1β, IL-6, and TNF-α), leading to cellular infiltration of macrophages and lymphocytes and further cartilage breakdown (77-80). Therefore, the tightly regulated equilibrium between anabolic and catabolic processes are disrupted due to the stimulation of the inflammatory mechanism and the release of cytokines. Moreover, the proteolytic degradation of matrix proteins results in destabilization of the macromolecular framework of the cartilage. This architectural disruption

reduces the compressive stiffness of the cartilage, making it vulnerable to further mechanical damages (81). Therefore, OA is more than a simple process of wear and tear, but rather an imbalance in the homeostasis of the joint tissue driven by the host inflammatory mediators within the joint.

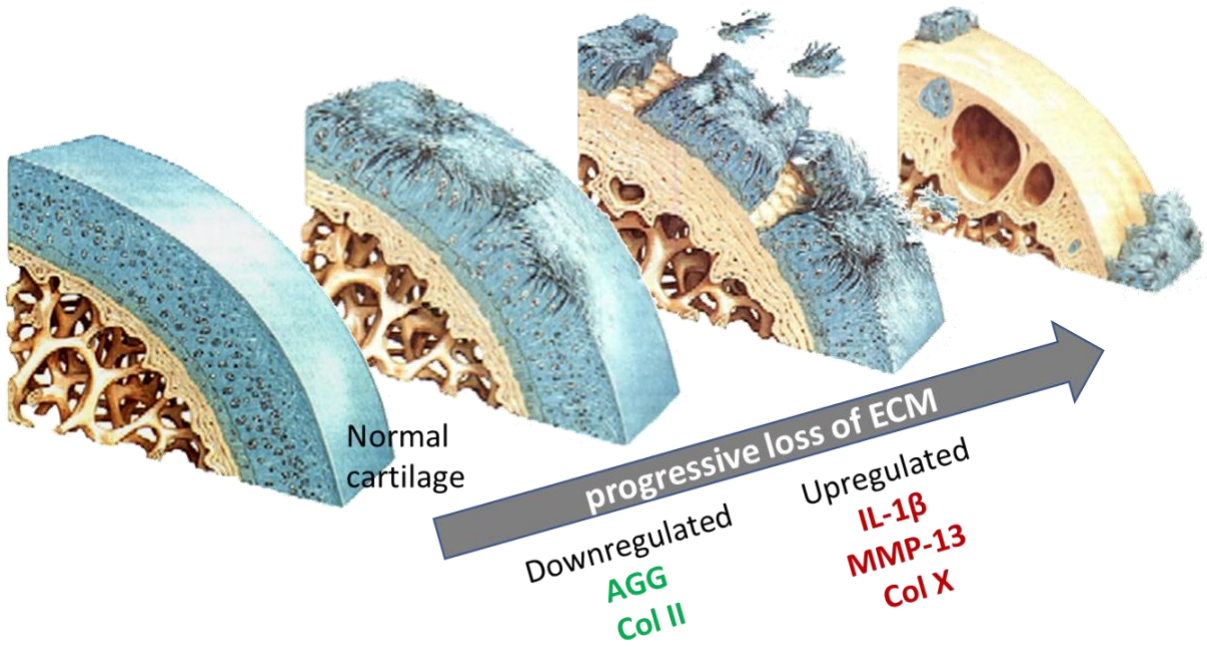

Figure 10: OA progression. Normal articular cartilage (left side) is smooth and strong. With the onset of the OA, AC starts to break down. Factors leading to cartilage degeneration: the upregulation of IL-1β and MMPs, synthesis suppression of Agg and Col II. (Figure obtained with permission from OrthoLab)

As OA progresses, subchondral bone also experiences also multiple alterations. It is still not clear if these changes occur as a response to the cartilage damage or if they have it has a driving

role in cartilage degradation (82). At the early stages of OA, there is an increase in bone remodeling, especially in the area underlying the cartilage damage. It starts with bone loss in the subchondral zone, resulting in increased porosity and reduced thickness of the subchondral bone (83). These changes cause a modification in joint shape and load transmission to the cartilage.

It is well known that AC has a limited capacity for repair. The relatively low number of chondrocytes in the tissue and their senescence are the main factors responsible for this low metabolic rate (84, 85). Additionally, unlike other connective tissues, cartilage has no blood vessels and nerve supply, making it susceptible to slow healing and insensitivity to early injuries. With aging, many molecular pathways become downregulated in the joint, affecting the cartilage homeostasis and its ability to regenerate, causing a disruption in its ECM architecture and therefore a progressive deterioration of its biomechanical properties (83). Although aging is a well-known risk factor for developing OA, gender, obesity, genetic predisposition, and anatomical factors have also been associated with OA development (86, 87).

2.3. Anabolic/Catabolic Balance in Pathogenesis of OA: Identifying Molecular Targets

Articular chondrocytes respond to any structural changes of the surrounding matrix, releasing anabolic factors and catabolic cytokines, which act in an autocrine-paracrine manner. OA is a set of complex processes leading to progressive degeneration of joints. But, contrary to what it may seem to be, it is not only a set of catabolic effects. Apart from them, anabolic anti-inflammatory processes also occur continually. Evident imbalance in metabolism observed in OA is the final manifestation of the dysregulation within the so-called cytokine network between anti-

inflammatory and inflammatory cytokines. Therefore, understanding the basic molecular mechanisms responsible for tissue degeneration is essential to identifying and developing means to efficiently prevent, block or reverse the progression of OA.

2.3.1. Anabolic Factors

The anabolism is vital for cartilage to maintain its integrity as the AC is subjected to a constant mechanical stress and/or increased catabolic proteolysis (88). Growth factors are stimuli and regulators of AC structure and function. They stimulate the anabolic activity, and in some cases, they work indirectly by inhibiting the catabolic activity.

Bone Morphogenetic Proteins (BMPs) are among the most potent anabolic factors for AC. BMPs are recognized as a subclass of Transforming Growth Factor-β (TGF-β) superfamily. They play a dominant role in joint development and appendicular skeleton growth (89). Multiple BMPs including BMP-2, BMP-7, and BMP-14 promote the differentiation of hypertrophic chondrocytes and support the differentiation of mesenchymal precursors into chondrocytes. Many studies showed the capability of BMP-2, -4, -6, -7, -9 and -13 to stimulate the synthesis of aggrecan and collagen type II (90-93). Some BMPs, like BMP-7, have the additional effect of reversing many of the catabolic responses induced by IL-1β (94). Due to their ability to promote the synthesis of aggrecan and collagen type II, they can be used in cartilage tissue engineering and to improve cartilage repair (94, 95). However, they can stimulate chondrocyte hypertrophy and promote endochondral ossification (96).

Transforming Growth Factor-β (TGF-β) 1, 2, and 3 are also potent anabolic inducers of PG and collagen type II synthesis (97). The microarray analysis of articular chondrocytes showed that

TGF-β has a preventative role in cartilage injury by counteracting the expression of many IL-1β – induced genes (98). In animal models, intra-articular injection of adenovirus-mediated delivery of TGF-β showed a robust stimulation of PG synthesis and minimized cartilage damage. However, osteophyte formation, synovial hyperplasia and swelling were also observed (99, 100). *In vitro*, TGF-β induces the expression of ADAMTS-4 promoting aggrecan degradation in human chondrocytes (101). Blocking the activity of TGF-β receptor (TGF-βRII) or latency-associated peptide-1 (LAP-1), resulted in the loss of cartilage PG (102, 103). These studies highlighted the importance of a balance between TGF-β family and the natural antagonist to have an either protective or harmful effect.

Insulin-Like Growth Factor-1 (IGF-1) induces the upregulation of aggrecan and type II collagen synthesis and plays a significant role in supporting chondrocyte survival (104, 105). OA chondrocytes are hyporesponsive to IGF-1 and this is thought to be due to the increased level of IGF-binding protein (IGFBP) that might interfere with the free level of IGF-1(106). The desensitization phenomenon of IGF-1 could be related to IL-1 induced suppression of cytokine signaling 3 (SOCS3) which interferes with IGF-1 signaling by reducing the phosphorylation of the insulin receptor substrate-1 (IRS-1) (107).

Fibroblast Growth Factors (FGFs), are part of a broad superfamily that includes ligands like FGF-2, -4, -8, -9, -10, and -18, and receptors such as FGFR1, 2, and 3. They play a dominant role in coordinating cell proliferation during chondrogenesis and endochondral ossification of a newly developed skeleton (108). Among the FGF superfamily, FGF-2 is heavily studied in AC as it showed a robust regenerative capability for AC. Recent studies have shown that FGF-2 stored in the adult cartilage matrix is released on physiological joint loading or mechanical injury to the

tissue (109, 110). During injury or OA progression, excessive release of FGF-2 may contribute to the increase in chondrocyte proliferation and the reduction of its anabolic activity (111-113).

2.3.2. Catabolic Factors

The ability of chondrocytes to degrade ECM constituents depends on their capacity to synthetize and secrete proteinases. Many studies have established a close association between molecular destruction of the cartilage matrix and an increased activity of proteinases (Figure 11) (114-116). Over a decade, the primary focus was on the enzymatic breakdown of cartilage matrix and the catabolic enzymes responsible: the MMPs and the so-called aggrecanases of the ADAMTS-family of proteinases (117). These enzymes are induced by catabolic cytokines such as IL-1β or TNF-α, but also by matrix degradation products and other non-physiologic stimuli which the cells are exposed to (94).

MMPs in AC, MMPs have a dominant role in cleaving collagen during matrix turnover. However, many recent studies have shown that these MMPs have other roles as well, such as in regulating chondrocyte environments and modulating multiple bioactive molecules at the cell surface which influence cell behavior (118). These include MMP-1 (collagenase 1), MMP-8 (collagenase 2) and MMP-13 (collagenase 3), MMP-3 (stromelysin 1), MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MT1-MMP (membrane type I) (119, 120).

In OA, the expression of MMPs in the joint anatomical structures is commonly related to disease progression and tissue destruction (119, 121). Inflammatory cytokines stimulate the production of MMPs which degrade matrix proteins. MMP-1 produced by chondrocytes, fibroblasts, and macrophages, is responsible for collagen degradation while MMP-13 has a dual

effect giving its ability to digest collagen and, PG and aggrecan molecule. Other MMPs, like MMP-2, MMP-3, MMP-8, MMP-9, and MMP-19, are also elevated in OA and can degrade non-collagen matrix proteins of the joints (122).

Although several MMPs are capable of cleavage and degradation of PGs, ADAMTS-4 and ADAMTS-5 are the principal degrading enzyme showing high affinity for aggrecan molecule degradation (104, 123).

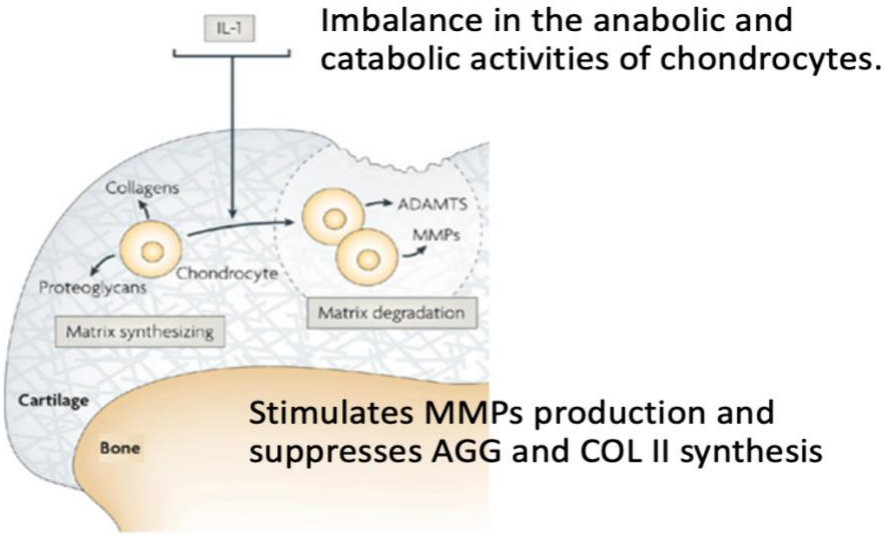

Figure 11: Cartilage degradation is a multistep process based on the release of matrix-degrading enzymes. The cytokine-matrix metalloproteinase relationship contributes to the intrinsic process of cartilage degeneration. Interleukin-1 (IL-1) induces a switch in the synthesis pattern of chondrocytes from matrix molecules to matrix-degrading enzymes. (Reproduced from McInnes, I. B., & Schett, G. (124).

ADAMTS is a subfamily (19 metalloproteinases) of metalloendopeptidases. These multidomain extracellular enzymes are involved in the developmental and homeostatic processes (125) In AC, ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) have high specificity to aggrecan degradation. They cleave the aggrecan core protein at the Glu^{373}- Ala^{374} bond in the IGD region (126). Other cleavage sites have been described, including Glu^{1480}–Gly^{1481}, Glu^{1667}–Gly^{1668}, Glu^{1771}–Ala^{1772} and Glu^{1871}–Leu^{1872} (127, 128).

A precise regulation of theses aggrecanases is essential to achieve a fine balance between synthesis and degradation of aggrecan molecules (129). In healthy cartilage, endogenous inhibitors like tissue inhibitor of matrix metalloproteinase (TIMP-3) are involved in regulating aggrecan catabolism, however, in OA, the balance between ADAMTS-4 and TIMP-3 synthesis is disturbed in favor of catabolism (130). Several studies using a neutralizing antibody against ADAMTS-4 (131) and ADAMTS-5 (132) demonstrated their important role in aggrecanolysis. Furthermore, an *in vivo* study demonstrated that mice deficient in ADAMTS-5 are protected from early aggrecan loss and cartilage erosion, in non-inflammatory and inflammatory models of arthritis (116, 133).

Catabolic Cytokines: Chondrocytes secrete and release catabolic cytokines into synovial fluid (134). Further, they respond to these inflammatory mediators by an increased production of prostaglandins, nitric oxide (NO) and many matrix proteinases to break down the surrounding cartilage matrix (135-139). At low-levels, these cytokines are essential for normal cartilage homeostasis and turnover. During OA the balance is shifted toward catabolism.

An enlarging body of research evidence indicates the crucial role of IL-1β and tumor necrosis factor alpha (TNF-α), in the initiation and progression of OA. These catabolic cytokines can stimulate the secretion of multiple degradative enzymes, like MMPs (140), aggrecanases (ADAMTS-4 and ADAMTS-5)(126, 141), cathepsins (B, L, and D) and Prostaglandin E2 (PGE

2)(142). The action of these catabolic cytokines can be amplified by specific degradation products of the AC matrix, such as fibronectin fragments, which have been demonstrated to increase the synthesis of catabolic cytokines, resulting in initiation or magnification of cartilage matrix destruction (143). *In vivo* studies demonstrated that intra-articular injection of recombinant preparations of IL-1β stimulates the degradation of AC while co-injection of both TNF- α and IL-1β resulted in more severe damage in comparison to injection of either cytokine alone.

2.4. Interleukin-1β

IL-1β is thought to be one of the most important catabolic cytokines synthesized during OA. Chondrocytes secrete IL-1β at a concentration that stimulates the expression of aggrecanases, MMPs, and other catabolic factors (144). In OA, IL-1β level is elevated in many joint tissues including subchondral bone, synovial fluid and membrane, and cartilage (145). IL-1β activates chondrocytes through its specific cell surface receptor IL-1 receptor type I (IL-1RI). OA chondrocytes express increased levels of IL-1RI as compared to normal chondrocytes (146).

An enlarging body of research demonstrated that IL-1β produces a plethora of effects in chondrocytes including: 1) downregulation of the expression of matrix proteins by inhibiting the anabolic activity of chondrocytes (147-150); and 2) upregulation of main catabolic proteinases involved in cartilage degradation (MMP-1,-3 -13 and ADAMTS-4) (151) . However, no changes in ADAMTS-5 expression was noted (132). Although IL-1β is considered a catabolic factor, the deletion of IL-1β gene accelerated the progression of OA lesion in a mouse model, proving the complex role of IL-1β in maintaining cartilage homeostasis (152).

2.5. IL-1β Signaling Pathways

IL-1β has the potential to activate four classic cellular signaling pathways, three of which belong to the mitogen-activated protein kinase (MAPK) pathways: c-Jun NH2-terminal kinase (JNK), 38-kd protein kinases (p38), and extracellular signal-regulated kinase (ERK). Mechanical stress and cartilage matrix degradation products may also activate these pathways using integrin and other receptor-mediated events (153).

The MAPKs phosphorylate a large variety of transcription factors such as c-jun and c-fos in a relatively specific manner. There are at least four p38 MAPK inhibitors (154) and three forms of JNK-1, -2, and -3.

Most of the MAPK pathway inhibitors demonstrated anti-catabolic activity in animal models of inflammation (153-156). Previous reports have shown the presence of p38 mediators in vitro in both normal and OA chondrocytes. However, only two preliminary *in vivo* studies suggest that the expression and activation of JNK is present only in OA cartilage but not in normal chondrocytes (157). The JNKs have been targeted for development of specific inhibitors (158). For instance, SP600125 is a potent JNK1/2 inhibitor, which showed an anti-inflammatory effect in a model of rheumatoid arthritis (159). The selected JNK inhibition resulted in a decrease of cytokine- induced chondrocyte response (160).

The fourth signaling pathway that mediates IL-1 signaling involves Nuclear Factor NF-kappa-B (NF-κB).

2.6. NF-κB Signaling Pathway

The NF-κB proteins are a family of ubiquitously expressed transcription factors that contribute to stress response, inflammatory diseases, immunity, cell proliferation and cell death (161). In normal unstimulated conditions, the NF-κB dimers are situated in the cytoplasm as inactive form and bound to IκB kinase (IKK) complex. However, once stimulation of the cell occurs by various mechanical and chemical signals, phosphorylation of IκBs happens, leading to their subsequent degradation through the ubiquitin-proteasome system (161). Subsequent to IκB degradation, NF-κB hetero-dimers are disengaged to translocate into the nucleus and trigger the expression of a wide range of chemokines, cytokines, proteinases, an angiogenic factor, immunomodulatory proteins, and proliferation or apoptosis-related molecules (162).

Two main pathways can activate NF-κB signaling cascades (Figure 12). 1) The classical (or canonical) pathway, which is stimulated by Toll-like, TNF and T-cell receptors (TL-R, TNF-R, and TC-R, respectively), involves the activation of IKKα/IKKβ/IKKγ-NEMO complex which leads to phosphorylation and degradation of IκB proteins using the ubiquitin-proteasome system. 2) The second pathway, termed alternative (or non-canonical) pathway, includes the activation of CD40, lymphotoxin β (LTβ) receptors and B-cell activating factor (BAFF) and it uses NF-κB inducing kinase (NIK), which in turn activates the IKKα kinase through phosphorylation. Once IKKα kinase activated, it results in phosphorylation-dependent proteolysis of the p100 precursor NF-κB species that will lead to a release of mature p52 protein which can turn on the transcription of target genes (161, 162).

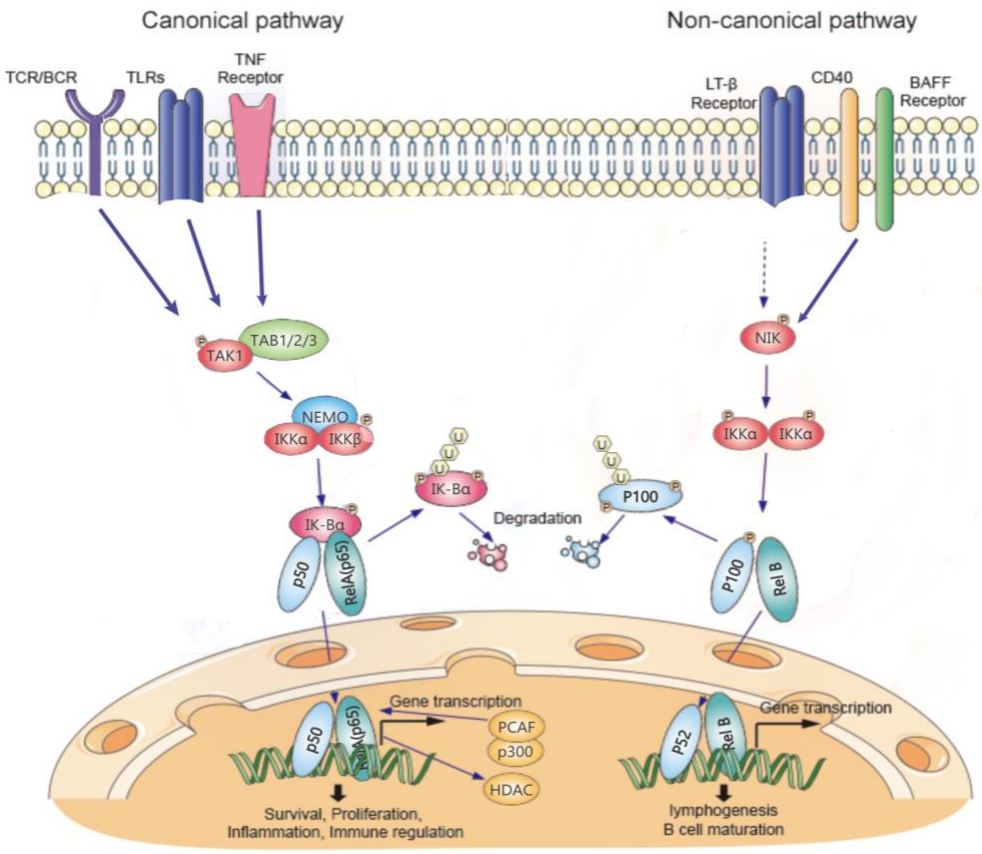

Figure 12. Canonical and non-canonical NF-κB signaling pathways start at the cell surface receptors stimulated by their ligands. Once stimulation of the cell occurs, it causes the activation of the IkB kinase (IKK) complex. This complex consists of a heterodimer of IKKα and IKKβ catalytic subunits and an IKKγ regulatory subunit. The IKKγ unit is also called NEMO for NF-kB. The activated IKK complex, predominantly acting through IKKβ in an IKKγ-dependent manner, catalyzes the phosphorylation of IkBs which lead to polyubiquitination and subsequent degradation by the 26S proteasome. The released NF-κB dimers translocate to the nucleus and activate the downstream gene transcription. The non-canonical pathway is independent of IKKβ and IKKγ, but dependent on IKKα dimer instead. The NIK protein then phosphonates the IKKα homodimmers. The target for IKKα homodimers is NF-kB2/p100 which is phosphorylated at two C-terminal sites. Once IKKα kinase is activated, it results in phosphorylation-dependent

proteolysis of the p100 precursor NF-κB species, leading to a release of mature p52 protein which is capable of turning on the transcription of target genes (161, 162).

Articular chondrocytes have multiple receptors on their surface that are capable of stimulation of NF-κB molecules; these receptors include cytokine receptors (TNF-R and TL-R) and mechanoreceptors. Upon stimulation of these receptors by mechanical stress, aging factors, fibronectin fragments, or inflammatory cytokines like IL-1β or TNF-α, activation of NF-κB signaling occurs (163). Activation of NF-κB transcription factors leads to stimulation of many degradative enzymes including the aggrecanases ADAMTS-4 and ADAMTS-5 and the MMPs, mainly MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP13, resulting in ECM degradation (164).

Glucosamine hydrochloride has shown the ability to block the IL-1β -mediated activation of NF-κB (165), while thalidomide prevents the induction of the NF-κB pathway by TNF-α (162). In addition, IL-1β and TNF-α receptor antagonists, such as IL-1 receptor antagonist (IL-1Ra) and TNF-R respectively, were able to decrease NF-κB activity (166).

Nonetheless, some of these inhibitors are not specific for NF-κB signaling pathway, which results in many side effects. Therefore, a novel targeted therapy that aims for a selective inhibition of the NF-κB signaling pathway is needed.

2.7. Current State and Bottlenecks in OA Treatment

In early stages of OA, pain and stiffness dominate the other symptoms. Therefore, the goal of the treatments is primarily palliative using analgesics and nonsteroidal anti-inflammatory drugs

(NSAIDs), and some attempt to control structural deterioration in the affected joints using physical therapy. In addition to drug administration, intra-articular injection of long-acting glucocorticoids is an effective treatment of inflammatory flares of OA. Hyaluronic acid has varying effectiveness when used for intra-articular injections for the treatment of OA of the knee. However, none of these agents are disease modifying, and often their effects are short-lived as many patients continue to suffer from pain symptoms.

With the progression of OA, where the cartilage defect is still small (area $<2-3cm^2$) microfracture (MF) marrow stimulation is considered medical option. MF is a minimally invasive procedure which involves removing the damaged cartilage and then drilling into the surface of the underlying bone to allow blood and bone marrow to come through to the bone/cartilage interface, where the mesenchymal stem cells contribute to the formation and repair of the cartilage and bone. However, the regenerated cartilage is mainly fibrocartilage and is not expected to have the same durability as the articular hyaline cartilage. This type of cartilage is mostly type I collagen, fibrocytes and a disorganized matrix that lacks the biomechanical and viscoelastic characteristics of normal hyaline cartilage and can fail with high shear forces in the joint, leading to an ongoing articular surface irregularity and subsequent OA.

If the joint damage is extensive and the cartilage defect has progressed to the stage where the patient's quality of life has significantly decreased, the non-surgical treatments are no longer effective and joint replacement surgeries (arthroplasties) are performed. This major surgical procedure often does not restore the full function of joints and, as with any surgery, includes short- and long-term complications: risk of infection, blood clots, bearing surface wear and particle driven osteolysis, leading to high rate of implant failure (10-15% in the first 10 years) requiring revision (167). Alternative approaches are needed.

2.8. Regenerative Medicine

The unsatisfactory effects associated with traditional OA drugs warrant a continued search for potential new medications. A promising non-surgical approach is to facilitate cartilage repair *in situ* by injection of bioactive factors to stimulate cartilage production. However, no drugs are available to modify OA disease progression, and current clinical OA management is mainly concerned with symptom reduction, e.g. pain and joint swelling/stiffness, with oral NSAIDs as pharmacological treatment at mid-stage of the disease, and arthroplasty, an irreversible procedure, as the final solution to maintain joint function. Timely intervention at early stage in OA progression is critical for preventing or delaying disease development and a key target in preserving proper joint function.

Although few of the new therapies have received the regulatory approval for routine clinical use, and a variety of new OA drugs have shown promising results in clinical trials (Table 1), there is no available treatment or disease modifying drug for managing OA (DMOADs). Based on the potential therapeutic targets, the new DMOADs can be classified as chondrogenesis inducers, osteogenesis inhibitors, matrix degradation inhibitors, apoptosis inhibitors, and anti-inflammatory cytokines (Table 1).

Table 1. New OA drugs and therapeutics investigated in clinical or preclinical studies (summarized from ClinicalTrials.gov)

Mode of action	Targets	Potential therapeutics	Clinical study
Chondrogenic differentiation	BMP-7	rhBMP-7 (OP-1)	x
	FGF	rhFGF-18 (sprifermin)	x
	PRP (containing several kinds of growth factors)	Autologous PRP	x
Inhibition of hypertrophy & ossification	PTH/PTHrP receptor	rhPTH (1–34) (teriparatide, Forteo), rhPTHrP (1–40)	
	Hedgehog signaling	Smo inhibitor (HhAntag, LDE223)	
Inhibition of matrix degradation	MMP13	MMP13 inhibitor (CL82198)	
	Adamts-5	Adamts-5 inhibitor (114810)	
	Syndecan-4	Syndecan-4-specific antibody	
Inhibition of inflammation	IL-1β	IL-1β receptor antagonist and IL-1β receptor antibody (AMG108)	x
	HSA	a o5-kDa ultrafiltrate of HSA (Ampion)	x
	Methotrexate	Methotrexate	x
Reduction in pain	β-NGF	Monoclonal antibody against β-NGF (Tanezumab)	x
Subchondral bone	TGF-β	TGF-β type I receptor inhibitor (SB-505124), TGF-β antibody (1D11)	
	Wnt/b-catenin	Wnt antagonist (Dkk-1)	

β-NGF, β-nerve growth factor; BMP-7, bone morphogenetic protein-7; OP-1, osteogenic protein-1; Dk
factor; HSA, human serum albumin; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related

2.9. Link N for AC Repair

Proteolytic fragmentation of LP1 or LP2 occurs in the N-terminal region of the disulfide-bonded loop, generating LP3 and a 16 amino acids N-terminal peptide called Link N (DHLSDNYTLDHDRAIH) (61-63). Early studies showed that Link N was able to stimulate the synthesis of PG and collagen in cartilage chondrocytes (168), indicating that Link N, can serve as a growth factor to counteract the increased proteolytic activity in the OA cartilage, restoring the homeostasis balance toward a synthesis of new matrix protein.

The regenerative potential of Link N has also been demonstrated *ex vivo* intact human IVDs (169) and *in vivo* in a rabbit annular puncture model (170). Some evidence showed an interaction of Link N peptide with BMP type II receptor, upregulating the BMP-7 expression through the Smad1/5 signaling pathway in IVD, of growth factor (171). In addition, Link N was demonstrated to suppress markers of pain in annulus fibrossus IVD cells (172). Recent evidence showed that Link N-peptide is not only effective in increasing the synthesis of PG, but it has an extended effect on collagen type II expression as a marker of chondrogenesis (173). Moreover, Link N also decreases the expression of type X collagen, a marker of late-stage chondrocyte hypertrophy (174, 175). Therefore, Link N may provide a therapeutic benefit not only to degenerating discs but also to AC because of the similar composition of the ECM. Although Link N can activate the BMP pathway, it does not stimulate bone formation as has been demonstrated with direct application of BMP growth factors (176). The purpose of the present study was to determine whether Link N can regulate human OA cartilage under inflammatory conditions.

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Link N Suppresses Interleukin-1β-Induced Biological Effects on Human Osteoarthritic Cartilage

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Keywords:	Osteoarthritis, Cartilage Repair, Bioactive Peptides, Link N, NF-κB, Interlukin-1
Abstract:	Osteoarthritis (OA) is a disease of diarthrodial joints associated with proteolytic degradation of the extracellular matrix (ECM) under inflammatory conditions, pain and disability. Currently, there is no therapy to prevent, reverse or modulate the course of the disease. Present study aims to evaluate the regenerative potential of Link N (LN) in human OA cartilage in an inflammatory milieu and determine if LN could affect pain-related behavior in a mouse injury model of knee OA. OA cartilage-bone explants and OA chondrocytes isolated from various donors were treated with LN in the presence of IL-1β to simulate an OA

	environment. Quantitative PCR and western blotting were performed to determine the effect of LN on matrix protein synthesis, catabolic enzymes, cytokines and neurotrophic factor NGF expression. Partial Medial Meniscectomy (PMM) was performed on the knee of C57BL/6 mice. Twelve weeks post-surgery, mice were given a 5 µg intra-articular injection of LN or PBS and a Von Frey test was conducted to measure the mechanical allodynia in the hind paw. LN supressed the IL-1β-induced upregulation of cartilage degrading enzymes and inflammatory molecules in OA chondrocytes, including ADAMTS-4, MMP-3, IL-1β, IL-6, and NGF. LN also modulated proteoglycan and collagen synthesis in human OA cartilage through inhibition of IL-1β-induced biological effects. Upon investigation of the canonical signaling pathways IL-1β, NF-□B, LN significantly inhibited its activation in a dose dependent manner. In addition, LN suppressed mechanical allodynia in an OA PMM-mouse model. These results support the concept that LN administration could provide therapeutic potential in OA

Link N Suppresses Interleukin-1β-Induced Biological Effects on Human Osteoarthritic Cartilage

*Motaz Alaqeel[1,2], *Michael P Grant[2], Laura M Epure[2], Omar Salem[2], Ahmed AlShaer[1,2], Olga L Huk[1,2], Stephane G Bergeron[1,2], Ranjan Kc[3], Hee-Jeong Im[4,5], Arivarasu Natarajan Anbazhagan[6], John Antoniou[1,2] and δFackson Mwale[1,2]

[1]*Department of Surgery, McGill University, Montreal, Canada*

[2]*Orthopaedic Research Laboratory, Lady Davis Institute for Medical Research, SMBD-Jewish General Hospital, McGill University, Montreal, Canada*

[3]*Division of Orthopaedic Surgery, The Children's Hospital of Philadelphia Research Institute, Philadelphia, PA 19104*

[4]*Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60612. USA.*

[5]Jesse *Brown Veterans Affairs Medical Center (JBVAMC), Chicago, IL 60612, USA*

[6]*Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA*

* these authors share equally the first authorship

δ **Corresponding author:** Fackson Mwale, PhD, FIOR

Orthopaedics Research Laboratory, Lady Davis Institute for Medical Research, SMBD-Jewish General Hospital

Department of Experimental Surgery, Faculty of Medicine, McGill University

3755 Chemin de la Côte-Sainte-Catherine, Montréal, QC, H3T 1E2, Canada

Phone: +1 (514) 340 8222 ext 22948

Email: fmwale@jgh.mcgill.ca

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ABSTRACT

Osteoarthritis (OA) is a disease of diarthrodial joints associated with proteolytic degradation of the extracellular matrix (ECM) under inflammatory conditions, pain and disability. Currently, there is no therapy to prevent, reverse or modulate the course of the disease. Present study aims to evaluate the regenerative potential of Link N (LN) in human OA cartilage in an inflammatory milieu and determine if LN could affect pain-related behavior in a mouse injury model of knee OA.

Osteo-chondro OA explants and OA chondrocytes isolated from various donors were treated with LN in the presence of IL-1β to simulate an osteoarthritic environment. Quantitative PCR and western blotting were performed to determine the effect of LN on matrix protein synthesis, catabolic enzymes, cytokines and neurotrophic factor NGF expression. Partial Medial Meniscectomy (PMM) was performed on the knee of C57BL/6 mice and twelve weeks post-surgery, mice were given a 5 µg intra-articular injection of LN or PBS. A Von Frey test was conducted over 24 hours to measure the mechanical allodynia in the hind paw.

LN modulated proteoglycan and collagen synthesis in human OA cartilage through inhibition of IL-1β-induced biological effects. LN also supressed the IL-1β-induced upregulation of cartilage degrading enzymes and inflammatory molecules in OA chondrocytes. Upon investigation of the canonical signaling pathways IL-1β, NF-κB, LN significantly inhibited its activation in a dose dependent manner. In addition, LN suppressed mechanical allodynia in an OA PMM-mouse model. These results support the concept that LN administration could provide therapeutic potential in OA.

INTRODUCTION

Articular cartilage (AC) is a unique connective tissue, covering the bony parts of diarthrodial joints, which, in conjunction with lubricating functions provided by the synovial fluid, allows the frictionless motion of the joint. It has no blood supply, lymphatics, or nerves, and its properties are related to the composition and structure of an abundant extracellular matrix (ECM). The major matrix proteins in cartilage are the proteoglycans and collagen. Aggrecan is the key proteoglycan responsible for the resiliency of the tissue, while type II collagen provides tensile strength (Roughley, 2006). Cartilage responds to a complex multitude of autocrine and paracrine factors that can be either anabolic or catabolic, that regulate gene expression and protein synthesis in chondrocytes, there are responsible for the synthesis and maintenance of ECM. When the catabolic processes overtake anabolic activities, the cartilage degeneration occurs with the onset of OA (Lotz and Loeser, 2012). Unfortunately, unlike most tissues in the body, cartilage does not regenerate.

Osteoarthritis (OA) is a chronic incurable degenerative joint disorder characterized by two fundamental pathological changes: inflammation which may be driven by cytokines (Kapoor *et al.*, 2011) and destruction of the AC (mediated by proteolytic enzymes). Pain is the major symptom in OA and one of the leading causes of impaired mobility in the elderly population. Aging, obesity, and joint injuries are associated with increased risk of OA (Felson *et al.*, 2000; Loeser, 2010). The most recent update of the Global Burden of Disease declared OA the 11th most debilitating disease around the world. By 2050, 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease (Hunter and Bierma-Zeinstra, 2019). OA is resistant to effective therapy and the relief from severe OA pain remains an unmet medical need and a major reason for seeking surgical intervention. Over $185 billion a year are the estimated direct costs of OA in the United States (Evans, 2018).

Several cytokines have been implicated in OA pathogenesis including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, and other common c-chain cytokines (IL-2, IL-7, IL-15, and IL-21) (Goldring and Berenbaum, 2004; Kapoor et al., 2011). These factors produced by synovial cells and chondrocytes result in the upregulation of members of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families of enzymes. Interleukin-1β (IL-1β) is considered one of the key cytokines involved in OA progression by disrupting chondrocyte metabolism.

There is general agreement that, since inflammatory processes plays a fundamental role in the pathogenesis of various rheumatic diseases such as OA and rheumatoid arthritis (RA), selective inhibition of inflammatory activities is vital for therapy, and that the family of NF-κB transcription factors (Marcu *et al.*, 2010; Poulet *et al.*, 2012; Rigoglou and Papavassiliou, 2013) plays a prominent role in this process. Thus, several studies have been directed towards the pharmacologic modulation of the NF-κB pathways using non-steroidal anti-inflammatory drugs, corticosteroids, nutraceuticals, antisense DNA therapy, RNA interference, and anti-rheumatic drugs. However, there is a need for new generations of NF-κB –targeting anti-inflammatory agents that are specific, efficacious, and cost-effective. Despite significant progress in understanding the molecular mechanisms involved in the pathogenesis of OA, there are currently no disease-modifying agents for its treatment. Current treatment methods are generally not effective and involve either symptomatic relief with non-steroidal anti-inflammatory drugs and physical therapy or surgery when conservative treatments fail. The lack of adequate analgesia for OA pain contributes to the opioid epidemic.

Link N (DHLSDNYTLDHDRAIH, LN) is the naturally occurring N-terminal peptide of link protein that stabilizes the proteoglycan aggregates in both disc and cartilage. This peptide is

generated *in vivo* by MMPs during tissue turnover (Nguyen *et al.*, 1989). There is an abundance of pre-clinical data confirming that LN can stimulate synthesis of proteoglycans and collagens in explant cultures of normal human articular cartilage (McKenna *et al*., 1998; Liu *et al.*, 1999; Liu *et al.*, 2000). LN can also preferentially stimulate the synthesis of proteoglycan over collagen by bovine IVD cells *in vitro*, without any effect on cell division (Mwale *et al.*, 2003).

Two studies confirm the repair potential of LN in the intervertebral disc, in a rabbit model of disc degeneration (Mwale *et al.*, 2018; Mwale *et al.*, 2011). Recently we have shown that LN inhibits the expression of neurotrophic factors in human disc cells (Noorwali *et al.*, 2012). LN was also found to modulate inflammation and stimulate the repair of arthritis-mediated temporomandibular joint disruption *in vivo* (Yang *et al.*, 2019). Delivery of synthetic LN encoding mRNA into primary human chondrocytes and mesenchymal stromal cells resulted in an enhanced expression of aggrecan, Sox 9, and type II collagen (Tendulkar *et al.*, 2019). Similarly, LN stimulated the chondrogenic differentiation of cartilage stem/progenitor cells (He *et al.*, 2018).

To date, however, there have been no reports on the effect of LN on OA cartilage under inflammatory conditions and on pain-related behaviors associated with progressive joint damage. The purpose of the present study was to determine whether LN can regulate human OA cartilage under inflammatory conditions and if LN could affect pain-related behavior in an established inflammatory-mechanical murine model of OA (Glasson *et al.*, 2007).

MATERIALS AND METHODS

Peptide Synthesis - Link N (DHLSDNYTLDHDRAIH) was synthesized with a purity > 98% by CanPeptide (Pointe Claire, QC, Canada).

Antibodies - Anti-collagen II antibody (Abcam, Cat# ab34712), anti-phospho-NF-κB p65 antibody (Cell Signaling Technology, Danvers, MA) and anti-G1 aggrecan antibody (generated by Dr. Peter Roughley) (Roughley and Mort, 2012).

OA Cartilage - OA cartilage was obtained from ten donors ranging from 45 – 65 years of age undergoing total knee arthroplasty with informed consent. Samples were collected immediately after surgery and processed accordingly.

Cartilage explant preparation and cell culture – Osteo-chondro explants were prepared from donors and included cartilage with the cortical bone. Explants were cut using a circular saw and bone cutters to approximately 1 cm^2 in size. Explants were washed thrice in PBS containing penicillin-streptomycin (Wisent Bioproducts, Montreal, Canada) prior to incubation with 0.125% Trypsin (Wisent Bioproducts, Montreal, Canada) for 30 min in DMEM. Explants were washed twice in regular culture medium (low glucose DMEM supplemented with 10% FBS serum, 1% Pen-Strep and 1% amphotericin) (Wisent Bioproducts, Montreal, Canada) and maintained for 6 days in regular culture medium under standard culture conditions (humidified atmosphere and 5% CO_2). Culture medium was changed every three days.

OA chondrocytes were recovered from the cartilage of each knee by sequential digestion with 0.125% Pronase followed by 0.2% Collagenase. After isolation, the cells were expanded in DMEM supplemented with 10% heat-inactivated FBS and 1% Streptomycin, with culture medium changed every three days.

Osteochondral explant treatments

After 6 days, the explants were exposed to either 5 ng/ml IL-1β (Sigma-Aldrich, St. Louis, MO), 1 μg/ml LN, or co-exposed to IL-1β + LN for 21 days. Explants cultured in DMEM without supplementation were used as control. Culture medium was changed every three days.

Histological assessment

After 21 days of treatment, explants were fixed in Accustain (MilliporeSigma, Burlington, MA) and decalcified in Osteosoft before paraffin embedding. Sections were rehydrated prior to immunohistochemistry or staining by deparaffinization in xylene followed by sequential incubation in decreasing concentrations of alcohol and water. Five-micrometer sections were then stained with 0.1 % Safranin O for 5 min, rinsed in water, dehydrated by sequential alcohol concentrations and xylene, and mounted in Permount (Thermo Fisher Scientific, Waltham, MA). Images were captured using an Optika light microscope (FroggaBio, Toronto, Canada).

Aggrecan extraction

Cartilage plugs of 3 mm diameter (approximately 30 - 60 mg) obtained from different sites for each explant were extracted with 15 volumes (v/w) guanidinium chloride buffer (4 M guanidinium chloride, 50 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid, pH 7.4) for 72 hours as previously described (Roughley and Mort, 2012). Purified tissue extracts were digested with Keratinase [0.2 mU/μL buffer: 50 mM Tris-HCl, pH 7.4] for 1 h at 37°C followed by Chondroitinase digestion [10 mU/uL buffer: 100 mM sodium acetate and 100 mM Tris-HCl, pH 7.4] for 16 h at 37°C. Aggrecan content was assessed in the extracts by Western blotting. Briefly, extracts were electrophoresed on 4-20% gradient gels (Bio-Rad, Hercules, CA) and transferred to PVDF membrane. Blots were blocked in 5% bovine serum albumin (BSA) in PBS and 0.1% Tween for 1 h, and probed with anti-G1 [1:2000] for detection of aggrecan in antibody solution

(TBS, 1% BSA and 0.1% Tween). Blots were developed by incubation with anti-rabbit-HRP secondary antibodies and Amersham ECL Prime chemiluminescent detection reagent (GE Healthcare, Piscataway, NJ). Images were captured on a Molecular Imager VersaDoc (Bio-Rad, Hercules, CA).

Proteoglycan and Collagen Content

Cartilage plugs were digested with Proteinase K solution [1.5 mg/mL Proteinase K, 50 mM Tris, pH 7.4] in a ratio of 10 µL/mg tissue and incubated overnight at 55°C. To determine proteoglycan content in explant cartilage, the sulfated glycosaminoglycan (GAG, predominantly aggrecan) content in the tissue was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay (Farndale *et al.*, 1986). Shark chondroitin-6-sulphate (Sigma-Aldrich, St. Louis, MO, USA) dissolved at a concentration of 0-40 mg/mL in serum-free culture medium was used to generate the standard curve.

The hydroxyproline assay was used to determine collagen content. Briefly, 10 µL of digested sample was incubated in an equal volume of sodium hydroxide (7 N) at 120°C for 30 min. An equal volume of sulfuric acid (3.5 N) was added along with PBS to a final volume of 100 µL. Samples were then incubated with 50 µL of chloramine T solution for 20 min at RT. Perchloric acid was added to the samples followed by 50 µL of p-Dimethylaminobenzaldehide solution and incubated for 20 min at 60°C. Samples were transferred to a 96-well plate and analyzed by a spectrophotometer at an absorbance of 570 nm (BMG LABTECH, Ortenberg, Germany).

NF-κB signaling

OA human chondrocytes (n = 4 donors) were transferred to 6-well plates and cultured to 90% confluency. Cells were serum deprived overnight and incubated in culture medium containing

IL-1β (5ng/ml), LN (1μg/ml) or combination of the two for 10 minutes at 37°C. Cells were lysed in RIPA (radio immuno-precipitation assay) buffer and protease cocktail II (Sigma-Aldrich, St. Louis, MO) and phosphatase (Thermo Fisher Scientific, Waltham, MA) inhibitors. The lysate was electrophoresed on a 4-20% gradient gels (Bio-Rad, Hercules, CA) under reducing conditions and transferred to 0.2 um PVDF membranes. Blots were probed with anti-phospho-NF-κB p65 antibody (Cell Signaling Technology, Danvers, MA), NF-κB (Cell Signaling Technology, Danvers, MA) and GAPDH (Sigma-Aldrich, St. Louis, MO) for normalization.

RNA extraction and quantitative real-time PCR

OA human chondrocytes (n=4 donors) were cultured as micro-pellets at a density of $3x10^5$ cells/pellet and treated for 6 days with 0.5 mL medium supplemented with 0.5 μg LN, 5ng/mL IL-1β, 0.5 μg LN + 5ng/mL IL-1β, or with vehicle (PBS) alone as a control. Total RNA was extracted using a total RNA mini-kit (Geneaid Biotech Ltd., Taiwan) following manufacturer instructions. Complementary DNA was synthesized using a superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR of human was quantified by an ABI 7500 fast light cycler using CYBR green master mix (Thermo Fisher Scientific, Waltham, MA) and specific primers (Table 1). Relative mRNA expression level was normalized against GAPDH as previously described (Antoniou *et al.*, 2012). The one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test was used to assess differences in gene expression. P values ≤0.05 were deemed statistically significant.

Induction of OA in Mouse Knee Joint by Partial Medial Meniscectomy (PMM).

The protocols described here were approved by the Institutional Animal Care and Use Committee at Rush University Medical Center (Glasson et al., 2007). Mice were anaesthetized by 5% isoflurane (Abbott Laboratories) in oxygen, the left hind limb was shaved and swabbed with

70% ethanol, the animal positioned on a dissecting microscope and the leg draped. A medial para-patella arthrotomy was performed and the patella luxated laterally. The anterior fat pad was dissected to expose the anterior medial meniscotibial ligament, which was elevated and severed using microsurgical knife. For PMM surgery, cranial horn of the medial meniscus was resected following the severance of the medial meniscotibial ligament. Throughout surgery the cartilage was kept moist with sterile saline. The patella was repositioned, the knee was flushed with saline and the incision closed in 3 layers – simple continuous 8/0 vicryl in the joint capsule, simple continuous 8/0 vicryl subcutaneously and tissue glue for the skin. In sham operations, the meniscotibial ligament was exposed but not cut and the joint flushed and closed as above (Glasson et al., 2007)

Link N Injections and von Frey Assay

Mice were given a 5 µg injection of LN or PBS intra-articularly 12 weeks post-surgery and monitored for pain-related behaviors longitudinally over 24 hrs using the von Frey assay. Hind-Paw Mechanical Allodynia (Secondary Allodynia) was determined with von Frey fibers using the up-down staircase method and expressed as paw weight threshold (PWT) (Chaplan *et al.*, 1994; Das *et al.*, 2018). Mice were placed on a perforated metal grid floor within small Plexiglas cubicles, and a set of eight calibrated von Frey fibers (Stoelting Touch Test Sensory Evaluator Kit #2 to #9) were applied to the plantar surface of the hind paw until the fiber bows and held for 3 seconds. The threshold force required to elicit paw withdrawal (median 50% withdrawal) was determined twice per hind paw per testing day (Van de Weerd *et al.*, 2001).

RESULTS

To determine if LN can supress IL-1β-induced biological effects on human OA cartilage, we investigated the proteoglycan content in osteo-chondro explants from OA donors cultured for a 3-week period with LN, IL-1β, or LN and IL-1β (Figure 1). Treatment of explants with IL-1β significantly decreased ($p = 0.0418$) the GAG content by approximatively 50%, when compared to control (explants in regular culture media), indicative of proteoglycan loss. In contrast, when LN was co-administered with IL-1β, the decrease in GAG content was abolished. Interestingly, no statistical significance was observed between control and LN treated osteochondral explants. This suggests that during cartilage degeneration, LN has the potential to maintain the proteoglycan content in an inflammatory environment.

Proteoglycan deposition and distribution in the accumulated cartilage matrix was visualized by staining with with Safranin-O. Staining of tissue sections confirmed a loss of proteoglycans in the cartilage of IL-1β treated explants, where little Safranin O (red) staining was found in the superficial and middle zones (Figure 2). In contrast, LN supplementation led to an enhanced Safranin-O staining throughout the osteocohondral explants. This pattern was also present when LN was administered alone. Thus, LN can stimulate proteoglycan synthesis in an inflammatory cartilage millieux.

Maintaining proteoglycan content also requires aggrecan retention. To address this, we next determined the effect of LN on the synthesis and retention of aggrecan in the tissue using an antibody against the G1 domain. In the absence of treatment, OA cartilage already presented with a depletion and a multi-component pattern of aggrecan (Figure 3). After culturing the osteochondral explants for 21 days in the presence of LN, the content of aggrecan fragments bearing the G1 domain was increased significantly ($p = 0.0005$). When explants were treated with IL-1β, the content of aggrecan fragments bearing the G1 domain was comparable to the control.

Analysis of LN co-administered with IL-1β, results revealed that proteoglycan fragments containing the G1 domain are enhanced and are comparable to LN alone.

The function of articular cartilage is dependent on mainly proteoglycan and collagen composition in the ECM. During OA progression, degradation of collagen impairs cartilage integrity. To determine if LN could maintain or restore collagen content following IL-1β exposure, we quantified for hydroxyproline. When osteochondral explants were treated with IL-1β it was apparent that the hydroxyproline content was significantly lower ($p = 0.0384$) when compared to the controls (Figure 4). In contrast, LN supplementation in the presence of IL-1β blunted the decrease in hydroxyproline content observed by treatment of IL-1β alone. There were no significant changes in hydroxyproline content when explants were incubated with LN.

Type II collagen (Col II) is the most abundant collagen in cartilage. IL-1β is known to decrease Col II content through downregulation of gene expression and upregulation of catabolic enzymes, collagenases such MMP-3 and MMP-13. To further, determine if LN could affect Col II content in an OA environment, we performed western blotting on extractable collagen from osteochondral explants treated with LN, IL-1β and IL-1β + LN (Figure 5). When the explants were treated with IL-1β, the Col II content was significantly decrease ($p = 0.0331$). Although we did not observe significant changes in the cartilage of LN treated explants, LN significantly reversed the effects of IL-1β on Col II content ($p = 0.0088$).

To determine if LN can supress IL-1β-induced biological effects on human OA chondrocytes, we first performed qPCR to quantify changes in the expression of matrix proteins, catabolic enzymes, inflammatory cytokines, and NGF, known factors regulated by IL-1β. After culturing OA chondrocytes for 6 days supplemented with LN, the cells showed significant increased aggrecan mRNA (ACAN) when compared to the control ($p = 0.0001$) (Figure 6).

Although, IL-1β treatment did not increase expression of ACAN, in combination with LN, ACAN expression was significantly enhanced ($p = 0.0013$). Surprisingly, mRNA levels for type II collagen (COL2A1) were not significantly altered by any of the treatments, despite the effect of LN on IL-1β induced changes in Col II content (Figure 5 and Figure 6).

To determine if LN can regulate catabolic factors associated with collagen and proteoglycan degradation, we measured changes in the aggrecanases ADAMTS-4 and ADAMTS-5, and collagenases MMP-3 and MMP-13. IL-1β significantly upregulated the expression of ADAMTS-4 ($p = 0.0045$) and MMP-3 ($p = 0.0001$) in OA chondrocytes. In addition, there was a trend towards an increased expression of catabolic enzymes ADAMTS-5 and MMP-13 (Figure 6). When chondrocytes were co-incubated with IL-1β and LN, upregulation of these catabolic factors was blunted.

In addition to catabolic enzymes, IL-1β is also known to upregulate inflammatory markers such as IL-6 and itself (Kushner, 1993). Similar to its effect on catabolic enzymes, LN also inhibited the IL-1b-induced upregulation of IL-1 ($p = 0.0001$) and IL-6 ($p = 0.0311$) (Figure 6).

IL-1β is known to upregulate NGF (Manni and Aloe, 1998), one of the main factors associated with pain. NGF was significantly upregulated in IL-1β treated chondrocytes ($p = 0.0467$), however, its expression was blunted when LN was present. Treatments of chondrocytes with LN alone had no effect on NGF expression.

NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) is a transcription factor that regulates the inducible expression of a wide range of proinflammatory mediators (Rigoglou and Papavassiliou, 2013; Saklatvala, 2007). Since enhanced abnormal NF-κB activation is one of the characteristic features of OA pathophysiology (Rigoglou and Papavassiliou, 2013), we next examined the effect of various concentration of LN on NF-κB activation by IL-1β in OA chondrocytes (Figure 7). Stimulation of phosphorylated NF-kappaB RelA (p65) was determined

by western blotting using antibodies specific to P-P65(NF-κB). After culturing chondrocytes cells for 10 minutes in the absence of IL-1β, chondrocytes show no P-P65(NF-κB) protein (Figure 7, CTL). With LN supplementation, no effect on P-P65(NF-κB) protein was observed, either (Figure 7, lane 2). As expected, P-P65(NF-κB) is prominent after stimulation with IL-1β ($P<0.0001$) (Figure 6, lane 3). LN significantly inhibited IL-1β stimulated P-P65(NF-κB) in a dose-dependent manner. The 100 ng of LN was similar to 1000 ng LN.

As cytokine-induced activation of NF-κB pathway has been associated with pain (Ahmed *et al.*, 2019; Yan *et al.*, 2019) and LN was found to suppress neurotrophins (Noorwali et al., 2012), Von Frey test was performed to determine whether LN could affect pain-related behaviors in a mouse model of knee OA, induced by partial medial meniscectomy (PMM) (Figure 8). PMM results in OA-induced symptoms, showing mechanical allodynia after 12 weeks. In the sham group, mouse withdrew the paws after 4.5g of force. In PMM group, the pain threshold is significantly decreased as the mouse withdraws the paws with less than 1g force (~ 80% decrease in pain threshold). In contrast, a single intra-articular injection of 5 μg LN in to the mouse knee joint, caused a fairly rapid increase in pain threshold which was statistically significant after only 3 hours. The threshold continued to increase 6 hours post injection and was maintained throughout the 24 h-assessment period, suggesting its pharmacological properties, and therapeutic efficacy in chronic OA pain by LN.

DISCUSSION

AC architecture is kept intact and functional through anabolic and catabolic factors, which act on the chondrocytes that in turn maintain tissue homeostasis by balancing synthesis and degradation. Degradation and loss of collagen and aggrecan, subchondral bone remodeling, and inflammation of the synovial membrane characterize OA, as the balance shifts to catabolism. Moreover, inflammatory mediators are thought to inhibit the compensatory synthesis of matrix macromolecules by chondrocytes, thereby exacerbating the problem (Goldring, 2006).

A drug that slows the rate of joint destruction would be labeled a DMOAD (disease-modifying osteoarthritis drug). Currently, there are no U.S. Food and Drug Administration (FDA)–approved DMOADs. It has previously been reported that LN promotes matrix production in explant cultures of normal human AC (McKenna *et al*., 1998; Liu *et al.*, 1999; Liu *et al.*, 2000). Our recent *in vivo* studies have provided evidence that LN could prevent OA lesion development or progression *in vivo* in a rabbit model of OA (Antoniou *et al.*, 2019). LN was also found to modulate inflammation and stimulate the repair of arthritis-mediated temporomandibular joint disruption *in vivo* (Yang et al., 2019). Delivery of synthetic LN encoding mRNA into primary human chondrocytes and mesenchymal stromal cells resulted in an enhanced expression of aggrecan, Sox 9, and type II collagen (Tendulkar et al., 2019). Similarly, LN stimulated the chondrogenic differentiation of cartilage stem/progenitor cells (He et al., 2018). It is, however, not known if LN can restore proteoglycan and collagen content in OA cartilage in an inflammatory milieu.

This work intended to determine whether LN can stimulate matrix production in an inflammation-induced human cartilage ECM and determine the potential mechanisms involved. Our findings indicate that LN stimulates aggrecan expression, when it is administered to human chondrocytes. In addition to stimulating aggrecan gene expression, LN is also able to downregulate ADAMTS-4, MMP-3, IL-1 and IL-6 gene expression in human OA chondrocytes stimulated with

IL-1b. LN also maintained total proteoglycan and collagen content in osteo-chondro explants following prolonged IL-1 exposure. Interestingly, our data indicates that LN suppresses the activation of NF-κB, the canonical signaling pathway of IL-1β. LN also supressed IL-1β-induced upregulation of NGF, a neurotrophic factor regulated by NF-κB activation. Furthermore, since NF-κB signaling has been associated with pain (Ahmed et al., 2019; Cao *et al.*, 2019; Xu *et al.*, 2018) and LN suppressed the upregulation of neurotrophins (Noorwali et al., 2012), a Von Frey test was performed to determine whether LN could affect nociception in a PMM mouse model of knee OA. Our *in vivo* studies demonstrated that a single intra-articular injection of LN significantly reduced advanced stage of chronic OA pain within 3 hours, and this reduced pain symptom was sustained over 24 hours of the experimental period, suggesting its dual effects: (i) improvement of joint pathology and (ii) rapid pain reduction. Therefore, LN supplementation could be a viable option for treating OA lesions.

LN, a bioactive factor, has been demonstrated to have the potential to stimulate disc repair (AlGarni *et al.*, 2016; Bach *et al.*, 2017; Gawri *et al.*, 2013; Gawri *et al.*, 2014; Mwale et al., 2003; Mwale et al., 2018; Mwale et al., 2011; Mwale *et al.*, 2014; Wang *et al.*, 2013; Yeh *et al.*, 2018). It has been identified using isolated IVD cells *in vitro,* to induce collagen and proteoglycan message levels and has been reported to increase incorporation of radioactive $^{35}SO_4$ into newly synthesized proteoglycans (Gawri et al., 2013; Gawri et al., 2014). Indeed, LN injection into intact human IVDs *ex vivo* (Gawri et al., 2013) resulted in increased incorporation of radioactive $^{35}SO_4$ in newly synthesized proteoglycans and led to partial restoration of disc height when injected into rabbit discs in a stab model of disc degeneration (Mwale et al., 2018; Mwale et al., 2011). Extending upon previous studies, our work reports that LN can stimulate proteoglycan and collagen expression in chondrocytes from OA patients, consistent with a functional role in restoring the functional properties of cartilage.

NF-κB signaling pathways play active roles in the development and progression of arthritis *in vivo* (Marcu et al., 2010; Rigoglou and Papavassiliou, 2013). Indeed, our studies showed the activation of NF-κB in articular chondrocytes following stimulation with IL-1β. However this activation was suppressed by the coincubation with LN.

NF-κB expression was correlated with collagenase-3 (MMP-13) and stromelysin 1 (MMP-3) levels (Liacini *et al.*, 2003). Also, a shift to nuclear NF-κB localization was shown in chondrocytes during cartilage destruction in the early stage of arthritis in DBA/1 mice immunized with type II collagen (Eguchi *et al.*, 2002). Our study shows that stimulation with IL-1β causes stimulation of NF-κB activation in articular chondrocytes. It has been shown that in chondrocytes, the NF-κB and the MAP kinase pathways mediate inhibition of type II collagen and link protein gene expression by TNF-α (Saklatvala, 2007). Other studies have also shown that NF-κB, as well as MAP kinases, mediate MMP-1, MMP-3 and MMP-13 expression induced by TNF-α or IL-1β in human OA chondrocytes (Liacini et al., 2003; Liacini *et al.*, 2002). The MAP kinase pathway is the signaling pathway described as a partner for NF-κB in this regard. In this study, we did not look at the MAP kinase pathway. However, in a previous study, LN decreased the phosphorylation of p38 in nucleus pulposus cells (Petit *et al.*, 2011).

The potential use of inhibitors of NF-κB to reduce AC degradation by MMPs in arthritis has been described (Liacini et al., 2002; Vincenti and Brinckerhoff, 2002). Favorable results using nonsteroidal anti-inflammatory drugs (NSAIDs) (Tegeder *et al.*, 2001), and glucocorticoids (Yamamoto and Gaynor, 2001) demonstrate decreased NF-κB activation. However, the use of NSAIDs can result in gastrointestinal side effects and the lack of specificity in antisense and transcription factor decoy strategies present a big challenge when targeting gene expression in only a single organ. The problems of protein delivery, immunogenicity, and cost of treatment have

limited the realistic prospect of whole proteins for therapy. Being a synthetic peptide, LN has considerable financial benefits for clinical use over recombinant proteins. Furthermore, being a small peptide, it has the potential to selectively block specific signaling pathways that are dependent on NF-kB activation without affecting all NF-κB –dependent functions. Thus, it may be an ideal candidate for therapy.

Finally, we must accept that while inhibition of NF-κB by pharmacological agents as treatments for certain rheumatic diseases, such as OA and RA, may be attractive, the systemic and indiscriminate blockade of its numerous beneficial effects remains a concern. It is commonly believed that the NF-κB proteins play an essential and beneficial role in normal physiology. Thus, a means to improve specificity is needed.

CONCLUSIONS

LN can stimulate proteoglycan and collagen production in OA cartilage when it is administered in an inflammatory milieu. In addition, LN is also able to suppress metalloproteinase activity and NF-κB expression in the degenerated cartilage explants and chondrocytes from OA patients. These features are vital for any agent designed to become an effective therapy for joint diseases. In principle, systemic therapy by LN supplementation could be a viable option for treating cartilage degeneration and OA pain management.

ACKNOWLEDGEMENTS

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DISCLOSURES

The authors have nothing to disclose.

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FIGURE LEGENDS

Figure 1. GAG content in human OA cartilage treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 µg/mL], IL-1β [5 ng/mL], IL-1β + Link N, or vehicle (Control). GAG content was measured by the DMMB assay and quantified as µg per mg of cartilage tissue wet weight. The results are represented as Means ± SD, n = 4 donors; ANOVA posthoc Tukey's, multiple comparison test; *, $p < 0.05$ (compared to control); #, $p < 0.05$.

Figure 2. Proteoglycan content in human OA cartilage/bone explants treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 µg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. Histological sections demonstrating safranin O staining for proteoglycan content. Scale bar = 350 µm.

Figure 3. Western blot of aggrecan in human OA cartilage. Cartilage/bone explants in medium supplemented with LN [1 µg/mL], IL-1β [5 ng/mL], both IL-1β + LN and control cartilage. A) Representative blot demonstrating of intact aggrecan core protein with a molecular weight of about 250 kDa and fragments of aggrecan. B) Densitometry on blots presented in A). The results are represented as mean ± SD of three donors. ANOVA posthoc Tukey's multiple comparison test. ***, $p < 0.001$, comparison with control.

Figure 4. Hydroxyproline content in human OA cartilage treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 µg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. Hydroxyproline (HYP) was measured by the Hydroxyproline assay and quantified as µg per mg of cartilage tissue wet weight. Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test; *, $p < 0.05$, comparison with control.

Figure 5. Western blot of type II collagen in human OA cartilage. Cartilage/bone explants were treated with Link N [1 µg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. A) Representative blot demonstrating type II collagen (Col II). B) Densitometry on blots presented in A). Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test. *, $p < 0.05$, comparison with control; ##, $p < 0.01$, comparison with IL-1β.

Figure 6. Effect of Link N on IL-1β-induced gene expression in human OA chondrocytes. Chondrocyte pellets were treated with Link N [1 µg/mL] or PBS (CTL) for 6 days in the absence or presence of IL-1β [5 ng/mL]. Gene expression was measured by qPCR. Means ± SD; n = 4 donors; ANOVA, posthoc Tukey's multiple comparison test. ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, comparison with control; #, $p < 0.05$, comparison with IL-1β.

Figure 7. Dose-dependent effect of Link N on IL-1β signalling in human chondrocytes. Human osteoarthritic chondrocytes were treated with Link N [1 µg/mL] (LN), IL-1β [5 ng/mL], or IL-1β with the indicated concentrations of Link N [0.01, 0.1 or 1.0 µg/mL]. A) Representative blot demonstrating phosphorylated NFκB (P-NFκB) and GAPDH as a loading control. B) Densitometry on blots presented in A). Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test. ***, $p < 0.001$, ****, $p < 0.0001$, comparison with control; ##, $p < 0.01$, ####, $p < 0.0001$, comparison with IL-1β.

Figure 8. Effect of Link N on pain behavior in a mouse model of knee OA. Twelve weeks post-surgery mice were injected intra-articularly with PBS or Link N (5 µg) and monitored for pain-related behaviors longitudinally over 24 hrs using the von Frey assay. PWT (Paw weight threshold in grams). Means ± SDs; n = 8–10 animals. ANOVA, posthoc Tukey's multiple comparison test. ****, $p < 0.0001$ (PBS and Link N vs Sham); *** $p < 0.001$ (PBS vs Sham); ##, $p < 0.01$, (PBS vs Link N).

Table 1: Primers for gene expression

Genes	Primer sequence
ACAN	**F:** 5'-TGAGTCCTCAAGCCTCCTGT-3' **R:** 5'-CCTCTGTCTCCTTGCAGGTC-3'
COL1A1	**F:** 5'-GAGAGCATGACCGATGGATT-3' **R:** 5'-CCTTCTTGAGGTTGCCAGTC-3'
COL2A1	**F:** 5'-ATGACAATCTGGCTCCCAAC-3' **R:** 5'-CTTCAGGGCAGTGTACGTGA3'
ADAMTS4	**F:** 5'-TCCTGCAACACTGAGGACT-3' **R:** 5'-GGTGAGTTTGCACTGGTCC-3'
ADAMTS5	**F:** 5'-ACAAGGACAAGAGCCTGGAA-3' **R:** 5'-ATCGTCTTCAATCACAGCACA-3'
MMP1	**F:** 5'-AAAGGGAATAAGTACTGGG-3' **R:** 5'-GTTTTTCGAGTGTTTACCTCAG-3'
MMP3	**F:** 5'-GGCAGTTTGCTCAGCCTATC-3' **R:** 5'-GAGTGTCGGAGTCCAGCTT-3'
MMP13	**F:** 5'-TAAGGAGCATGGCGACTTC-3' **R:** 5'-GGTCCTTGGAGTGGTCAAG-3'
TIMP1	**F:** 5'-AATTCCGACCTCGTCATCAG-3' **R:** 5'-GTTGTGGGACCTGTGGAAG-3'
TIMP2	**F:** 5'-AAGCGGTCAGTGAGAAGGAA-3' **R:** 5'-CTTCTTTCCTCCAACGTCCA-3'
TIMP3	**F:** 5'-CTGACAGGTCGCGTCTATGA-3' **R:** 5'-AGTCACAAAGCAAGGCAGGT-3'
IL1	**F:** 5'-ACCTATCTTCTTCGACACATG-3' **R:** 5'-ACCACTTGTTGCTCCATATCC-3'
IL6	**F:** 5'-AACCTTCCAAAGATGGCTGAA-3' **R:** 5'-TGTACTCATCTGCACAGCTCT-3'

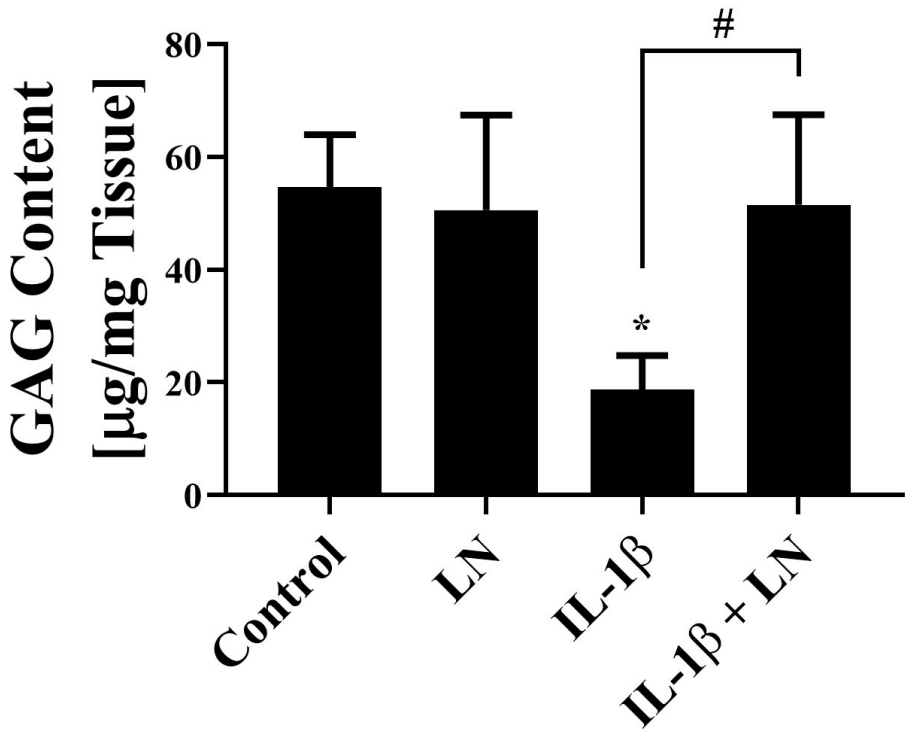

Figure 1. GAG content in human OA cartilage treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 µg/mL], IL-1β [5 ng/mL], IL-1β + Link N, or vehicle (Control). GAG content was measured by the DMMB assay and quantified as µg per mg of cartilage tissue wet weight. The results are represented as Means ± SD, n = 4 donors; ANOVA posthoc Tukey's, multiple comparison test; *, $p < 0.05$ (compared to control); #, $p < 0.05$.

112x90mm (300 x 300 DPI)

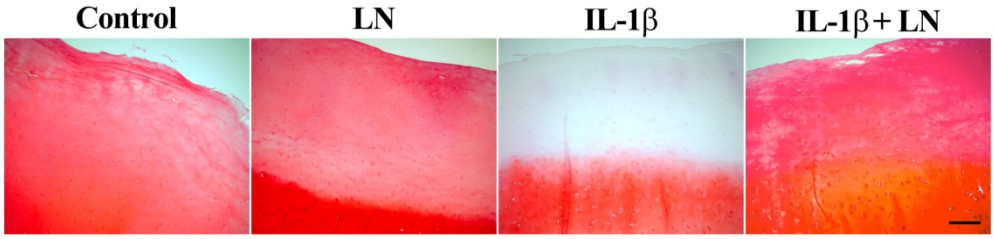

Figure 2. Proteoglycan content in human OA cartilage/bone explants treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 µg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. Histological sections demonstrating Safranin-O staining for proteoglycan content. Scale bar = 350 µm.

176x43mm (300 x 300 DPI)

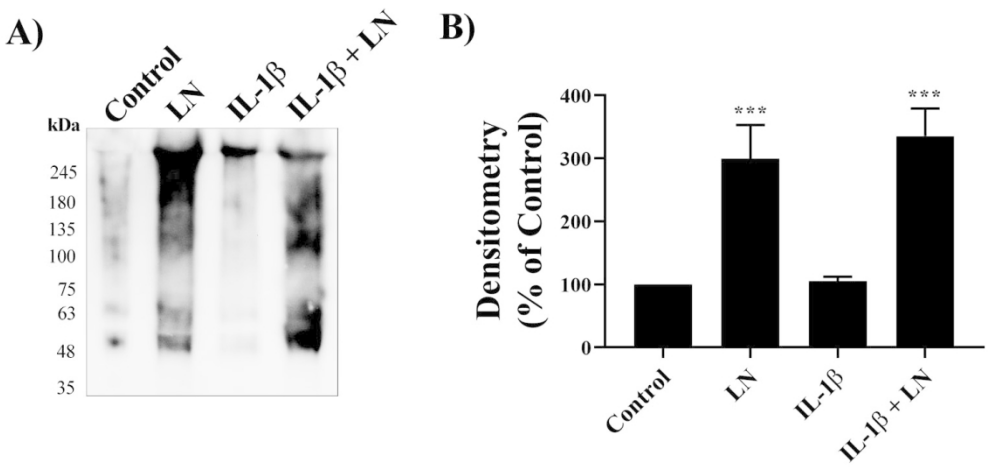

Figure 3. Western blot of aggrecan in human OA cartilage. Cartilage/bone explants in medium supplemented with LN [1 µg/mL], IL-1β [5 ng/mL], both IL-1β + LN and control cartilage. A) Representative blot demonstrating of intact aggrecan core protein with a molecular weight of about 250 kDa and fragments of aggrecan. B) Densitometry on blots presented in A). The results are represented as mean ± SD of three donors. ANOVA posthoc Tukey's multiple comparison test. ***, $p < 0.001$, comparison with control.

130x62mm (300 x 300 DPI)

Figure 4. Hydroxyproline content in human OA cartilage treated with IL-1β and Link N. Cartilage/bone explants were cultured for 21 days in medium supplemented with Link N [1 μg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. Hydroxyproline (HYP) was measured by the Hydroxyproline assay and quantified as μg per mg of cartilage tissue wet weight. Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test; *, $p < 0.05$, comparison with control.

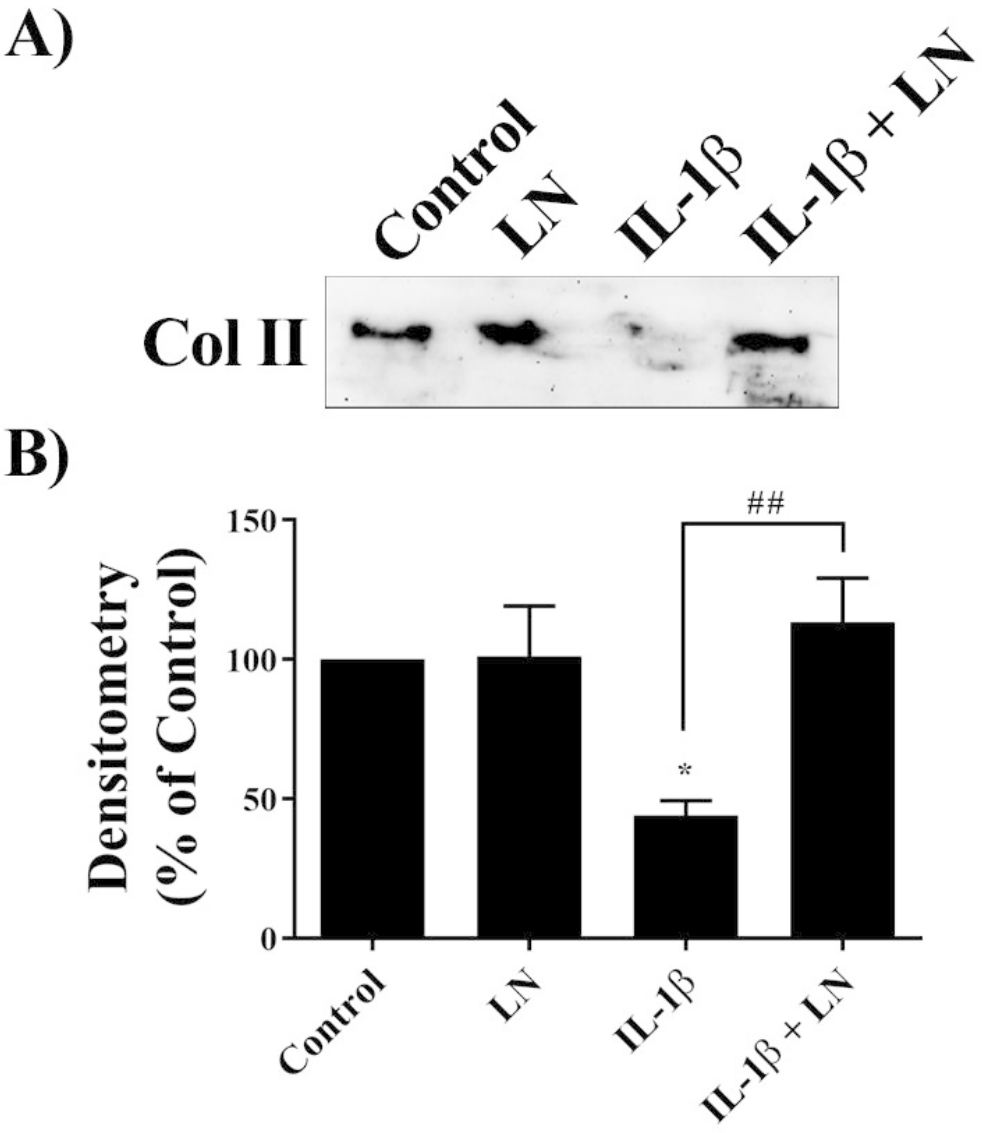

Figure 5. Western blot of type II collagen in human OA cartilage. Cartilage/bone explants were treated with Link N [1 μg/mL], IL-1β [5 ng/mL], or IL-1β + Link N. A) Representative blot demonstrating type II collagen (Col II). B) Densitometry on blots presented in A). Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test. *, $p < 0.05$, comparison with control; ##, $p < 0.01$, comparison with IL-1β.

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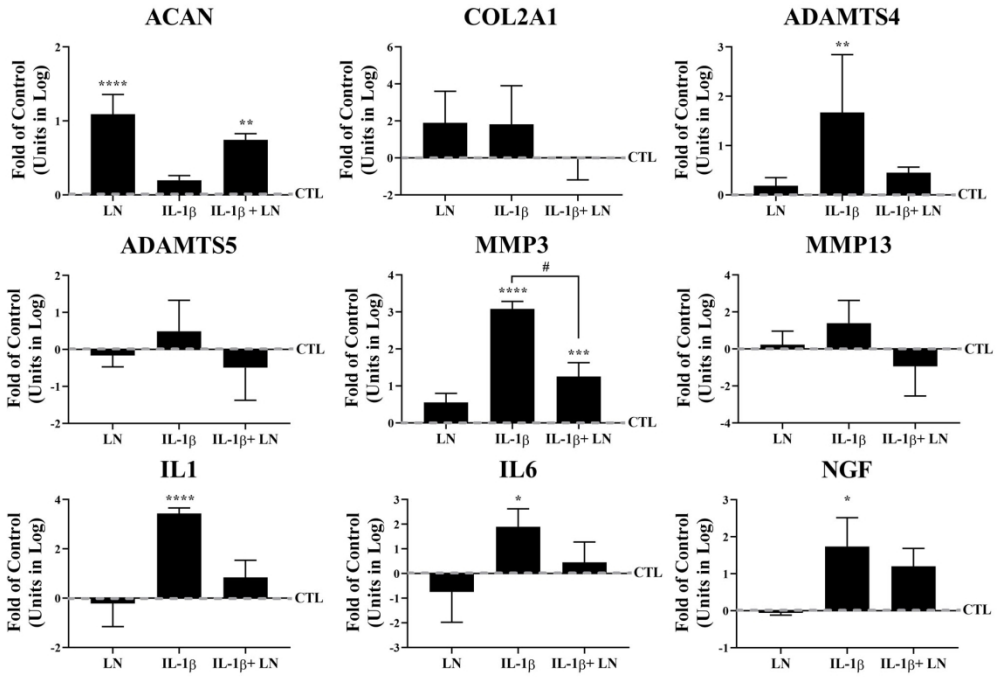

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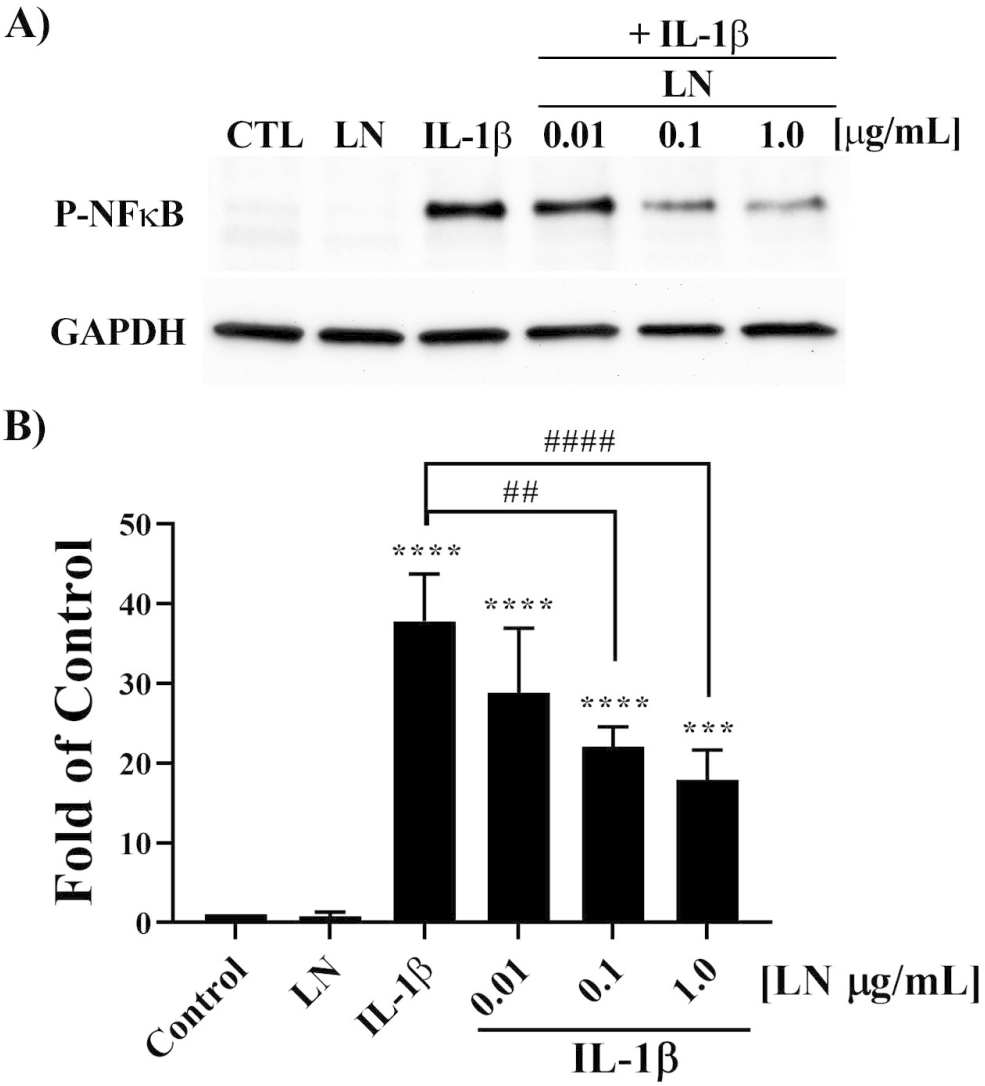
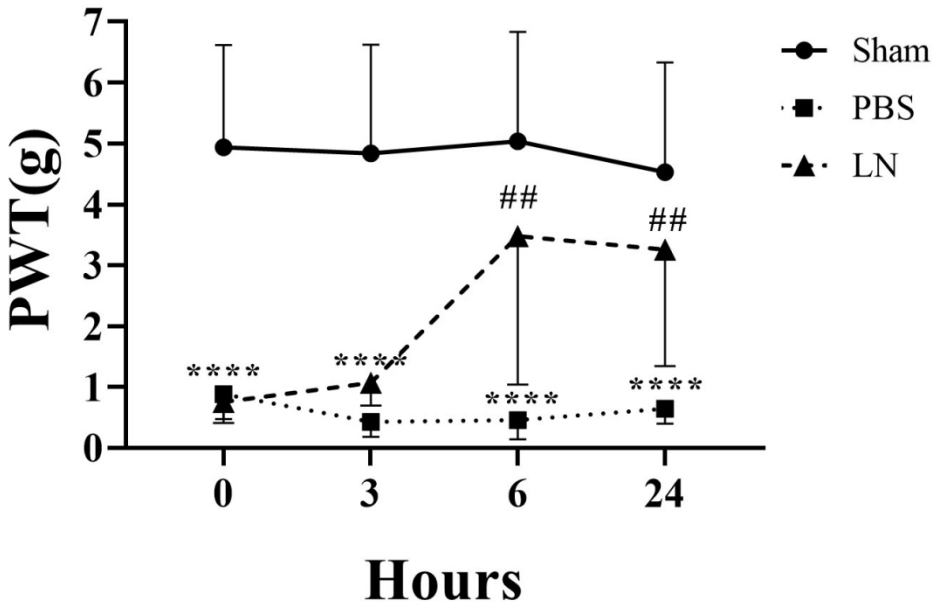
171x118mm (300 x 300 DPI)



Dose-dependent effect of Link N on IL-1β signalling in human chondrocytes. Human osteoarthritic chondrocytes were treated with Link N [1 μg/mL] (LN), IL-1β [5 ng/mL], or IL-1β with the indicated concentrations of Link N [0.01, 0.1 or 1.0 μg/mL]. A) Representative blot demonstrating phosphorylated NFκB (P-NFκB) and GAPDH as a loading control. B) Densitometry on blots presented in A). Means ± SD, n = 4 donors; ANOVA posthoc Tukey's multiple comparison test. ***, $p < 0.001$, ****, $p < 0.0001$, comparison with control; ##, $p < 0.01$, ####, $p < 0.0001$, comparison with IL-1β.

108x119mm (300 x 300 DPI)



Effect of Link N on pain behavior in a mouse model of knee OA. Twelve weeks post-surgery mice were injected intra-articularly with PBS or Link N (5 µg) and monitored for pain-related behaviors longitudinally over 24 hrs using the von Frey assay. PWT (Paw weight threshold in grams). Means ± SDs; n = 8–10 animals. ANOVA, posthoc Tukey's multiple comparison test. ****, $p < 0.0001$ (PBS and Link N vs Sham); *** $p < 0.001$ (PBS vs Sham); ##, $p < 0.01$, (PBS vs Link N).

119x82mm (300 x 300 DPI)

Section 3: Thesis Conclusion and References

Conclusion

Link N peptide can stimulate the production of proteoglycan and collagen synthesis in OA cartilage within an inflammatory milieu (IL-1β). In addition, it has the ability to suppress metalloproteinase activity.

One mechanism for Link N in preserving matrix protein synthesis may, in part, be due to its ability to rapidly suppress IL-1β-induced activation of NF-κB in the degenerated cartilage explants and chondrocytes from OA patients.

Further work is needed to determine whether Link N directly inhibits the IL-1β receptor or interferes with NF-κB activation through an independent pathway(s). Link N may be a cost-effective therapy in the treatment of OA by delaying the progression of the disease.

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List of Abbreviations

Agg:	Aggrecan
ADAMTS:	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
BAFF:	B-cell activating factor
BFGF	Basic Fibroblast Growth Factor
BMP:	Bone Morphogenetic Protein
Col	Collagen
CTL:	Control
CS:	Chondroitin Sulfate
DMEM:	Dulbecco's Modified Eagle's Medium
DMMB:	Dimethylmethylene Blue
DMOAD:	Disease Modifying Osteoarthritis Drug
ECM:	Extracellular Matrix
EGF:	Epidermal Growth Factor
ERK:	Extracellular Signal-Regulated Kinase
FGF:	Fibroblast Growth Factor
FBS:	Fetal Bovine Serum
GAG:	Glycosaminoglycans
GAPDH:	Glyceraldehyde 3-Phosphate Dehydrogenase

GAG:	Glycosaminoglycans
GD:	Globular Domain
GLUT:	Glucose Transporter
HA:	Hyaluronic Acid
HIF-1α	Hypoxia-Inducible Factor 1-Alpha,
HLA:	Human Leukocyte Antigen
IGD:	Interglobular Domain
IGF-1:	Insulin-like Growth Factor-1
IGFBP:	Insulin-Like Growth Factor Binding Protein
IKKs:	IκB Kinase
IRS-1:	Insulin Receptor Substrate-1
IL:	Interleukin
IL-1β:	Interleukin-1-β
IL-1RI:	IL-1 receptor type I
IVD:	Intervertebral Disc
KS:	Keratan Sulfate
LAP-1:	Latency-Associated Peptide-1
LP:	Link Protein
LPP:	Link Protein Peptide

MF:	Microfracture
MSCs:	Mesenchymal Stem Cells
MMPs:	Matrix Metalloproteinases
MPa:	Megapascal
MRI:	Magnetic Resonance Image
MT1-MMP:	Matrix metalloproteinases Membrane Type I
NO:	Nitric Oxide
NF-κB:	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NIK:	NF-κB Inducing Kinase
OA:	Osteoarthritis
PGE2:	Prostaglandin E2
PGs:	Proteoglycans
PDGF:	Platelet Derived Growth Factor
PTH:	Parathyroid Hormone
PRG4:	Proteoglycan 4 [Homo Sapiens (Human] - Gene
RCT:	Randomized Control Trial
SOCS3:	Suppression of Cytokine Signaling 3
TDR:	Total Disc Replacement
TGF:	Transforming Growth Factor

TGF-β:	Transforming Growth Factor-β
TGF-βRI:	TGF-beta receptor type-2 precursor
TIMP-3:	Tissue Inhibitor of Matrix Metalloproteinase 3
TNF:	Tumor Necrosis Factor
TNF-α:	Tumor Necrosis Factor-Alpha
VEGF:	Vascular Endothelial Growth Factor

Section 4: Contribution to Other Projects

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METHODS ARTICLE

Development of a Large Animal Long-Term Intervertebral Disc Organ Culture Model That Includes the Bony Vertebrae for *Ex Vivo* Studies

Michael Grant, PhD,[1,2] Laura M. Epure, MSc,[2] Omar Salem, MD,[1,2] Nizar AlGarni, MD,[1,2]
Ovidiu Ciobanu, DMD, PhD,[2] Motaz Alaqeel, MD,[1,2] John Antoniou, MD, PhD,[1,2] and Fackson Mwale, PhD[1,2]

Intervertebral disc (IVD) degeneration is a common cause of low back pain. Testing potential therapeutics in the regeneration of the disc requires the use of model systems. Although several animal models have been developed to investigate IVD degeneration, they are technically challenging to prepare, expensive, present with limitations when performing biomechanical studies on the disc, and are impractical in large-scale screening of novel anabolic and scaffolding agents. An IVD organ culture system offers an inexpensive alternative. In the current paradigm, the bony endplates are removed to allow for nutrient diffusion and maintenance of disc cell viability. Although this is an excellent system for testing biologics, it results in concave cartilage endplates and, as such, requires special platens for loading purposes in a bioreactor as flat ones can overload the annular disc region leading to improper loading. Furthermore, the absence of bone makes it unsuitable for applying complex cyclic loading, a topic of interest in the study of chronic progressive degeneration, as multiaxial loading is more representative of daily forces encountered by the IVD. We have developed and validated a novel long-term IVD organ culture model that retains vertebral bone and is easy to prepare. Our model is ideal for testing potential drugs and alternate-based therapies, in addition to investigating the long-term effects of loading paradigms on disc degeneration and repair.

Introduction

Chronic back pain is one of the main reasons for disability in the working population.[1] Although several factors have been associated with the development of chronic back pain, one factor that is strongly correlated is degeneration of the intervertebral disc (IVD).[2] IVDs are the largest avascular tissue in the body, composed of an inner nucleus pulposus (NP) and surrounded by an annulus fibrosus (AF). They are linked to adjacent vertebrae within the spine through contact with cartilaginous (C) and bony (B) endplates (EPs).[3] One key function of the IVD is to dissipate compressive loading on the spine. This is achieved primarily by the NP, a gelatinous matrix composed mostly of the proteoglycan, aggrecan, which attracts water molecules.[4,5] The AF, composed of concentric layers (lamellae) of fibrocartilage, are arranged to resist sheer forces, and together with the NP, impart the disc with hydrostatic pressure.[3,6] IVD nutrition is primarily achieved by diffusion through the capillary buds from the adjacent vertebrae that end at the cartilage endplate (CEP), the gateway for nutrient supply to the disc.[7]

IVD degeneration is influenced by a multitude of factors: poor nutrition, aging, biomechanical,[8–11] biochemical,[12–16] genetic factors,[17–20] and injury of the spine. Thus, mechanisms that are responsible for disc degeneration lead to biochemical changes in the composition and structure of the extracellular matrix due to both reduced synthesis and increased degradation, with a particularly pronounced net loss of aggrecan.[21]

Several models are employed to study the processes and treatments of IVD degeneration. They include *in vitro* 2- and 3-dimensional culturing of IVD cells, *in vitro* culturing of IVD tissues (NP and AF), whole-disc organ culturing of IVD, and *in vivo* animal models. Each system has its limitations. For instance, culturing IVD cells in isolation may be inexpensive, however, this system does not replicate the IVD environment, and mechanical studies are not possible. Small animals such as rabbits, rats, and mice have been used for metabolic studies[22–26] and disc repair.[27–29] Advantages of small animal models include affordability and a rapid degeneration process. However, there are issues with managing the small size of the IVDs and the NP cells that remain notochordal throughout life, unlike the human, and

[1]Department of Surgery, McGill University, Montreal, Canada.
[2]Orthopaedic Research Laboratory, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, Montreal, Canada.

many large animal models.[30,31] Alternatively, large animals such as dog and sheep have a disc structure, cell type, and size similar to the human,[32–34] they undergo degeneration slowly, are expensive, and not appropriate to establish new experimental conditions. Motion segment organ models, using larger discs that preserve the native IVD structure and adjacent vertebral bodies, have an invaluable role in developing new therapies, but loss of cell viability when cultured for long periods limits their usefulness.[35–37]

Although advancements in organ culture model techniques with larger discs have been achieved, they are hampered by the inability to achieve long-term cell viability due to hindrance in nutrient diffusion through the bony endplate (BEP).[34,37,38] IVD cell viability is maintained in a large animal disc by removal of vertebral bone and the adjacent calcified portion of the EP.[33,36,39,40] Although this partially improves problems with nutrient diffusion, it results in concave EPs that makes applying complex loading, such as torsion in a bioreactor, a major challenge.

Complex loading, which consists of axial compression, torsion, flexion, and extension within the spine, is becoming an increasingly important topic of investigation, as it has been shown to affect disc biology.[41–47] Ideally, a long-term disc organ culture model with a flat surface would be preferable to a curved one, although at present there is no established procedure to achieve this result beyond 3 weeks. Recently, a method was established that maintains a flat surface by retaining the BEP.[35] However, cell viability and proteoglycan synthesis declined after 21 days.

The objective of the present study was to develop a large animal disc organ culture model that maintains long-term cell viability while retaining the BEP and vertebral bone (vIVDs). To achieve this, we developed an easy-to-use media system named PrimeGrowth™ that when applied to isolated vIVD motion segments can extend and maintain viability for up to 5 months. Our media system for vIVD preparation and culture is highly reproducible, making it amenable for investigation of regenerative disc therapies and mechanobiology in a well-controlled environment.

Materials and Methods

Disc isolation and culture

Tails of 22- to 28-month-old steers were obtained from the local abattoir within 4 h of slaughter. Tails were immersed in Dovidine Solution (10% Povidone–Iodine) (Laboratoire Atlas, Inc.) before removal of the skin and excess soft tissue. The largest four IVDs were prepared for organ culture by parallel cuts through the adjacent vertebral bodies at ~1 cm from the EPs (vIVDs) using an IsoMet®1000 precision sectioning saw (Buehler) (Fig. 1). Sixteen vIVDs were washed in phosphate-buffered saline 1× (PBS) (Cat# 311-010-CL; Wisent, Inc.) and divided into two groups: eight Control (Dulbecco's modified Eagle's medium [DMEM]) and eight PrimeGrowth™. vIVDs were placed into sterile 60-mL LeakBuster™ Specimen Containers (Starplex Scientific, Inc.) followed by incubation in either 30 mL DMEM (Cat# 319-005-CL; Wisent, Inc.) or 30 mL PrimeGrowth Isolation Medium (Cat# 319-511-EL; Wisent, Inc.) for 1 h on an orbital rocker at 37°C. To neutralize the reaction with PrimeGrowth Isolation Medium, vIVDs were washed thrice for 2 min with PrimeGrowth Neutralization Medium (Cat# 319-512-CL; Wisent, Inc.). The control vIVDs were washed thrice for 2 min in DMEM. Both groups of vIVDs (DMEM and PrimeGrowth) were placed in newly prepared 0.2 μm filter-vented 60-mL LeakBuster Specimen Containers and cultured in 30 mL standard growth medium (DMEM, 10% heat-inactivated fetal bovine serum, 1× penicillin–streptomycin, and 50 μg/mL ascorbic acid) or 30 mL PrimeGrowth Culture Medium (Cat# 319-510-CL; Wisent, Inc.), respectively, under standard culture conditions (37°C, 5% CO_2). Medium was replaced every 3 days for each group of vIVDs. A summary of the procedure and a representative vIVD dissected demonstrating vertebral bone thickness and CEP, NP, and AF content is presented in Figure 2.

Live/dead assay and cell density

vIVDs cultured for 1 or 5 months were dissected to separate the NP, inner AF (iAF), and outer AF (oAF) regions. A 4 mm biopsy punch was used to prepare specimens for cell viability using a live/dead fluorescence assay (Live/Dead® Viability/Cytotoxicity Kit, Cat# L3224; Thermo Fisher Scientific, Waltham, MA). Bone sections were prepared by cutting a portion of the vertebral bone adjacent to the disc. Tissue punches and bone sections were washed once in PBS before incubation with the Live/Dead Viability/Cytotoxicity reagent for 30 min following the manufacturer's guidelines. Tissue punches were placed on a slide and visualized by confocal microscopy using a Zeiss LSM confocal laser-scanning microscope equipped with 488 and 543 nm laser lines. Ten Z-stacked images were merged representing 100 μm tissue thickness. Percent viability was determined by counting the total number of green (live) and red (dead) cells from the merged images using the cell counter function in ImageJ (National Institute of Health, Bethesda, MD) and calculating the ratio of the two (live/dead).

To determine cell density, NP, iAF, and oAF tissue punches of freshly prepared (Day 0) and 5-month PrimeGrowth® -cultured vIVDs were incubated with the Live/Dead Fluorescent Assay Kit as indicated above. Tissues were imaged by confocal microscopy using a 488 nm laser line and Z-stacked images representing a volume of 0.1 mm^3 were developed. Cells were counted using the cell counter function in ImageJ software (National Institute of Health, Bethesda, MD).

Glucose diffusion

vIVDs from either the DMEM or PrimeGrowth groups were cultured for 1 month. Discs were washed in PBS and incubated for 72 h in diffusion medium containing PBS (1×), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM), KCl (5 mM), 0.1% BSA, and 0.5 mM 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), a D-glucose fluorescent analogue. To demonstrate the diffusion of 2-NBDG in disc tissue, 3 mm biopsy punches were taken from dissected vIVDs and imaged using a Zeiss LSM confocal laser-scanning microscope equipped with a 488 nm laser line. Ten Z-stacked images representing an area 1 mm^2 and 100 μm depth were compiled using the 3D surface function in ImageJ software (National Institute of Health Research, Bethesda, MD). Average normalized intensities were taken from three different regions of either NP, iAF, or oAF over three vIVDs and plotted in 3D using ImageJ. Data were normalized by subtracting fluorescent intensity signals from the NP, iAF, or oAF tissues of dissected vIVDs incubated in

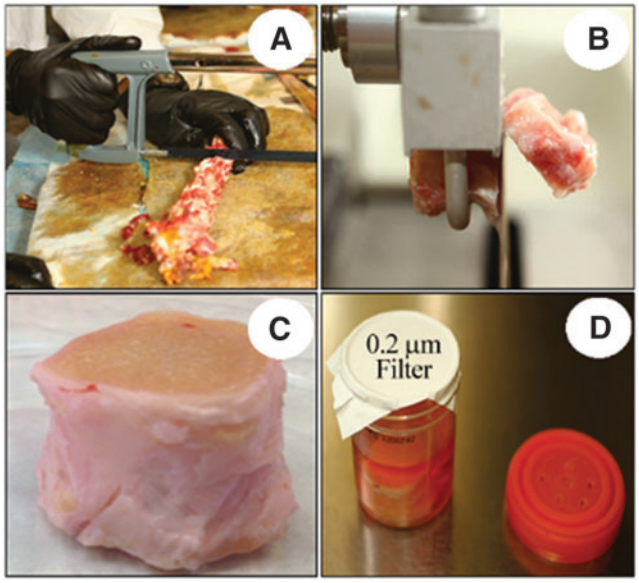

FIG. 1. Images demonstrating IVD preparation. **(A)** Bovine caudal tail processing and crude sectioning of IVD with a hand saw. **(B)** Sectioning of IVDs with an IsoMet®1000 precision sectioning saw leaving ~1 cm of vertebral bone. **(C)** Representative vIVD after cutting showing vertebral bone. **(D)** IVDs in culture after processing with PrimeGrowth™. IVD, intervertebral disc; vIVD, vertebral bone IVD.

diffusion medium containing no 2-NBDG. To determine the concentration of 2-NBDJ in vIVD tissue, discs were dissected and biopsy punches from the different regions (NP, iAF, oAF) were incubated in extraction buffer (4 M guanidinium chloride, 0.1 M Tris HCl, pH 7.5) for 48 h. Extracts were measured for fluorescence using a FLUOstar OPTIMA (BMG Labtech) spectrophotometer and calculated for concentration using a standard curve prepared from 2-NBDG titration.

Histology

IVDs were dissected from the vertebra, fixed in Accustain (Sigma-Aldrich), paraffin embedded, and 5 µm sections were prepared on slides. Sections were rehydrated before immunohistochemistry or staining by deparaffinization in xylene followed by sequential incubation in decreasing concentrations of alcohol and water. Proteoglycan content was visualized by staining tissue sections with Safranin O solution (0.1%) for 5 min. Slides were washed in water before mounting. To determine collagen content, specimens were stained with 0.1% Picrosirius red for 1 h and washed twice in acidified water (0.5% acetic acid). All sections were dehydrated by sequential alcohol concentrations and xylene, and mounted in Permount (Thermo Fisher Scientific). Slides were imaged using a Leica DM LB2 microscope (Leica Microsystems Inc.).

Glycosaminoglycan analysis

Sulfated glycosaminoglycans (GAGs) were quantified in tissue extracts by a modified dimethyl methylene blue (DMMB) dye-binding assay.[48,49] Samples were diluted to fall within the middle of the linear range of the standard curve.

Statistical analysis

Statistical analysis was performed using two-way analysis of variance followed by Bonferroni multiple comparison test with GraphPad Prism 5.0 software.

Results

Current methods of isolating and culturing IVDs for extended periods that retain vIVD result in extensive disc cell death. To determine if PrimeGrowth media system improves the viability of disc cells in vIVDs, we cultured vIVDs in PrimeGrowth or standard growth (Control) medium for 1 month. As shown in Figure 3A, when vIVDs were processed with PrimeGrowth, cell viability (green cells) was maintained in the IVD. Greater than 90% cell viability was achieved in all regions of PrimeGrowth-treated discs, compared with <25% in the NP, and <5% in the iAF and oAF, respectively, when vIVDs were cultured in control medium (Fig. 3B). Interestingly, PrimeGrowth also maintained the viability of cells in the vertebral bone of vIVDs (Fig. 3A, B). To determine if PrimeGrowth improved the nutrient availability of disc cells, we prepared vIVDs using either PrimeGrowth or control media, cultured them for 1 month in the respective media, followed by 72 h incubation with the stable fluorescent glucose analog, 2-NBDG. Accumulation of 2-NBDG in vIVDs suggests nutrient diffusion. Using 3D confocal Z-stack fluorescent intensity mapping of the NP, iAF, and oAF of dissected vIVDs, the presence of 2-NBDG was markedly reduced or even absent in various regions of the control discs. Contrarily, in the PrimeGrowth group, 2-NBDG was found to be widely diffused throughout the disc (Fig. 4A). Similarly, when vIVDs

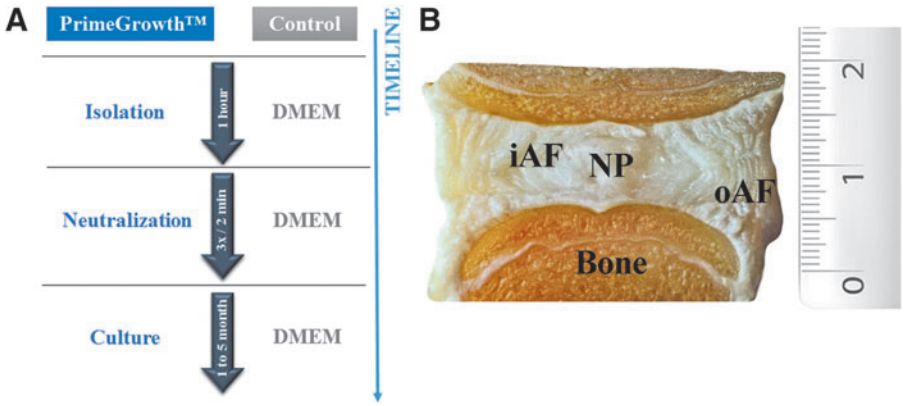

FIG. 2. Schematic of vIVD processing with PrimeGrowth. **(A)** vIVDs were cultured for 4 weeks in either PrimeGrowth culture medium or control medium (DMEM). **(B)** Representative image of a dissected vIVD following processing and 1 month culturing with PrimeGrowth culture medium. Imperial ruler demonstrating thickness of vertebral bone and disc. DMEM, Dulbecco's modified Eagle's medium.

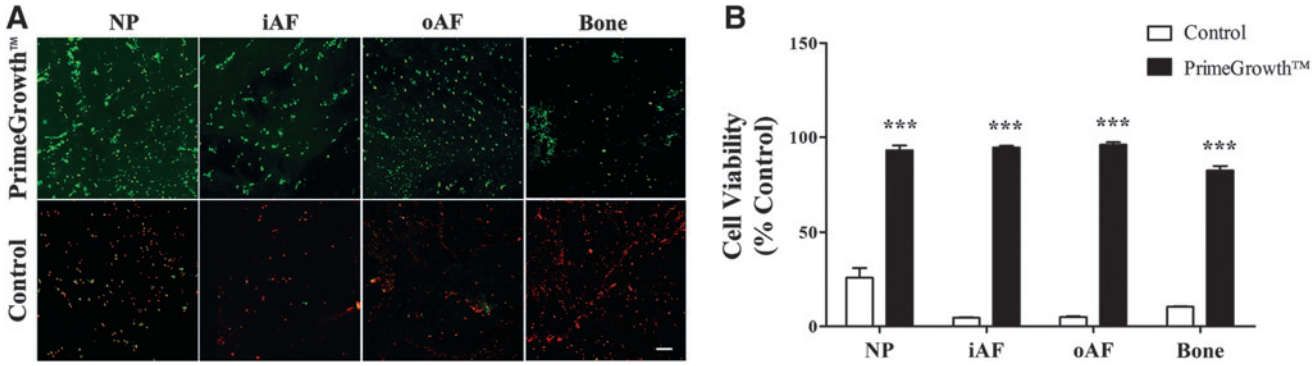

FIG. 3. Comparison of cell survival in discs isolated with PrimeGrowth or control medium. Cell viability was measured in a 1-mm-thick tissue section after vIVDs were incubated in serum-free medium containing fluorescent dyes (Live/Dead, Invitrogen). **(A)** Live (*green*) and dead (*red*) cells were visualized using an inverted confocal laser-scanning microscope after 1 month of culture. Cell viability is illustrated separately in the NP, iAF, oAF, and bone. **(B)** Comparison of percentage cell viability in discs treated with PrimeGrowth or control from images presented in **(A)**. Mean ± SEMs, $n = 6$ discs per group. Two-way ANOVA, *post hoc* Bonferroni, was used to compare sections from control and PrimeGrowth-treated vIVDs. ***, $p < 0.001$. iAF, inner annulus fibrosus; NP, nucleus pulposus; oAF, outer annulus fibrosus. ANOVA, analysis of variance.

were dissected and 2-NBDG was extracted from NP, iAF, and oAF tissues, and analyzed by fluorescent spectroscopy, significantly higher concentrations of 2-NBDG were found in all regions of the PrimeGrowth-treated vIVDs when compared with controls (Fig. 4B).

Monitoring regeneration of vIVDs requires extended periods of culturing, as the disc environment presents with reduced oxygen tension and nutrient permeability rates effectively altering the metabolism of IVD cells compared with other cell types.[50,51] To determine if PrimeGrowth-treated vIVDs can remain viable for extended periods of time, we cultured them for up to 5 months. As shown in Figure 5, cell viability persisted in all regions of the vIVD (NP, iAF, and oAF) similar to freshly processed vIVDs (Day 0). Notwithstanding cell viability, long-term culturing of vIVDs did not affect disc cellularity. There were no differences in NP, iAF, or oAF cell numbers between Day 0 and 5-month cultured vIVDs (Fig. 5C).

To determine if the matrix composition of PrimeGrowth-treated vIVDs was affected following extended culturing periods, we measured proteoglycan and collagen content. Histological examination of freshly prepared vIVDs (Day 0), and those cultured for 1 or 5 months in PrimeGrowth demonstrated no apparent difference in Safranin O staining for proteoglycans in either the NP or AF regions of the discs (Fig. 6A). Similarly, using the DMMB dye-binding assay to measure GAGs, an indicator of proteoglycan content in NP and AF tissues, no significant differences were determined

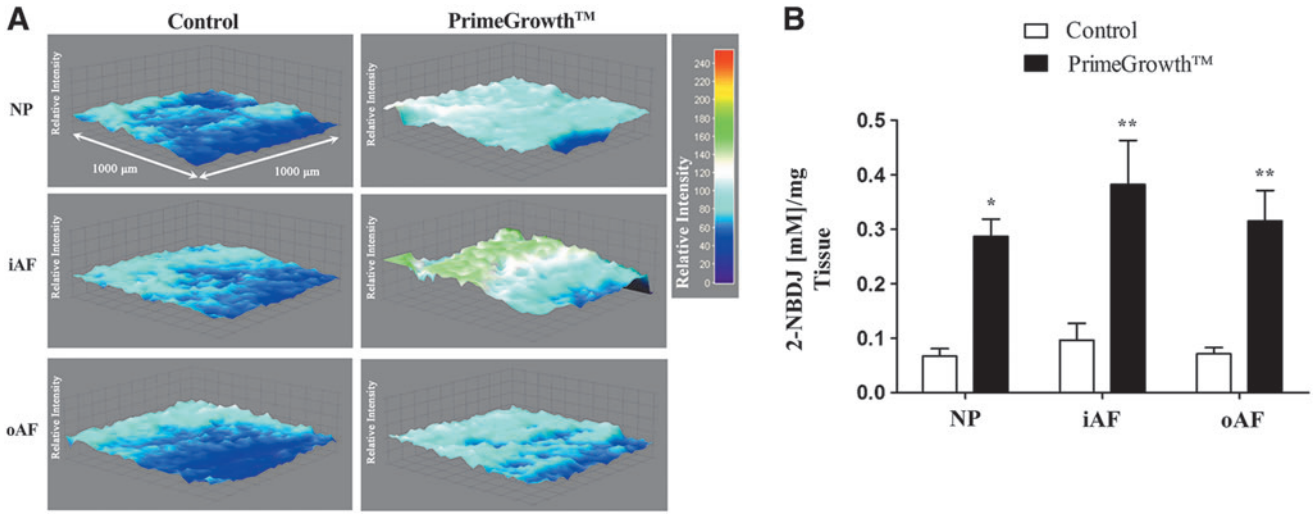

FIG. 4. Glucose diffusion in vIVDs treated with PrimeGrowth or control. Bovine IVDs with vertebrae were prepared with control or PrimeGrowth medium and cultured for 1 month. Discs were incubated for 72 h in diffusion medium containing 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), a D-glucose fluorescent analogue. **(A)** Fluorescent intensity mapping representing diffusion of 2-NBDG in NP, iAF, and oAF tissue from PrimeGrowth and control treated vIVDs. Averaged normalized intensity maps were prepared from three independent discs. Scale bar indicates increased signal intensity from *blue* to *red*. **(B)** Discs treated with 2-NBDG were dissected and regions (NP, iAF, oAF) were incubated in extraction buffer. Extracts were measured for fluorescence using a spectrophotometer. Mean ± SEMs, $n = 3$ discs per group. Two-way ANOVA, *post hoc* Bonferroni, was used to compare PrimeGrowth with the control group within each region (NP, iAF, oAF). *, $p < 0.05$; **, $p < 0.01$.

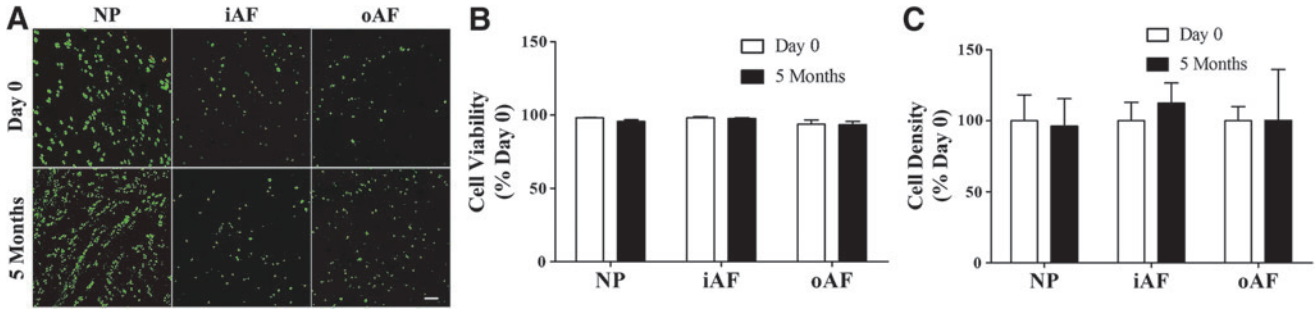

FIG. 5. Comparison of cell survival in discs isolated with PrimeGrowth after 5 months in culture and discs before culture (Day 0). Cell viability was measured in a 1-mm-thick tissue section after incubation in serum-free medium containing fluorescent dyes (Live/Dead, Invitrogen). **(A)** Live (*green*) and dead (*red*) cells were visualized using an inverted confocal laser-scanning microscope on day 0 and after 5 months of culture. **(B)** Cell viability is illustrated separately in the NP, iAF, and oAF. Note that the percentage cell viability was very similar on day 0 and after 5 months of culturing. Mean ± SEMs, $n = 3$ discs per group. No statistical differences were observed. Two-way ANOVA, *post hoc* Bonferroni, $p > 0.05$. **(C)** Cellularity of NP, iAF, and oAF tissues in Day 0 and 5-month PrimeGrowth-cultured vIVDs. No statistical differences were observed. Two-way ANOVA, *post hoc* Bonferroni, $p > 0.05$.

in freshly prepared (Day 0) compared with 1 or 5 month(s) cultured vIVDs, respectively (Fig. 6B). In addition to proteoglycan, collagen content, as determined by Picrosirius red staining, also remained similar in the NP and AF regions of Day 0, 1-, and 5-month cultured vIVDs (Fig. 6C).

Discussion

Large animal IVD organ culture models are an effective means of investigating regenerative therapies in disc degeneration and the biological responses of loading on disc cell physiology and matrix composition in a controlled environment. Although several organ culture models have been developed, limitations on the long-term culturing of IVDs that retain vertebrae persist.[34–37,52,53] The vertebrae, and particularly the BEP, are infiltrated with blood vessels that terminate at the CEP—the membrane barrier that filters nutrients entering the disc. It is believed that these vessels undergo clotting during IVD isolation, obstructing, and thereby limiting nutrient supply to disc cells eventually resulting in cell death.

In the present study, we characterize the use of a three-step media system (isolation, neutralization, and culture) named PrimeGrowth, which was specifically optimized for the isolation and culturing of vIVDs. Our unique media formulation was demonstrated to sustain greater than 90% NP and AF cell viability in free-swelling vIVDs cultured for up to 5 months. To our knowledge, this is the longest reported survival period of any IVD organ culture model retaining vertebral bone in the literature.

In earlier attempts at culturing bovine IVDs retaining BEPs for studying mechanobiology, cell viability was markedly decreased after 1 week.[37] To overcome drastic declines in cell viability in large animal IVDs retaining BEPs, Gantenbein *et al.*[34] injected sheep with heparin before sacrifice and isolation of their discs. IVD cell viability was greatly improved in this model; however, decreases were apparent after 7 days in culture. Although this method of preparing IVDs was possible in ovine, heparinization is not a feasible option for culturing bovine IVDs since tissues are often obtained from abattoirs.[33,35,37,39,40,46,47,54–57]

An alternative method derived to overcome barriers to nutrient diffusion by removing the vertebrae and the BEP all together, but retaining the CEP to prevent disc swelling, has been effective in maintaining long-term cell viability in cultured discs.[36,40] Although an excellent system for

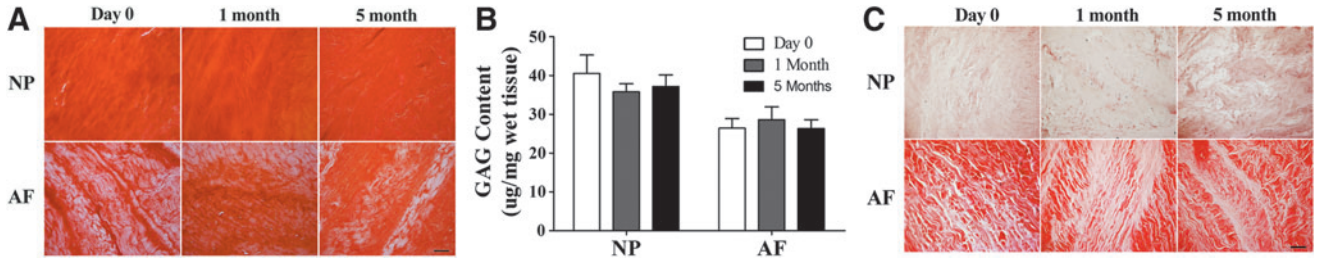

FIG. 6. Proteoglycan content in vIVDs following prolonged culturing. Discs isolated with the PrimeGrowth media were cultured for 1 or 5 months. **(A)** Representative images of NP and AF regions of vIVDs freshly prepared (Day 0), or cultured for 1 or 5 months in PrimeGrowth medium stained with Safranin O. Scale bar, 100 μm. **(B)** GAG content of NP and AF tissues from Day 0, 1-, and 5-month culture discs. The results are represented as mean ± SEMs of three discs from different bovine tails in each group. No statistical differences in GAG content were observed between NP or AF tissues of the vIVDs. Two-way ANOVA, *post hoc* Bonferroni, $p > 0.05$. **(C)** Histological images of NP and AF regions of Day 0, 1, and 5 months of PrimeGrowth-cultured vIVDs stained with Picrosirius red. Scale bar, 100 μm. AF, annulus fibrosus. GAG, glycosaminoglycan.

investigating biologics in disc repair,[57] this organ culture model is limited when considering various loading paradigms in repair strategies. Vertebral bone and the BEP provide flat surfaces amenable to static and dynamic loading paradigms. The use of this method in a bioreactor drew attention to the fact that the CEPs were concave and not ideal for flat platens, resulting in overloading of annular disc regions leading to cell death.[39] Thus, special platens and analysis of stress profilometry were required to evaluate load distribution in the discs with different geometries to find a shape that mimicked native load transfer.[39] Notwithstanding, the absence of bone makes it unsuitable for applying complex loading, a topic of interest in chronic progressive disc degeneration.[42,46,47,56]

Recently, Chan and Gantenbein-Ritter have demonstrated sustained NP and AF cell viability in bovine IVDs that retain both BEP and vertebral bone for a period of 2 weeks by adopting a surgical jet lavage system in their disc isolation method.[35] The success of their model was attributed to the penetrating abilities of the jet lavage capable of removing blood clots within the vertebral bone. Although this approach greatly improved the viability of both NP and AF cells in vIVD cultures, cell viability had begun to decline at the end of a 21-day culture period. Therefore, any experiments on these vIVDs must be performed within a 14-day period to be certain that the physiology and viability of IVD cells are not comprised due to culturing limitations.

In our organ culture model, treatment of vIVDs with PrimeGrowth isolation solution permitted diffusion of nutrients into the disc, as determined by incorporation of the glucose analog 2-NBDG in NP and AF tissues. When vIVDs were conditioned in PrimeGrowth isolation solution followed by culturing in PrimeGrowth culture medium, specifically optimized to represent the native physiological environment of the disc, long-term cell viability was achieved. Although long-term culturing of vIVDs may affect the integrity of disc tissue independent of cell viability, we found no significant differences in proteoglycan content upon either histological examination or GAG assay of NP and AF tissues. A similar result was obtained following histological examination of collagen fibers in vIVDs. PrimeGrowth also maintained viability of cells in the bony vertebrae. Taken together, these results suggest that our vIVD organ culture system not only retains the characteristic features of the disc for up to 5 months in culture, but maintains a viable vertebral bone. This organ model system provides a unique opportunity to investigate the interplay between the vertebrae and disc following long-term biomechanical loading.

In conclusion, we provide a novel ready-to-use disc preparation and culturing approach for vIVDs that can be used to investigate long-term biological repair of large animal discs in bioreactors, where complex loading paradigms can be applied.

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Disclosure Statement

A percentage of the sales of PrimeGrowth™ media will be used for research funding in Dr. Mwale's laboratory.

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Address correspondence to:
Fackson Mwale, PhD
Orthopaedic Research Laboratory
Lady Davis Institute for Medical Research
Sir Mortimer B. Davis-Jewish General Hospital
3755 chemin Cote Ste Catherine
Montréal H3T 1E2
Canada

E-mail: facksonmwale@yahoo.com

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The Effects of Naproxen on Chondrogenesis of Human Mesenchymal Stem Cells

John Antoniou, MD, PhD,[1,2] Hong Tian Wang, PhD,[1] Insaf Hadjab, BEng, MSc,[1,3] Sultan Aldebeyan, MD, MSc,[1,4] Motaz A. Alaqeel, MD, PhD,[1,5] Björn P. Meij, DVM, PhD, dipl ECVS,[6] Marianna A. Tryfonidou, DVM, PhD, dipl ECVS,[6] and Fackson Mwale, PhD[1,2]

Currently, there are no established treatments to prevent, stop, or even retard the degeneration of articular cartilage in osteoarthritis (OA). Biological repair of the degenerating articular cartilage would be preferable to surgery. There is no benign site where autologous chondrocytes can be harvested and used as a cell source for cartilage repair, leaving mesenchymal stem cells (MSCs) as an attractive option. However, MSCs from OA patients have been shown to constitutively express collagen type X (COL-X), a marker of late-stage chondrocyte hypertrophy. We recently found that naproxen (Npx), but not other nonsteroidal anti-inflammatory drugs, can induce collagen type X alpha 1 (*COL10A1*) gene expression in bone marrow-derived MSCs from healthy and OA donors. In this study, we determined the effect of Npx on *COL10A1* expression and investigated the intracellular signaling pathways that mediate such effect in normal human MSCs during chondrogenesis. MSCs were cultured in standard chondrogenic differentiation media supplemented with or without Npx. Our results show that Npx can regulate chondrogenic differentiation by affecting the gene expression of both Indian hedgehog and parathyroid hormone/parathyroid hormone-related protein signaling pathways in a time-dependent manner, suggesting a complex interaction of different signaling pathways during the process.

Introduction

Articular cartilage is a highly organized avascular and aneural tissue that covers the surfaces of the bony ends of all synovial joints in the human body. It provides a smooth surface for the bones to glide over each other, articulate and transmit loads, with minimal friction.[1] Chondrocytes in articular cartilage synthesize an extensive extracellular matrix whose primary constituents are water, aggrecan, and collagen type II. Aggrecan is a large proteoglycan with numerous glycosaminoglycan (GAG) chains attached to its core protein, which provides the osmotic properties needed to counter the effects of compressive forces. An organized network of cross-linked fibrils principally containing collagen type II provides the tensile strength.

Since articular cartilage lacks a blood supply, its capacity to self-repair is extremely poor, and if left untreated, localized cartilage defects due to injury or focal degeneration typically lead to osteoarthritis (OA) of the entire joint. OA is characterized by progressive degeneration of articular cartilage, subchondral bone, and other joint tissues, resulting in a major reduction in the quality of life and substantial economic costs. Symptomatic OA is characterized by cartilage deterioration and osteophyte formation, affecting primarily the knees, hips, and hands, and the predominant symptom is pain.[2,3] For this reason, patients are usually prescribed nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, diclofenac, naproxen (Npx), and celecoxib, as well as acetaminophen.[4] NSAIDs have been used because they can selectively inhibit cyclooxygenase-2 (COX-2), which is upregulated by interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α).[5] One drawback is NSAIDs can alter certain fundamental processes involved in the normal healing of injured tissues.[6] We recently showed that Npx can affect gene expression during osteogenic differentiation of mesenchymal stem cells (MSCs), which are also called mesenchymal stromal cells, and downregulate mineral deposition in the extracellular matrix through Indian hedgehog (IHH) signaling,[7] suggesting that Npx could affect MSC-mediated repair of subchondral bone in OA patients. However, little is known about the effects of Npx on articular cartilage repair in OA patients.

[1]Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University, Montreal, Quebec, Canada.
[2]Division of Orthopedic Surgery, McGill University, Montreal, Quebec, Canada.
[3]École Polytechnique, Montreal, Quebec, Canada.
[4]Department of Orthopaedic Surgery, King Fahad Medical City, Riyadh, Saudi Arabia.
[5]Department of Orthopedics, College of Medicine, King Saud University, Riyadh, Saudi Arabia.
[6]Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

In most cases, NSAIDs are not sufficient to delay the development of OA, and the chronic pain refractory to medication is the number one reason for joint replacement surgery. To delay the requirement for total joint replacement/arthroplasty as a result of OA, a number of conventional therapies such as microfracture and mosaicplasty are utilized. However, the long-term performance of these approaches is very poor. To reverse or retard the degeneration of articular cartilage and repair the subchondral bone, MSCs can be employed in the biological therapy of OA.[8] MSCs can be obtained from multiple tissues, including bone marrow, fat, and synovium. However, regardless of the source, MSCs have a propensity to express markers of hypertrophic chondrocytes,[9] an undesired characteristic in cartilage repair.[10,11]

Previous studies have shown that collagen type X, a marker of late-stage chondrocyte hypertrophy (associated with endochondral ossification), is constitutively expressed by MSCs isolated from OA patients,[9] and NSAIDs can affect the expression of both hypertrophic and osteogenic marker genes in MSCs,[7,12,13] with Npx showing stronger effects than other drugs. The effect of these drugs on the transcriptome of MSCs with inherent effects on their characteristics and differentiation can influence the MSC fate during the repair of OA. Thus, it is important to understand whether Npx affects the chondrogenesis of MSCs.

IHH regulates both chondrogenesis and endochondral bone formation.[14] HH ligands are secreted from the cells and bind to the receptor Patched (PTC) to relinquish smoothened, frizzled class receptor (SMO) from PTC suppression, resulting in the activation of the zinc finger transcription factor GLI family, which are used as markers for HH signaling activity, and are responsible for HH-induced lineage commitment during MSC differentiation.[15] Parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP), which share the same receptor, can affect the chondrogenesis of MSCs.[16,17] Chondrocyte differentiation is controlled at multiple steps by PTH/PTHrP and IHH through the mutual regulation of their activities.[18] Ihh acts independently or dependently on PTH-related peptide in a negative feedback loop to regulate early chondrocyte differentiation and entry to hypertrophic differentiation.[19,20]

The purpose of this study was to investigate if Npx can interfere with the chondrogenesis of MSCs through IHH and PTH/PTHrP signaling pathways. We also determined the mechanisms underlying the effect of Npx on hypertrophic differentiation of human MSCs cultured in chondrogenic differentiation media.

Materials and Methods

Source and expansion of MSCs

Human MSCs from three donors between 20 and 30 years old were obtained from Lonza (Mississauga, ON, Canada). According to the supplier, MSCs were harvested from healthy bone marrow and were positive for CD105, CD166, CD29, and CD44, but negative for CD14, CD34, and CD45. All cells were expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The culture medium and the supplement were from Wisent, Inc. (St-Bruno, QC, Canada). The cells were used within four passages.

Cell culture

MSCs have been shown to undergo chondrogenesis *in vitro* using pellet cultures,[21–23] which mimics the cellular condensation requirements for embryonic mesenchymal chondrogenesis and provides the physical and biochemical environmental factors conducive to cartilage formation. However, MSCs can also undergo chondrogenic differentiation in monolayer culture with a high density, when supplemented with a defined chondrogenic medium.[24,25] In this experiment, the monolayer culture was chosen to study the function of Npx. In every well of the six-well plate (Sarstedt, Saint-Leonard, QC, Canada), 5×10^5 MSCs were plated and cultured in the expansion medium overnight. The floating cells were removed and the attached cells were cultured until the confluence was more than 90%. Then the cells were cultured in the chondrogenic differentiation medium for 3 days to allow the cells to adapt to the new environment. Afterward, the cells were cultured in 3 mL/well of the chondrogenic differentiation medium with 0.5 μM Npx (Sigma-Aldrich, Oakville, ON, Canada). Since the bone marrow is well vascularized, the concentration of Npx in our experiments was similar to the blood circulating levels in patients taking Npx.[26] The cells that were cultured without Npx were used as control cells. The chondrogenic differentiation medium was prepared with high-glucose DMEM containing 1% ITS + 100 nM dexamethasone, 50 μg/mL ascorbic acid, 40 μg/mL proline, 10 ng/mL transforming growth factor beta 3 (TGFβ3), 100 units/mL penicillin, and 100 μg/mL streptomycin. The supplements for the chondrogenic differentiation medium were from Sigma-Aldrich, and penicillin and streptomycin were from Wisent, Inc.

Total RNA isolation

After MSCs were cultured for 3, 6, and 12 days, they were washed with phosphate-buffered saline (PBS) and total RNA was extracted using the TRIzol reagent (Invitrogen, Burlington, ON, Canada) as previously reported. Briefly, after centrifugation for 15 min at 12,000 *g* at 4°C, the aqueous phase was precipitated in one volume of isopropanol, incubated for 45 min at −20°C, and centrifuged again for 15 min at 12,000 *g* at 4°C. The resulting RNA pellet was washed with 75% ethanol and then centrifuged and air dried. Then, the pellets were suspended in 50 mL of diethylpyrocarbonate (DEPC)-treated water and assayed for RNA concentration by measuring A260 and purity by calculating A260/A280.

Reverse transcription and real-time PCR

First, 1 μg total RNA isolated from the cells was digested with DNase I (Invitrogen) according to the protocol of the supporter. Then, the purified RNA was reverse transcribed as described previously. Briefly, 1 μg RNA was mixed with random primers (final concentration 0.15 μg/μL), dNTP mixture (final concentration 0.5 mM), and DEPC-treated distilled water with a total volume of 12 μL. After the solution was incubated at 65°C for 5 min, it was mixed with a first-strand buffer, Dithiothreitol, RNaseOUT, and SuperScript II Reverse Transcriptase with a final volume of 20 μL. Then, the solution was incubated at 45°C for 50 min to perform the reverse transcription and then in 70°C for 15 min to inactivate the reverse transcriptase. For LightCycler real-time

Table 1. Primer Sequences

Genes	*Primers*	*Size (bp)*
ACAN	Forward (6708–6727): TGA GTC CTC AAG CCT CCT GT Reverse (6873–6892): CCT CTG TCT CCT TGC AGG TC	185
ADAMTS4	Forward (1702–1721): TCC TGC AAC ACT GAG GAC TG Reverse (1847–1866): GGT GAG TTT GCA CTG GTC CT	165
ADAMTS5	Forward (1399–1418): GGC CAT GGT AAC TGT TTG CT Reverse (1548–1567): GTA CCA CAG CAC ACC ACA GG	169
ALP	Forward (1397–1416): CCA CGT CTT CAC ATT TGG TG Reverse (1573–1592): AGA CTG CGC CTG GTA GTT GT	196
COL2A1	Forward (459–478): ATT TCA AGG CAA TCC TGG TG Reverse (657–676): GGC CTG GAT AAC CTC TGT GA	218
COL10A1	Forward (1670–1690): AAT GCC TGT GTC TGC TTT TAC Reverse (1779–1799): ACA AGT AAA GAT TCC AGT CCT	130
COMP	Forward (1674–1693): GGA GAT CGT GCA GAC AAT GA Reverse (1764–1782): GTC ATC CGT GAC CGT GTT C	109
GAPDH	Forward (113–133): TGA AGG TCG GAG TCA ACG GAT Reverse (273–293): TTC TCA GCC TTG ACG GTG CCA	181
GLI1	Forward (676–695): AAG CGT GAG CCT GAA TCT GT Reverse (845–864): CAG CAT GTA CTG GGC TTT GA	189
GLI2	Forward (199–218): CGA CAC CAG GAA GGA AGG TA Reverse (382–401): TGC ACA GAA CGG AGG TAG TG	203
GLI3	Forward (2285–2304): CTT TGC AAG CCA GGA GAA AC Reverse (2428–2447): TTG TTG GAC TGT GTG CCA TT	163
IHH	Forward (519–538): CGG CTT TGA CTG GGT GTA TT Reverse (718–737): AAA ATG AGC ACA TCG CTG AA	219
MMP3	Forward (36–55): GGC AGT TTG CTC AGC CTA TC Reverse (231–250): GAG TGT CGG AGT CCA GCT TC	215
MMP13	Forward (507–526): TAA GGA GCA TGG CGA CTT CT Reverse (687–706): GGT CCT TGG AGT GGT CAA GA	200
PTC1	Forward (1460–1479): TCA GCA ATG TCA CAG CCT TC Reverse (1688–1707): GTC GTG TGT GTC GGT GTA GG	248
PTH	Forward (158–177): AAT GGC TGC GTA AGA AGC TG Reverse (292–311): GCT TTG TCT GCC TCT CCA AG	154
PTHR1	Forward (569–588): TTT ACA CCG TGG GCT ACT CC Reverse (709–728): ACA GCG TCC TTG ACG AAG AT	160
SOX9	Forward (19–38): TTC ATG AAG ATG ACC GAC GA Reverse (174–193): CGC TCT CCT TCT TCA GAT CG	175
TIMP1	Forward (109–128): AAT TCC GAC CTC GTC ATC AG Reverse (284–303): GTT GTG GGA CCT GTG GAA GT	195
TIMP2	Forward (143–162): AAG CGG TCA GTG AGA AGG AA Reverse (305–324): CTT CTT TCC TCC AAC GTC CA	182
TIMP3	Forward (310–329): CTG ACA GGT CGC GTC TAT GA Reverse (455–474): AGT CAC AAA GCA AGG CAG GT	165

ACAN, aggrecan; *ADAMTS*, a disintegrin and metalloproteinase with thrombospondin motifs; *ALP*, alkaline phosphatase; *COL2A1*, collagen type II alpha 1; *COL10A1*, collagen type X alpha 1; *COMP*, cartilage oligomeric matrix protein; *GAPDH*, glyceraldehyde 3 phosphate dehydrogenase; *IHH*, Indian hedgehog; *MMP*, matrix metalloproteinase; *PTC1*, Patched 1; *PTHR1*, PTH/PTHrP receptor; *SOX9*, Sry-related high-mobility-group box 9; *TIMP*, tissue inhibitors of metalloproteinase.

polymerase chain reaction (PCR), a master mix of the following reaction components was prepared with the final concentrations as 10 μL SYBER PCR master mix (Qiagen, Mississauga, ON, Canada), 8 μL distilled water, 0.5 μL forward primer (final concentration 0.25 μM), and 0.5 μL reverse primer (final concentration 0.25 μM). To each 19 μL master mix, 1 μL of cDNA was mixed as a PCR template. The sequences of primers are in Table 1. The reaction conditions included one cycle of PCR initial activation step (95°C for 15 min, 20°C/s ramp rate), 45 cycles of amplification and quantification (94°C for 15 s, 57°C for 30 s, 72°C for 30 s), one cycle of melting curve (65–95°C with heating rate of 0.1°C/s with a continuous fluorescence measurement), and a final cooling step to 4°C. The crossing points (CPs) were determined by the LightCycler software 3.3 (Roche Diagnostics, Indianapolis, IN) and were measured at constant fluorescence level. The ratio of gene expression relative to glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) as the reference gene was determined by the following equation:

$$\text{Relative ratio} = \frac{2^{\Delta CP_{target}(\text{control-sample})}}{2^{\Delta CP_{reference}(\text{control-sample})}}$$

Alkaline phosphatase activity analysis

After MSCs were cultured in the chondrogenic differentiation medium with or without Npx for 6, 9, and 12 days, the cells were lysed and alkaline phosphatase (ALP) activity was assayed with the StemTAG™ Alkaline Phosphatase Activity Assay Kit (Colorimetric; Cell Biolabs, Inc., San

Diego, CA) according to the protocol from the supplier. Briefly, the cells were washed with cold PBS and then lysed with the lysis buffer. After incubating at 4°C for 10 min, the lysis solution was centrifuged at 4°C at 12,000 *g* for 10 min. The supernatant was collected as cell lysate and the protein concentration was measured with the BCA assay (Thermo Scientific, Burlington, ON, Canada). Then 50 μL of cell lysate was added to a 96-well plate, while the well containing 50 μL cell lysis buffer was applied as a blank control. The reaction was initiated by adding 50 μL of the StemTAG AP Activity Assay Substrate, and the plate was incubated at 37°C for 15 min. Then the reaction was stopped by adding 50 μL of 1× stop solution and mixed by placing the plate on an orbital plate shaker for 30 s. Finally the absorbance value of each well was read at 405 nm. To analyze pNP concentration produced by the reaction, a serial twofold dilution of pNP standard was prepared in 1× stop solution, and the absorbance of each dilution was measured at 405 nm. Then pNP concentration in the reaction buffer was calculated by comparing with the standard curve. Alp activity was calculated with the formula below:

$$\text{Alp activity} = \text{pNP quantity}/(\text{protein quantity} \times 30\ \text{min})$$

where 30 min is the reaction time.

GAG and DNA analysis

During the chondrogenesis of MSCs, the culture media were changed every 3 days. The media were collected and stored in −20°C for sulfated GAGs analysis. GAGs were quantified in culture media by a modified dimethylmethylene blue dye-binding assay.[27,28] Samples were diluted to fall within the middle of the linear range of the standard curve. To compare the number of cells at different time, DNA was measured on day 3 and 21 by using a Quant-iT dsDNA High-Sensitivity Assay Kit (Invitrogen), following the manufacturer's instructions.[25]

Statistical analysis

Statistical analysis was performed using analysis of variance, followed by Fisher's protected least significant difference *post hoc* test using StatView (SAS Institute, Inc., Cary, NC).

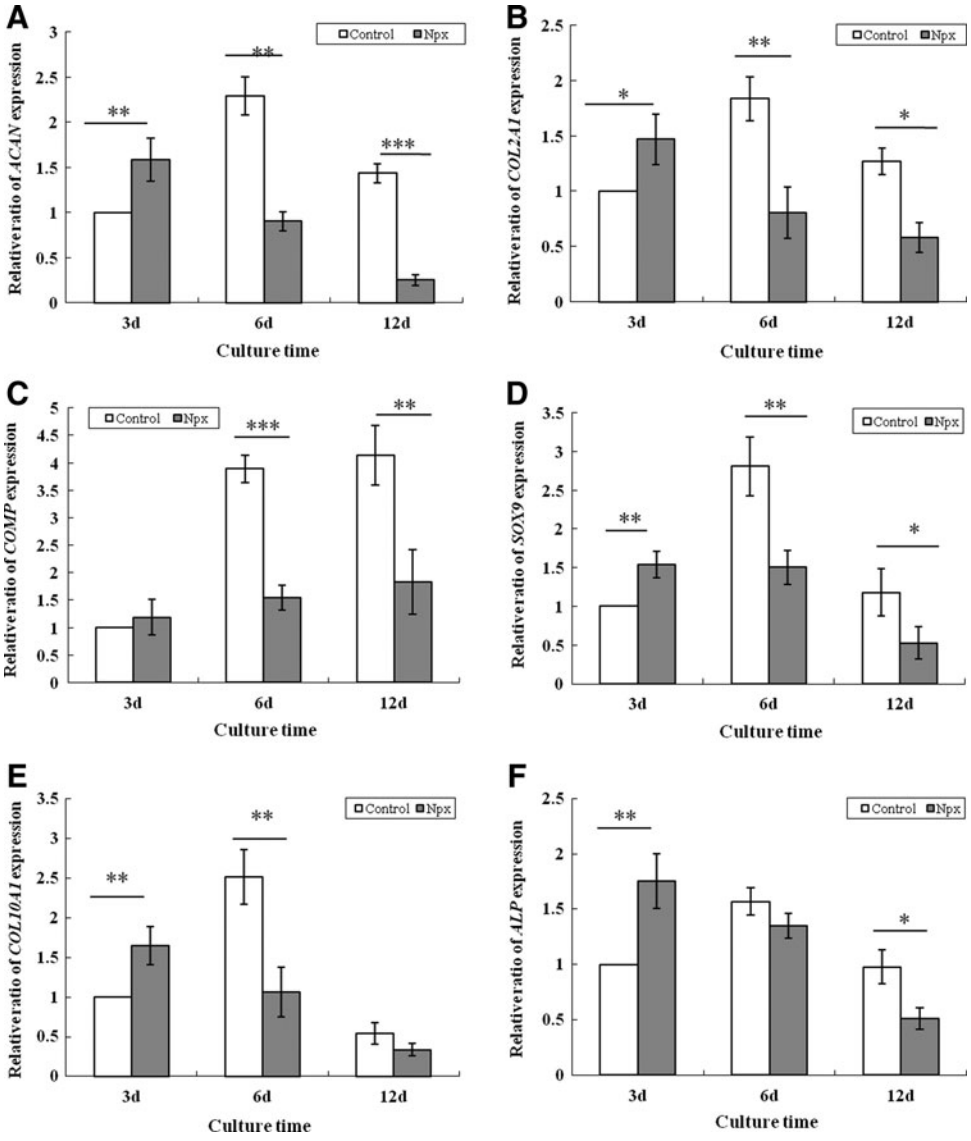

FIG. 1. The effect of naproxen (Npx) on the expression of aggrecan (*ACAN*) **(A)**, collagen type II alpha 1 (*COL2A1*) **(B)**, cartilage oligomeric matrix protein (*COMP*) **(C)**, Sry-related high-mobility-group box 9 (*SOX9*) **(D)**, collagen type X alpha 1 (*COL10A1*) **(E)**, and alkaline phosphatase (*ALP*) **(F)** in mesenchymal stem cells (MSCs) cultured in chondrogenic differentiation medium without (control) or with 0.5 μM Npx for different periods. The results are shown as mean ± standard deviation (SD) of three independent experiments with MSCs from three different donors. Day 3 control samples were used as a reference and set at 1. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control.

The results of three experiments with MSCs from three different donors were assessed on 80% power to detect a difference in gene expression. Results are presented as the mean ± standard deviation of three experiments. Differences were considered statistically significant with $p < 0.05$.

Results

Aggrecan (*ACAN*), collagen type II alpha 1 (*COL2A1*), and cartilage oligomeric matrix protein (*COMP*) are important genes that define the chondrocyte phenotype.[29,30] SOX9 is a transcription factor belonging to the Sry-related high-mobility-group box (Sox) proteins family, essential for chondrogenesis of MSCs.[31,32] The effect of Npx on the expression of these genes was therefore assessed after culturing MSCs in chondrogenic differentiation media for 3, 6, and 12 days. At day 3, the expression of *ACAN* ($p = 0.009$), *COL2A1* ($p = 0.012$), and *SOX9* ($p = 0.005$) was increased significantly, while no significant effect was found for *COMP*, when compared to controls (Fig. 1). In contrast, when MSCs were cultured with Npx for 6 and 12 days, the expressions of *ACAN* ($p = 0.005$ on day 6, $p < 0.001$ on day 12), *COL2A1* ($p = 0.003$ on day 6, $p = 0.012$ on day 12), *COMP* ($p < 0.001$ on day 6, $p = 0.007$ on day 12), and *SOX9* ($p = 0.007$ on day 6, $p = 0.032$ on day 12) decreased significantly when compared to controls. This indicates that at early time points Npx has the potential to stimulate chondrogenic differentiation, but with a longer period, Npx can suppress the chondrogenesis of MSCs.

Collagen type X alpha 1 (*COL10A1*) is a marker gene for hypertrophic chondrocyte differentiation, a process that is undesirable for effective cartilage repair.[33,34] When MSCs were cultured in a chondrogenic differentiation medium with Npx, the expression of *COL10A1* was significantly upregulated on day 3 ($p = 0.009$), but was significantly downregulated on day 6 ($p = 0.006$), while no significant difference was observed on day 12 (Fig. 1E). Thus, Npx appears to decrease hypertrophic differentiation when MSCs were cultured for 6 days, while stimulating hypertrophy in the short term. Alkaline phosphatase (ALP) plays an active role in initiating the calcification process after chondrocytes have become hypertrophic.[35] When MSCs were cultured in a chondrogenic differentiation medium with Npx, the expression of *ALP* was significantly upregulated on day 3 ($p = 0.006$), but was significantly decreased on day 12 ($p = 0.011$), while no significant difference was observed on day 6 (Fig. 1F).

Proteoglycan production during chondrogenic differentiation can also be monitored with GAG analysis, and this was used to assess the effect of Npx on MSC differentiation. After MSCs were cultured in a chondrogenic differentiation medium with Npx for 21 days, the quantity of GAG secreted into the culture medium was significantly lower than that in the control medium (Fig. 2). Since the DNA content of the cultures were not significantly different at day 3 and 21, the decreased GAG synthesis is likely the result of decreased production by each cell rather than a consequence of less cells due to cell death.

During the chondrogenic differentiation, ALP can also be monitored by its activity and this was used to assess the effect of Npx on MSC differentiation. Even though the mRNA level could be detected on day 3 through real-time PCR, the enzyme activity was too weak to be detected. So we determined the activities of ALP on days 6, 9, and 12. After MSCs were cultured in a chondrogenic differentiation medium with Npx for 9 and 12 days, ALP activity was significantly lower than that in the control medium, although no significant effect was observed on day 6 (Fig. 3).

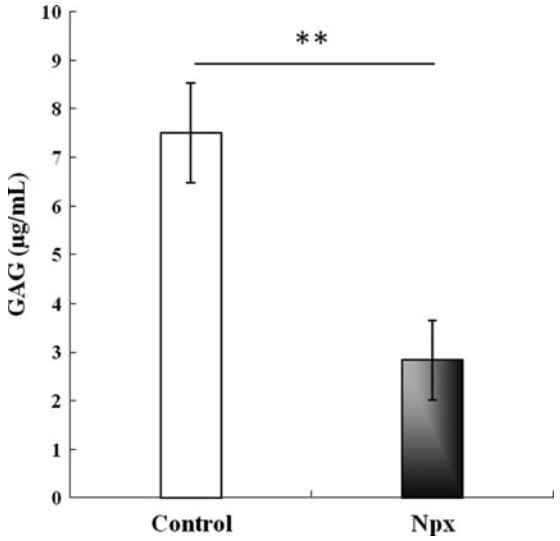

FIG. 2. Glycosaminoglycan (GAG) concentration in pooled culture media of MSCs cultured for 21 days in chondrogenic differentiation medium without (control) or with 0.5 μM Npx. The results are shown as mean ± SD of three independent experiments with MSCs from three different donors. $^{**}p < 0.01$ versus control.

Matrix metalloproteinases (MMPs) are essential for chondrogenic differentiation of adult human MSCs.[36] However, while the function of ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I motifs) family does not appear to be required for cartilage development in mice,[37] its

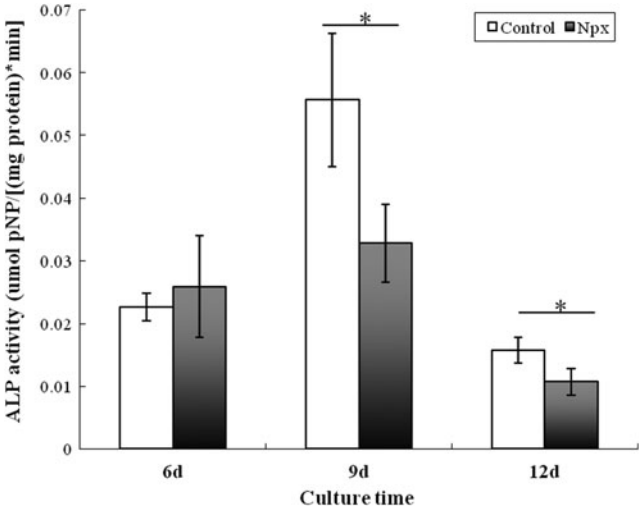

FIG. 3. ALP activity in MSCs cultured in chondrogenic differentiation medium without (control) or with 0.5 μM Npx for different periods. The results are shown as mean ± SD of three independent experiments with MSCs from three different donors. $^{*}p < 0.05$ versus control.

role in chondrocyte homeostasis is vital.[38] The effect of Npx on the expression of these genes was therefore assessed.

After the cells were cultured for 6 days in the chondrogenic differentiation medium, MMP3 expression with Npx increased significantly compared to controls ($p = 0.006$) (Fig. 4A). However, no significant effect was observed compared with control cells at both day 3 and 12 (Fig. 4A). In contrast, the expression of *MMP13* was increased significantly on day 3 ($p = 0.007$), but was decreased significantly on day 6 ($p < 0.001$) and day 12 ($p < 0.001$) (Fig. 4B). Compared with control cells, there was no significant effect of Npx on the expression of *ADAMTS4* (Fig. 4C), while the expression of *ADAMTS5* was increased significantly on day 3 ($p = 0.012$) and day 12 ($p < 0.001$) (Fig. 4D). Thus Npx appeared to affect MMP3, MMP13, and ADAMTS5 expressions during the chondrogenic differentiation of MSCs.

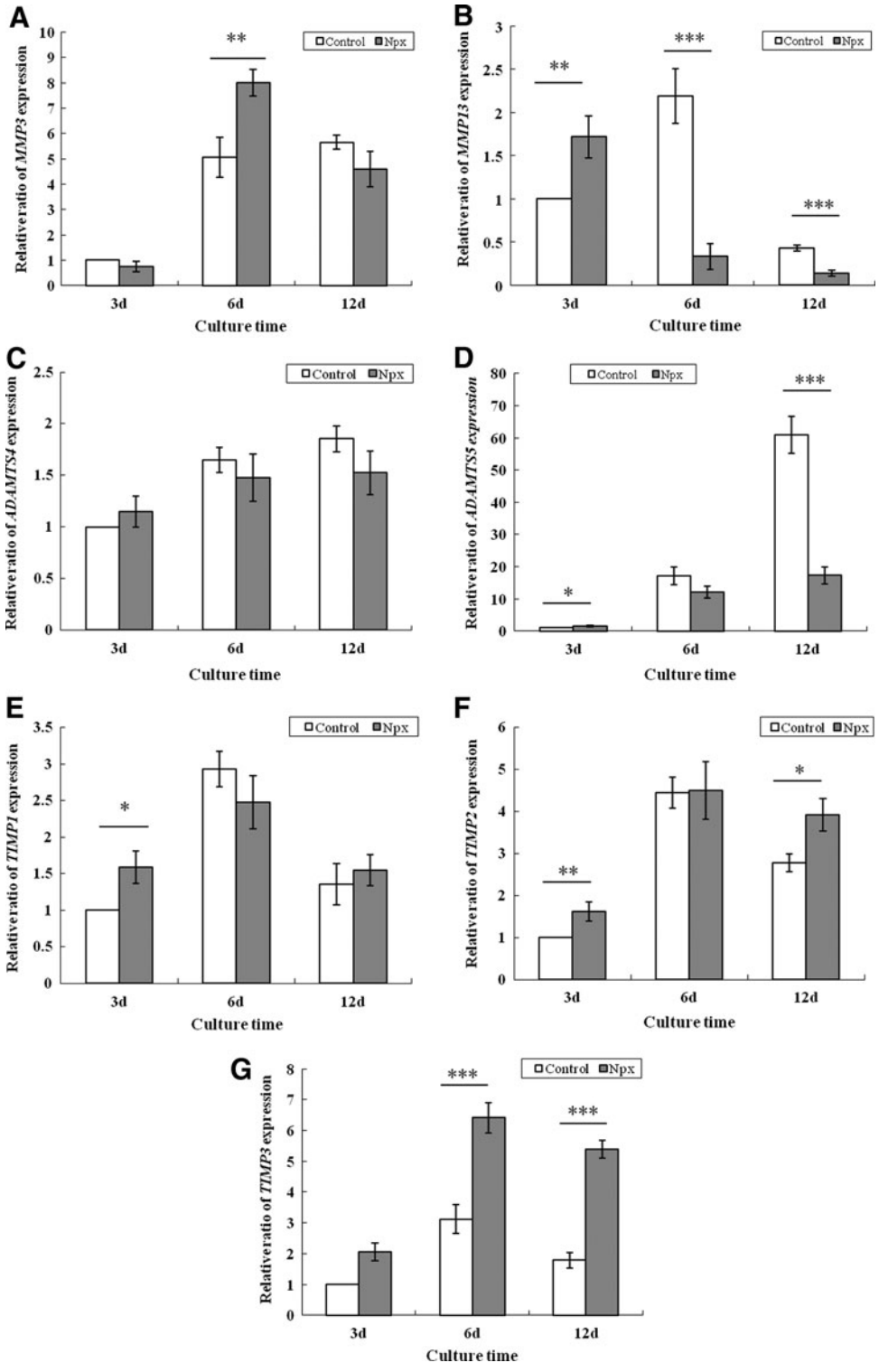

FIG. 4. The effects of Npx on the expression of matrix metalloproteinase (*MMP*)*3* **(A)**, *MMP13* **(B)**, a disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*)*4* **(C)**, *ADAMTS5* **(D)**, tissue inhibitors of metalloproteinase (*TIMP*)*1* **(E)**, *TIMP2* **(F)**, and *TIMP3* **(G)** in MSCs cultured in chondrogenic differentiation medium without (control) or with 0.5 μM Npx for different periods. The results are shown as mean ± SD of three independent experiments with MSCs from three different donors. Day 3 control samples were used as a reference and set at 1. $*p < 0.05$ versus control; $**p < 0.01$ versus control; $***p < 0.001$ versus control.

Striking changes in the expression of matrix remodeling enzymes in control cultures over time was observed, which have previously been observed in MSCs during chondrogenic differentiation.[25]

We next examined the expression of MMPs endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). MSCs have been shown to secrete high levels of TIMPs.[39] After culturing the cells for 12 days in the absence of Npx, MSCs show *TIMP1*, *TIMP2,* and *TIMP3* gene expression, with the highest expression at day 6 (Fig. 4E–G). With Npx supplementation, the expression of *TIMP1* was increased significantly on day 3 compared to controls ($p = 0.012$). This effect was not observed at day 6 and 12 (Fig. 4E). Npx supplementation increased the expression of *TIMP2* significantly on day 3 ($p = 0.009$) and day 12 ($p = 0.011$) (Fig. 4F), but not on day 6. After the cells were cultured for 3 days in the chondrogenic differentiation medium with Npx, *TIMP3* expression significantly increased compared to controls ($p = 0.003$), and the increase was also observed after 6 days ($p = 0.001$) and 12 days ($p < 0.001$) (Fig. 4G). Thus Npx appeared to stimulate the expression of *TIMPs* during chondrogenic differentiation of MSCs.

We have previously shown that Npx affected the expression of *IHH*, *PTC1*, *GLI1*, and *GLI2* genes belonging to the Indian hedgehog signaling pathway when MSCs were cultured in the osteogenic differentiation medium.[7] The effect of Npx on the expression of *PTC1*, *IHH*, *GLI1*, *GLI2*, and *GLI3* when MSCs were cultured in chondrogenic differentiation medium was therefore assessed (Fig. 5). When the cells were cultured in this medium with Npx, the expression of *PTC1* significantly decreased on day 6, compared with that in control cells (Fig. 5A). This difference was not observed on day 3 and 12. The expression of *IHH* significantly increased on days 3 and 6, compared with that in control cells, but its expression significantly decreased on day 12 compared with that in control cells (Fig. 5B). Npx supplementation significantly increased the expression of *GLI1* on day 3 ($p = 0.001$), but its expression was significantly decreased on day 6 ($p = 0.024$) (Fig. 5C). There was a tendency toward

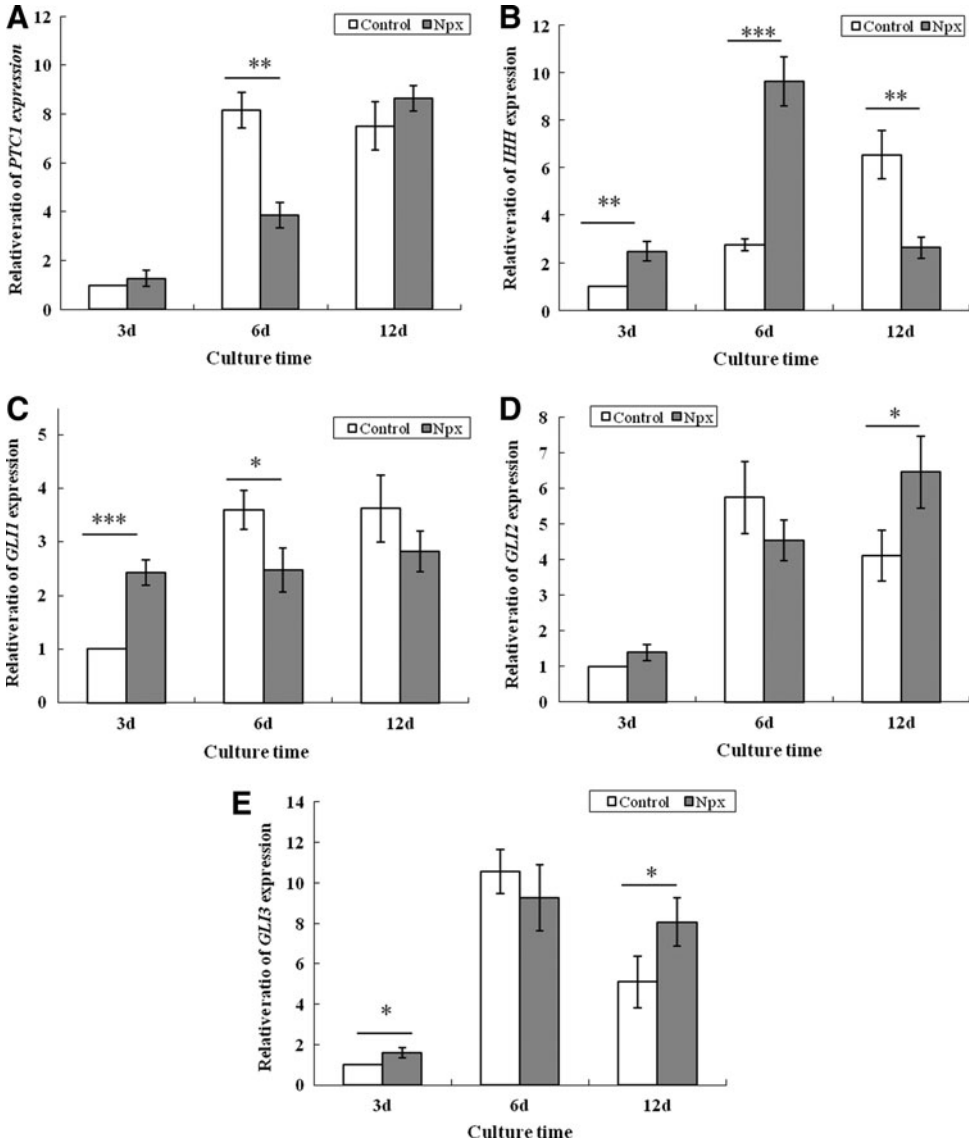

FIG. 5. The effects of Npx on the expression of genes Patched 1 (*PTC1*) **(A)**, Indian hedgehog (*IHH*) **(B)**, *GLI1* **(C)**, *GLI2* **(D)**, and *GLI3* **(E)** in MSCs cultured in chondrogenic differentiation medium without (control) or with 0.5 μM Npx for different periods. The results are shown as mean ± SD of three independent experiments with MSCs from three different donors. Day 3 control samples were used as a reference and set at 1. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control.

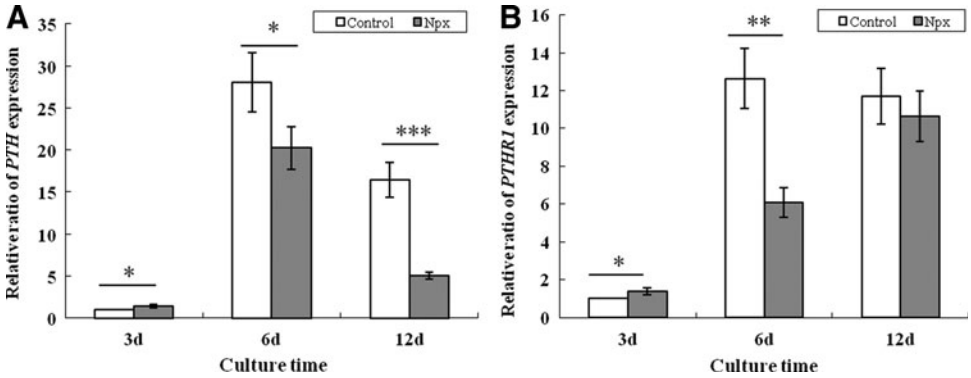

FIG. 6. The effects of Npx on the expression of parathyroid hormone (*PTH*) **(A)** and *PTHR1* **(B)** in MSCs cultured in chondrogenic differentiation medium without (control) or with 0.5 μM Npx for different periods. The results are shown as mean ± SD of three independent experiments with MSCs from three different donors. Day 3 control samples were used as a reference and set at 1. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control.

a decrease of *GLI1* expression on day 12 ($p = 0.08$). The expression of *GLI2* significantly increased on day 12 ($p = 0.031$), while no significant difference was observed on days 3 and 6 (Fig. 5D) when cells were supplemented with Npx. Finally, the expression of *GLI3* significantly increased on day 3 ($p = 0.017$) and day 12 ($p = 0.044$), while no significant effect of Npx on the expression of *GLI3* was observed on day 6 (Fig. 5E).

To study whether Npx can affect PTH/PTHrP signaling, the effects of Npx on the expression of *PTH* and PTH/PTHrP receptor (*PTHR1*) were studied. Npx increased the expression of *PTH* significantly on day 3 ($p = 0.04$) and day 12 ($p < 0.001$), but its expression was significantly decreased on day 6 compared with that in control cells ($p = 0.035$) (Fig. 6A). The expression of *PTHR1* was significantly increased on day 3 ($p = 0.045$), but significantly decreased on day 6 ($p = 0.003$) (Fig. 6B). No significant effect of Npx on the expression of *PTHR1* was observed on day 12. Thus Npx not only suppresses the expression of *PTH*, but also the expression of PTH/PTHrP receptor *PTHR1*.

Discussion

In the present study, we used Npx as an NSAID supplement and determined that it can interfere with chondrogenesis of MSCs. In a clinical context this indicates that NSAIDs present in the synovial fluid may have an influence on the ability of endogenous and exogenous MSCs to differentiate and stimulate cartilage repair.

Previous work has demonstrated a potential of Npx to interfere with MSC differentiation.[7,11,12] Studies utilizing MSCs isolated from the bone marrow of patients with OA or normal donors cultured with NSAIDs showed that Npx could induce the expression of *COL10A1*, *ACAN*, *COL1A1*, as well as *ALP*, bone sialoprotein (*BSP*), osteocalcin (*OC*), and Runt-related transcription factor 2 (*RUNX2*).[11,12] In addition, these results indicated that Npx can affect gene expression during osteogenic differentiation of MSCs through IHH signaling.[7] Elevated basal *COL10A1* expression in OA MSCs also involved the activation of MAPK pathway and stimulation of the 5-lipoxygenase signaling pathway by Npx.[13] The present study is the first to demonstrate that Npx can regulate chondrogenic differentiation by affecting both the IHH and PTH/PTHrP signaling pathways. Further studies are underway to determine if the gene expression observed with IHH is translated to protein. This is important to determine the role of Npx in IHH signaling pathways related to the process of chondrogenesis and terminal chondrocyte differentiation.

Culturing MSCs in chondrogenic differentiation media for 3, 6, and 12 days in the presence of Npx used in the present study mimic short-term, intermediate, and long-term intake of Npx by patients with OA, where it can be present in the synovial fluid and influence the ability of MSCs to differentiate and stimulate cartilage repair. For long-term use, Npx is preferred to other NSAIDs in patients because it is associated with less vascular risk than other NSAIDs.[40] Previous studies, in line with the findings of this study, showed that NSAIDs have the potential to suppress chondrogenic differentiation by MSCs.[41] We showed that Npx was also capable of suppressing osteogenic differentiation.[7] However, despite its extensive use as a prescription and over-the-counter medication, it still needs to be established at what dosage and duration it affects the repair of skeletal tissues.

The effect of Npx on the expression of genes in MSCs was time dependent, suggesting a complex interaction of different proteins involved in IHH signaling. Additionally, the independent functions of IHH and PTH/PTHrP signaling,[19,20,42,43] and the interaction between IHH and PTH/PTHrP signaling on the chondrogenesis of MSCs, can also lead to the complex results of gene expression.[17,18] In skeletogenesis, IHH regulates chondrogenesis during endochondral ossification, while Gli zinc finger proteins regulate IHH signaling.[44] Previously, we showed that Npx did not affect the expression of *GLI3* when MSCs were cultured in osteogenic differentiation media.[7] In the present study, the expression of *GLI3* increased significantly on days 3 and 12. The difference may be caused by the different culture media.

In this study, for most of the genes, only mRNA but not protein expression levels were studied. Although the fluctuation of mRNA expression level usually is parallel with protein level, the effects of Npx on protein level are on the way for further confirmation. Furthermore, IHH gene expression was not consistent, increasing on day 6 and decreasing at day 12 in the presence of NPx. Although decreasing levels of *IHH* may

be explained by the antihypertrophic effects of NPx during long-term treatment, it is possible that the variability in the cells' response to NPx could be due to differences in the cell sources. According to the supplier, these cells were harvested from healthy donors aged between 20 and 30 years and were positive for CD105, CD166, CD29, and CD44, but negative for CD14, CD34, and CD45. Altogether, the present data indicate that Npx can directly interfere with MSC chondrogenesis potentially through the IHH and PTH/PTHrP signaling pathways. Other studies showed that Npx could alter MAPK signaling, in particular p38-MAPK, in the induced expression of *COL10A1* in human MSCs.[12] Furthermore, Npx appears to be capable of affecting chondrogenesis and hypertrophic differentiation of MSCs depending on different culture time.

Our studies and those of others raise the question whether bone marrow, adipose, and synovium are the best sources of MSCs for the repair of permanent cartilages such as articular cartilage, since MSCs derived from these tissues consistently undergo hypertrophic differentiation during *in vitro* chondrogenic differentiation.[8] An alternative source would be articular cartilage stem cells (ACSCs), which do not differentiate down the endochondral pathway through hypertrophy *in vitro* in chondrogenic assays.[45,46] Although some methods, such as polymer substrates surface modified by glow discharge plasma,[11,47–49] can result in a decrease of hypertrophic markers in differentiated cells, they do not reach the levels found in stable (permanent) chondrocytes.[50] Conversely, ACSCs differentiate into chondrocytes that do not have the hypertrophic phenotype. However, it remains to be determined if Npx supplementation can influence ACSC hypertrophic differentiation.

Conclusions

Npx interferes with chondrogenesis of MSCs. At 12 days of culture, Npx negatively affects matrix production by the MSCs in the chondrogenic lineage and it seems to suppress termination of hypertrophic differentiation. These effects may be mediated by the interplay of PTH and IHH signaling pathways. Therefore, in principle, Npx supplementation could interfere with the expected outcome of medical treatment of degenerative lesions in articular cartilage.

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Disclosure statement

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Address correspondence to:
Fackson Mwale, PhD
Lady Davis Institute for Medical Research
Jewish General Hospital
McGill University
3755 Chemin de la Cote Sainte Catherine
Montreal, QC H3T1E2
Canada

E-mail: fmwale@ldi.jgh.mcgill.ca

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The Role of the Extracellular Calcium-Sensing Receptor in Osteoarthritis

Michael Philip Grant, PhD[1], Laura Mery Epure, Eng. MScA[2], Motaz Alaqeel, MD[3], Omar Salem, MD[4], Olga L. Huk, MD, MSc[1,3], John Antoniou, MD, PhD[3,1], Fackson Mwale, Ph.D[3,1].
[1]Lady Davis Institute for Medical Research - SMBD Jewis General Hospital, Montreal, QC, Canada, [2]Lady Davis Institute for Medical Research - SMBD-Jewish General Hospital, Montreal, QC, Canada, [3]McGill University, Montreal, QC, Canada, [4]Lady davis Institute for Medical Research, Montreal, QC, Canada.

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Introduction: Osteoarthritis (OA) affects millions of individuals, and, although, the mechanism s) of OA onset is unclear, the biological outcome is cartilage degradation. The degradation of cartilage is typified by the progressive loss of extracellular matrix components, such as the proteoglycan aggrecan, and type II collagen, resulting from the upregulation of catabolic enzymes aggrecanases a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS-) 4 and 5 as well as matrix metalloproteinases (MMPs) and type X collagen. There is currently no treatment that will prevent or repair joint damage. The extracellular calcium-sensing receptor (CaSR) is a G protein-coupled receptor (GPCR). It is the principle regulator of parathyroid hormone (PTH) synthesis and secretion, and functions to maintain calcium homeostasis. Recently, tissue-specific and inducible chondrocyte knockouts of CaSR have provided evidence for an important role in chondrogenesis, however, its role in human chondrocytes, and more specifically, its contribution to the pathology of OA remains unclear. We aim to determine the role of CaSR in the pathological development of OA

Methods: Articular cartilage was isolated from 5 donors undergoing total hip replacement. Cells were recovered from the cartilage of each femoral head or knee by sequential digestion with Pronase followed by Collagenase, and expanded in DMEM supplemented with 10% heat-inactivated FBS. OA and normal articular chondrocytes (**PromoCell**, Heidelberg, Germany) were transferred to 6-well plates in culture medium containing various concentrations of calcium (0.5, 1.0, 2.5, and 5.0 mM $CaCl_2$), allosteric agonist (cincalcet, 1 uM) and CaSR antagonist (antagonist, 1 uM), and replenished every third day for a duration of 7 days. Cartilage explants were prepared from the same donors, and included cartilage with the cortical bone approximately 1 cm^2 in dimension; bovine articular cartilage (6 months) was used as a control. Explants were cultured in the above mentioned media; however, the incubation period was extended to 21 days. Immunohistochemistry was performed on cartilage explants to measure CaSR expression and markers of OA (ColX, MMP-13, IL-1R). The sulfated glycosaminoglycan (GAG, predominantly aggrecan) content of cartilage was analyzed using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay, and aggregan fragmentation was determined by Western blotting using antibody targeted to its G1 domain. Western blotting was also performed on cell lysate from both OA and normal chondrocytes to measure CaSR expression, MAPK and MMP-13 activity.

Results: CaSR expression was markedly increased in superficial cartilage of OA donors when compared to bovine articular cartilage (Fig. 1A). Proteoglycan content of the cartilage explants decreased as a function of calcium, as determined by the DMMB assay and Western blotting of aggrecan. The expression of CaSR was also higher in OA versus normal human articular chondrocytes (Fig. 1B), a property that was reflected by the degree of MAPK activity following stimulation with calcium. When normal chondrocytes were cultured in medium supplemented with high calcium (5 mM Ca^{2+}), a modest increase in the activity of MMP-13 was observed; however, this activity was exaggerated in OA chondrocytes (Fig. 1C). When compared to normal cells, OA chondrocytes demonstrated a reduced capacity to synthesize proteoglycan. This property was reversed when OA cells were incubated with the CaSR antagonist.

Discussion: We provide evidence that prolonged activation of CaSR in human chondrocytes decreases proteoglycan synthesis and degradation by increasing the activity of MMP-13.

Significance: Inhibition of CaSR may support a role in cartilage regeneration.

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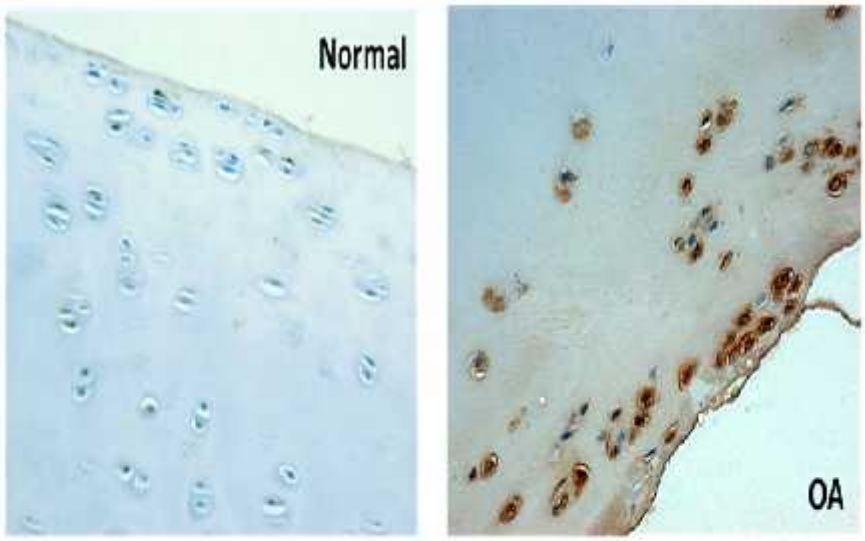
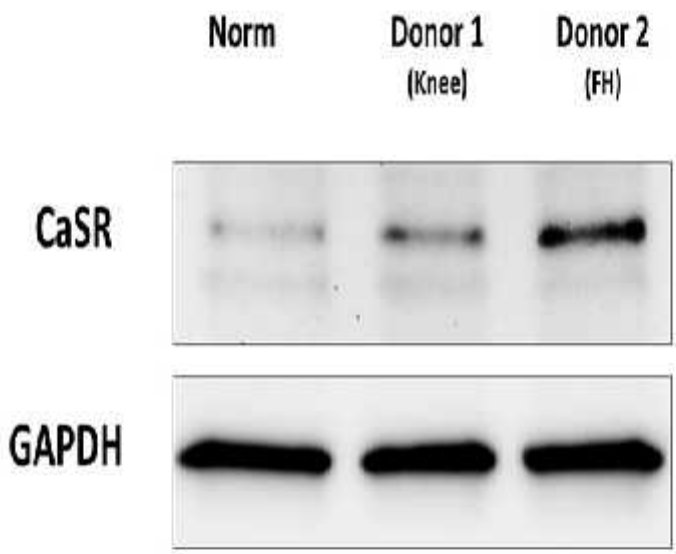


(A) Immunohistochemistry of CaSR expression in OA and bovine (6 month) cartilage



(B) Western blotting of CaSR in lysate from OA and normal articular chondrocytes