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The role of stress related proteins in osteoarthritis

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Table of content

1. Introduction	9
1.1. Main joint tissue components	11
1.2. Inside osteoarthritis	18
1.3. Role of SLRP in degenerative joint diseases	25
1.4. Role of heat shock proteins in stromal tissue of the joints	32
1.5 References	50
2. Research objective	69
3. Results	79
3.1. Distinct dysregulation of the small leucine-rich repeat protein (SLRP) family in osteoarthritic labrum compared to articular cartilage.....	81
3.2. Downregulation of small heat shock proteins in articular cartilage: a novel protective mechanism against osteoarthritis	103
4. General discussion and summary	125
4.1. sHsp downregulation, a rescue mechanism for OA joint remodeling	128
4.2. SLRP expression contributes to ECM synthesis.....	130
4.3 References	133
5. Samenvatting	137
6. Dankwoord	143
5. Curriculum vitae	147

List of abbreviations

ACLT	anterior crucial ligament transection
ADAMTS	a disintegrin and metalloproteinase domain with thrombospondin motifs
APC	antigen presenting cell
APSN	asporin
AS	ankylosing spondylitis
ATP	adenosine triphosphate
BMP	bone morphogenetic protein
CAIA	collagen antibody induced arthritis
C/EBP β	CCAAT/enhancer binding protein β
CIA	collagen induced arthritis
COMP	cartilage oligomeric matrix protein
DAMP	danger associated molecular pattern
DC	dendritic cell
DMM	destabilization of the medial meniscus
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular regulated kinase
ER	endoplasmic reticulum
FLS	fibroblast like synoviocyte
GAG	glycosaminoglycan
HLA	human leukocyte antigen
HMGB1	high mobility group box chromosome protein 1
HSF1	heat shock factor 1
HSP	heat shock protein
Ihh	Indian hedgehog protein
IL	interleukin
JNK	Jun-N-terminal kinase
LMW-HA	low molecular weight hyaluronic acid
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen activated protein kinase
MIP-2	macrophage inflammatory protein
MMP	metalloproteinase
MSC	mesenchymal stem cell

NF κ B	nuclear factor kappa B
NK	natural killer cell
NLR	nod like receptor
NO	nitric oxide
OA	osteoarthritis
OGN	osteoglycin
OMD	osteomodulin
PCM	pericellular matrix
PG	proteoglycan
PRR	pattern recognition receptor
RA	rheumatoid arthritis
RF	rheumatoid factor
sHsp	small heat shock proteins
SLE	systemic lupus erythematoses
SLRP	short leucine rich repeat proteoglycans
TGF β	tumor growth factor beta
TLR	toll like receptor
TNF α	tumor necrosis factor alpha

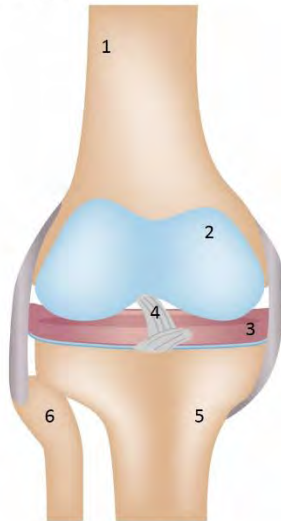
1. Introduction

Osteoarthritis (OA) is one of the most common joint diseases worldwide with an estimated prevalence of 15%^{1,2}. It is a degenerative joint disease, mainly targeting knee, hip, hand, feet and/or spine. OA is characterized by a gradual degeneration of the cartilage eventually leading to failure of the synovial joints³. Loss of cartilage matrix and joint space alterations, such as subchondral bone sclerosis and new bone formation by osteophytes are common clinical manifestations of the disease. Even though age is an important risk factor for the development of OA it cannot be considered as a simple degenerative process in elderly. The current paradigm looks at OA as a disease involving the whole joint^{4,5}. The joint is an organ in which several tissues and cell types are in close proximity and show important functional interactions with each other: failure of one component will influence the others and can affect the function of the whole joint⁶.

1.1. Main joint tissue components

The knee and hip joints are the two largest weight bearing synovial joints of the human body (figure 1). The synovial membrane, a semi permeable soft tissue structure lining the joint spaces, encases the synovial fluid and provides nutrition to the chondrocytes in the cartilage. The healthy synovium is populated with a small number of macrophages and synovial fibroblasts which heavily proliferate during inflammation⁷⁻⁹. In this thesis cartilage and fibrocartilage have been the main focus.

1. Femur
2. Articular cartilage
3. Medial meniscus
4. Anterior cruciate ligament
5. Tibia
6. Fibula



1. Pelvis
2. Acetabular labrum
3. Femoral head
4. Femur

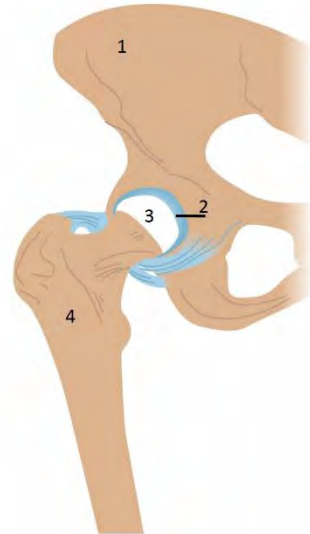


Figure 1: basic anatomical structure of the human knee (left) and hip (right) joint

Hyaline cartilage covers the articular joint surfaces and protects against biomechanical impact by distributing loading evenly and reducing friction by lubrication¹⁰. Both knee and hip joints have a fibrocartilagenous structure incorporated in the joint capsule: the menisci in the knee and the acetabular labrum in the hip. These are similar in shape and structure. Even though they have different functions, they both aim to protect the articular cartilage from degradation. Loss of function of these structures, for example after meniscus resection, increases the risk for OA development severely as reviewed by Englund and colleagues¹¹. Animal OA models such as destabilization of the medial meniscus (DMM) and anterior crucial ligament transection (ACLT) are based hereon and widely used in OA

research. The menisci are specialized shock absorbers, they facilitate load transmission in the joint by providing a larger contact area between the round femoral condyles and the flat tibial plateau. The acetabular labrum is mainly a sealing structure that seals/covers the femoral head in the acetabulum and protects against fluid flow in or out the joint space. This maintains the fluid pressure in the joint, ensuring more uniform distribution of forces on the cartilage. In this way, the acetabular labrum might also protect the articular cartilage from degradation¹.

Cartilage

The articular cartilage is a hyaline cartilage. It is an avascular, alymphatic, aneural connective tissue composed of an extracellular matrix (ECM) populated by a sparse number of chondrocytes. Chondrocytes are the only resident cells in articular cartilage and although they only make up 2% of the total cartilage volume they are responsible for ECM synthesis and turnover^{10,13,14}. Each chondrocyte is surrounded by ECM (figure 2), the region adjacent to the chondrocytes is called the pericellular matrix (PCM). Together they are sometimes referred to as the chondron.

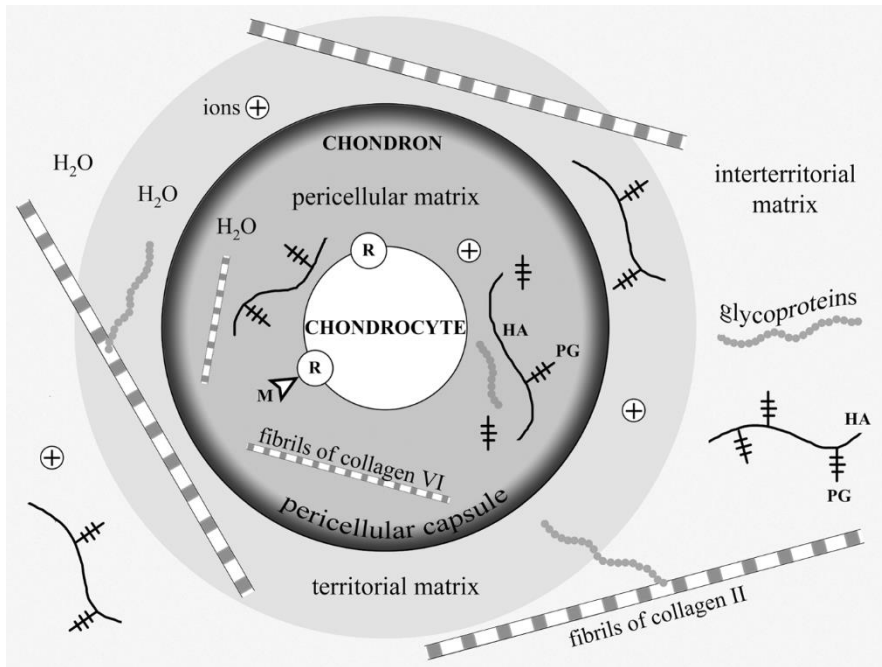


Figure 2: Schematic view of a chondrocyte and the surrounding ECM¹⁵. Chondrocytes are embedded in an extracellular matrix. The matrix can be subdivided according to the proximity to the chondrocyte. HA: hyaluronic acid; PG: proteoglycan; R: receptors

The PCM consists primarily of proteoglycans and is thought to play a critical role in transducing biomechanical and biochemical signals^{16–18}. Fine collagen fibrils provide a basketlike structure around the cells in the territorial region. The interterritorial region is the largest region and consist of large collagen fibril bundles which contribute to biomechanical cartilage properties and are arranged in specific patterns depending on which layer they are situated in^{10,14,19}. Articular cartilage can be divided in four different layers with distinct matrix structure and chondrocyte phenotype:

superficial/tangential zone, middle/transitional zone, deep zone and the calcified layer (figure 3).

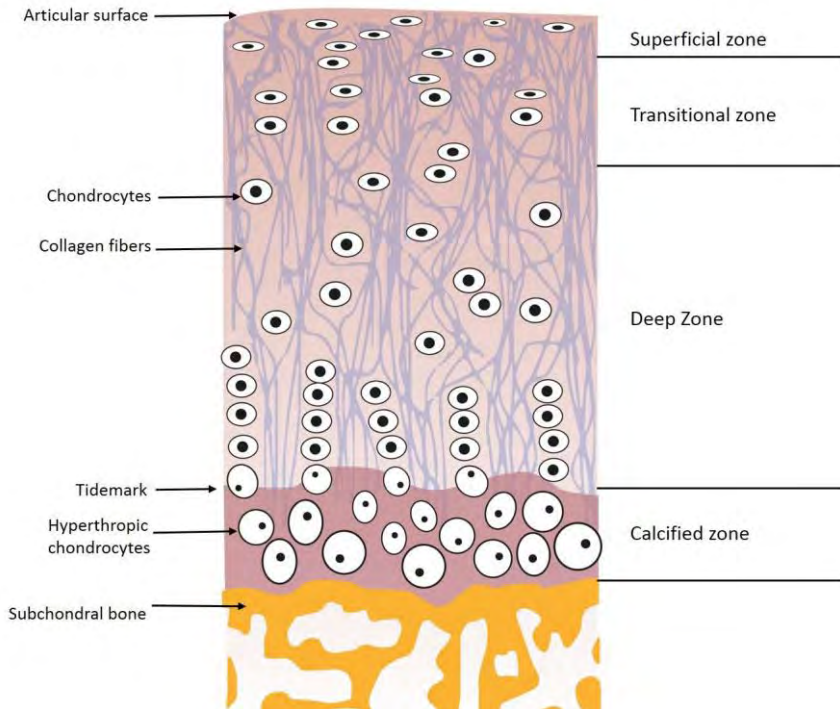


Figure 3: zonal compartmentalization of articular cartilage. Chondrocytes transition from randomly distributed and flat (superficial zone) to round shaped and column distributed (deep zone). Collagen fibrils change from parallel to surface (superficial zone) towards perpendicular to it (deep zone) and increase in thickness.

The superficial zone consists of collagen fibrils parallel orientated to the articular surface, abundantly populated with flat chondrocytes. In the transitional zone the orientation of collagen fibrils and chondrocytes, which are spheric here, changes to a more random distribution. It is the deep zone that provides most resistance to compressive forces. The collagen fibrils have a large diameter and are orientated perpendicular to the articular surface. Chondrocytes in this layer are typically

large, round shaped and arranged in columns parallel to the collagen fibrils. In the calcified zone the collagen fibrils are anchored into the subchondral bone, firmly attaching the cartilage to the bone. The layer is populated with a small number of hypertrophic chondrocytes^{10,13,19}.

The extracellular matrix is composed mainly of a large amount of water, collagens (mainly collagen type II), proteoglycans and a small number of other components (lipids, glycoproteins, non-collagenous proteins, SLRPs). Collagen type II forms triple helix structures stabilized by several other collagen types (type I, IV, V, VI, IX and XI). It is this triple helix matrix that largely contributes to the shear and tensile stress resistance of the articular cartilage^{10,20-22}. Proteoglycans are divided in large and small proteoglycans based on the size of their protein core. On the protein core one or several glycosaminoglycan (GAG) chains are attached. Aggrecan, a large proteoglycan, is the most abundant proteoglycan in articular cartilage. It consists of many GAG chains, mostly chondroitin sulfate and keratin sulphate. Aggrecan binds in large aggregates to hyaluronan. Furthermore, due to extensive negative charge, aggrecan is highly hydrophilic which attracts and retains water to the interfibrillar zones^{23,24}. The small proteoglycans are also known as small leucine rich repeat proteoglycans (SLRPs), because of several leucine rich repeats in their core protein^{25,26}. They are further divided in five classes base upon biological activity, structure and chromosomal organization: Class I (biglycan, decorin, asporin, ECM-2), class II (fibromodulin, lumican, keratocan,

osteomodulin, PRELP), class III (osteoglycin, opticin, epiphycan), class IV (chondroadherin, nyctolopin, tukushi) and class V (podocan, podocan-like protein 1)²⁵⁻²⁷.

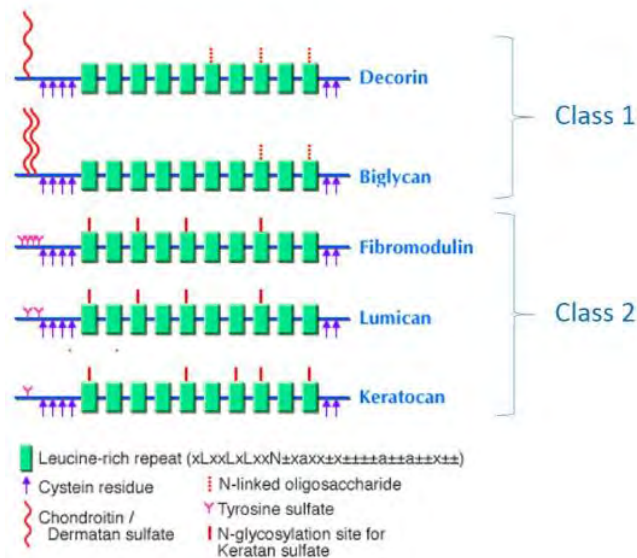


Figure 4: schematic overview of SLRP family members structure (adapted from glycoworld; www.glycoforum.gr.jp)

SLRPs are structural components of the ECM with binding sites for collagen, hereby protecting the collagen fibrils from degradation by proteases^{26,28}. However, increasing evidence indicates SLRPs are also involved in several signaling pathways as will be discussed later in this introduction²⁹⁻³².

Menisci and acetabular labrum

Both meniscus and acetabular labrum have a triangular crosssection³³. Histologically, the meniscus is divided in three zones: the superficial zone (in contact with the synovium), outer fibrous zone (resistant to tensile forces) and the inner zone

which resists mainly compressive forces^{34,35}. The acetabular labrum is divided into a dense connective tissue, the capsular side and the fibrocartilagenous articular side³⁶. Both labrum and meniscus are populated with a relative abundant number of spindle shaped fibrochondrocytes in contrast with the sparse, mostly round shaped, chondrocytes which populate the articular cartilage³⁷. These fibrochondrocytes are similar in menisci and acetabular labrum but distinct to chondrocytes not only in morphology but also in their protein expression pattern. Whereas the cartilage ECM contains large collagen type II fibrils, these fibrochondrocytes release more collagen type I in the ECM, giving rise to fibrocartilage rather than cartilage. Similar to chondrocytes, fibrochondrocytes will induce production of several matrix degrading proteins in response to pro-inflammatory stimuli and thus contribute to the homeostasis of the ECM in which they are embedded³⁷. Whereas the metabolism of chondrocytes in articular cartilage is very well studied, much less is known about the cellular homeostasis in fibrocartilagenous tissues such as meniscus and labrum.

1.2. Inside osteoarthritis

Disturbed cartilage homeostasis and low level inflammation

In the early years of OA research, OA was believed to be merely a disease of mechanical cartilage erosion. Later on, it became clear that an altered chondrocyte metabolism is crucial in the development of osteoarthritis. In consequence, the chondrocyte

metabolism was extensively studied during the last two decades. Nowadays, it has become clear that OA is a disease of the whole joint involving interactions between the synovium, components of the immune system, bone and cartilage.

In healthy adult cartilage a low turnover of ECM is maintained by resting chondrocytes with a half-life of a few decennia for aggrecan³⁸ and over 100 years for collagen³⁹. In OA the chondrocytes become activated, start to proliferate and shift to a hypertrophic phenotype. Their expression and secretion profile changes towards a catabolic environment^{3,40}. Although long believed to be a non-inflammatory arthritis, the current scientific opinion is that at least a low level of inflammation can be observed in OA patients^{41,42}. However, there still exists controversy whether this inflammation is primary or secondary to cartilage damage⁴³. Most of the current evidence suggests that initial mechanical damage or wear and tear triggers inflammatory responses in the joint tissue, which increases catabolic activity and further induces ECM degradation. These degradation products can upregulate inflammatory pathways and genes, generating a feedback loop which drives OA progression⁴⁴. Indeed, a low grade activation of the synovial membrane has been observed in OA patients, enforcing this hypothesis⁴⁵⁻⁴⁷.

Chondrocyte mediated cartilage degradation

In OA the balance between matrix synthesis and degradation is disrupted. Chondrocytes, the only resident cell type in articular

cartilage play an important role in this dysregulation. During the course of OA chondrocytes undergo a process of activation and transdifferentiation⁴⁸. They develop a phenotype characterized by a prominent Golgi apparatus and ER⁴⁹, to increase their secretory potential. Initially these transdifferentiated chondrocytes increase production of matrix components (e.g. collagen 2) in an attempt to restore the extracellular matrix. Gradually the chondrocytes protein secretion shifts away from repair, towards a degrading pattern, characterized by large amounts of proteoglycan degrading enzymes (e.g. aggrecanase) and collagen degrading matrix metalloproteinases MMP's (e.g. MMP13) are produced⁴⁸. This massive production and secretion of proteins induces severe cellular stress to the chondrocytes⁵⁰. A fraction of them will undergo apoptosis and die (also known as chondroptosis)⁵¹⁻⁵³. Since proteoglycans protect the collagen fibril from degradation, initial protection from proteoglycan depletion protects against OA progression since no collagen degradation can occur⁵⁴. Destruction of aggrecan, the major proteoglycan of the cartilage ECM, is mediated by two aggrecanases of the A disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS) family, ADAMTS-4 and ADAMTS-5^{54,55}. Both aggrecanases seem to be essential in aggrecan degradation however monoclonal antibodies against ADAMTS-5 have recently been shown to be more effective against OA cartilage damage^{56,57}. In a later stage of OA progression matrix degradation is focused on collagen cleavage⁵⁸. In humans this is mostly mediated by MMP's, most potently by MMP-13^{55,58,59}.

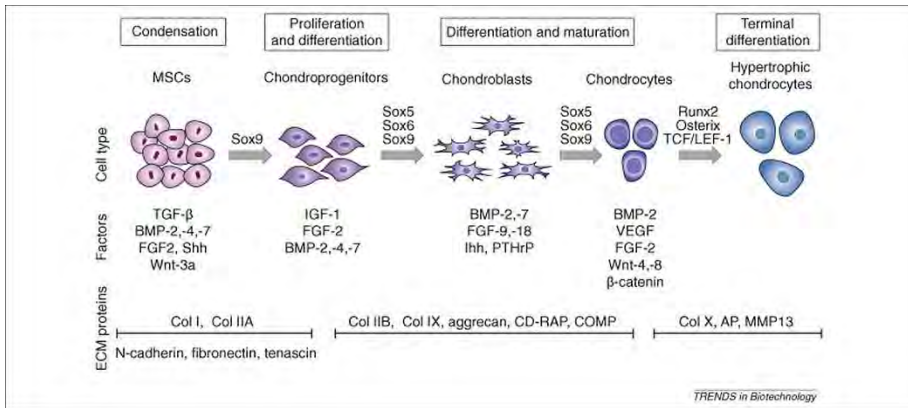


Figure 5: Overview of chondrocyte differentiation, involved growth factors and produced ECM proteins⁶⁰

The proteolytic enzymes described above are the main effectors of cartilage degradation in OA. Several cytokines and growth factors are known to drive the secretion of these effector enzymes. IL-1 β and TNF α are the most catabolic signaling molecules in OA⁶¹, stimulating the production of cartilage degrading enzymes by chondrocytes through MAPK and NF κ B signaling pathways^{3,62,63}. IL-1 β and TNF α are endogenously produced by chondrocytes but also by synovial fibroblasts. In addition to increased matrix catabolism, IL-1 β and TNF α inhibit anabolic matrix synthesis through downregulation of collagen type II and aggrecan^{64,65}. Growth factors also play an important role in regulating chondrocyte metabolism. Overactivation of pathways involved in endochondral bone development is strongly associated with OA progression. For example, Wnt/ β -catenin signaling is critical for chondrocyte homeostasis. Overactivation leads to chondrocyte dedifferentiation and increased MMP-13 expression^{66,67}. Activation of Indian

hedgehog protein (Ihh) induces ADAMTS-5 and MMP-13⁶⁸. TGF- β family signaling is essential in cartilage development but also contributes to OA pathology^{69,70}. BMP signaling is considered mostly anabolic in chondrocyte metabolism, moreover BMP-2 has been shown to be involved in OA osteophyte formation⁷¹⁻⁷⁴. Reports on TGF- β are even more complex and controversial^{70,75,76}. Distinct effects are exerted on chondrocytes, synovial fibroblasts and bone, furthermore even within one cell type different microenvironments may implicate different TGF- β effects⁷⁷. For example TGF- β is critical in osteophyte formation^{78,79} and it stimulates synovial fibroblasts to produce IL-1 β and TNF α ⁸⁰. On the other hand, TGF- β inhibits hypertrophic chondrocyte differentiation⁸¹ and mutations and SNP's associated with inhibition of TGF- β signaling are related to an increased susceptibility to OA^{82,83}. This demonstrates the duality of TGF- β signaling in OA. It is now hypothesized that canonical TGF- β signaling through SMAD2/3 pathway exerts TGF- β induced anabolic events whereas non canonical SMAD1/5/8 signaling is responsible for TGF- β -induced catabolism^{76,84-86}. Finally, ECM components itself can also exert catabolic effects, for instance fibronectin and collagen fragments activate ERK, JNK and p38 signaling through binding to the integrin receptor, thereby further inducing MMP13 expression^{87,88}.

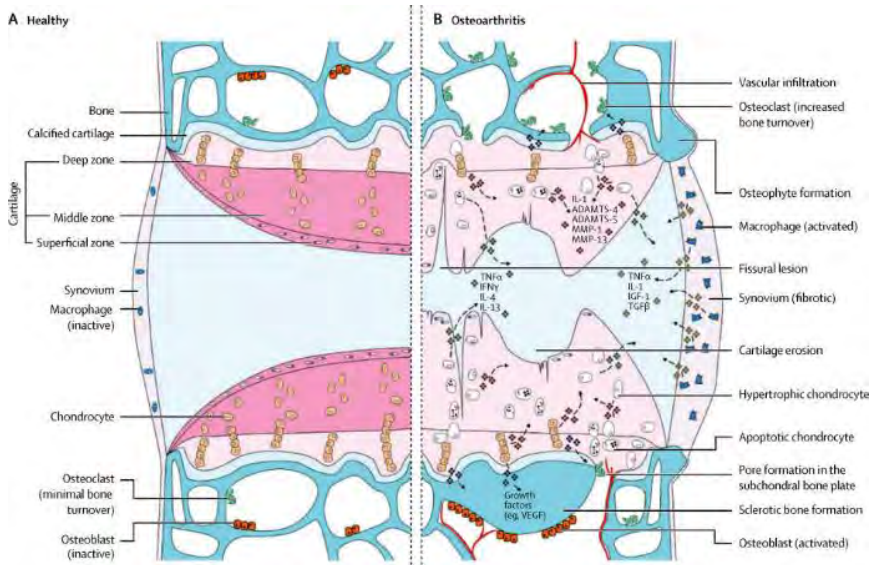


Figure 6: Overview of processes in joint degradation during OA (adapted from Glyn-Jones et al.⁸⁹). The osteoarthritic joint is characterized by several features such as cartilage erosions, osteophyte formation and chondrocyte hypertrophy.

Role for subtle inflammation on chondrocyte homeostasis and ECM destruction

Until the beginning of this century, the view on osteoarthritis could be summarized by the previous paragraph. OA was considered as a primarily chondrocyte driven imbalance of homeostasis. OA is not a prototype inflammatory arthritis, like rheumatoid arthritis or spondyloarthropathies. There is no autoimmune or autoinflammatory component in the disease etiology. However, in the past years the field has gradually accepted a role for a subtle inflammation in driving this imbalance as well as the involvement of the whole joint in the OA pathogenesis. For instance, synovitis has been observed in at least a significant subset of OA patients both early and late disease staged^{42,90}. Moreover mononuclear cell infiltration is

observed to be more abundant in early OA compared to late OA⁹¹. Additionally, toll like receptor 2 (TLR2) and TLR4 expression in OA chondrocytes are increased^{92,93}. These receptors respond not only to peptidoglycan or LPS but also to endogenous danger signals (DAMP's) released from stressed and dying cells. In OA these receptors have been shown to bind S100A4, S100A8, S100A9, low molecular weight hyaluronic acid (LMW-HA) and high mobility group box chromosome protein 1 (HMGB1) all of which are elevated in OA tissue. In addition it has recently been suggested that ECM components can act as DAMP's after being cleaved from the ECM. These DAMP's bind toll like receptors which promote cartilage degradation via MyD88 and subsequent NFκB signaling⁹⁴⁻⁹⁶.

Moreover, complement activation has recently been proven to be crucial in OA development. Complement activation and subsequent membrane attack complex (MAC) deposition has been observed in early stage OA joint tissue and is persistent throughout later OA disease stages. This activation of the complement cascade might be attributed to ECM components (e.g. fibromodulin, aggrecan, COMP) released after initial joint injury⁹⁷⁻⁹⁹. Deposition of MAC around OA chondrocytes will either be lytic and cause chondrocyte cell death or induce expression of matrix degrading enzymes and pro-inflammatory mediators. Interestingly, MAC deposition in the absence of cell death will also induce the expression of complement effectors in chondrocytes. This might be synergetic with the complement activation in OA synovial tissue and amplify complement

signaling. Treatment of mice with anti-C5 antibodies or fusion protein inhibiting C5 activation reduces OA development in murine OA models⁹⁷. This suggests complement cascade activation is crucial to OA pathology.

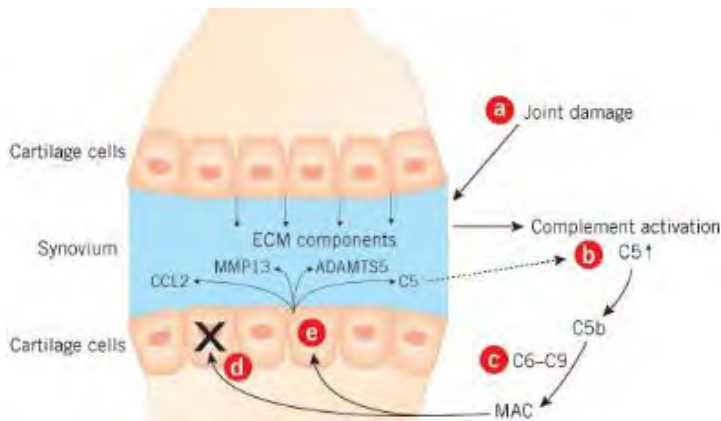


Figure 7: Role of complement activation in OA. ECM fragments released after initial damage activate the complement cascade, increasing membrane attack complex (MAC) deposition. This will lead to cell death or release of catabolic mediators in chondrocytes (adapted from Haas et al.¹⁰⁰)

In conclusion, the current paradigm suggests low level inflammation, induced upon initial cartilage damage, is essential in the progression from initial injury towards full OA development.

1.3. Role of SLRP in degenerative joint diseases

As mentioned above apart for collagen and large proteoglycans, the small proteoglycans and SLRP's are important structural components of the ECM as well. The family consists of 18 members divided in 5 classes based on shared activity^{22-24,27,29}. Their specific structure, consisting of a small protein core with

LRR's and variable glycosaminoglycan side chains makes them extremely well suited for protein-protein interactions. They are able to bind several cell surface receptors, growth factors and cytokines^{29,101}. Several SLRP knock out mice models have been shown to exhibit an OA-like phenotype¹⁰²⁻¹⁰⁹. The exact mechanism behind the suggested involvement of SLRP's in OA development still remains elusive but there might be a contribution of their ability to influence several signaling pathways. Concerning the role for SLRP's in degenerative joint diseases, different aspects of the SLRP should be considered. On the one hand they are important for ECM homeostasis, they function as a coating surrounding the collagen network, protecting it from cleavage by MMP's¹⁰¹. Moreover asporin influences collagen mineralization which might contribute to OA pathology¹¹⁰. On the other hand, protein-protein interactions may be, at least, equally as important in understanding how these SLRP could be involved in osteoarthritis.

SLRP-TGF β superfamily interactions

TGF- β family members are very important in chondrocyte biology and cartilage maintenance. Several SLRP's are known to signal via the superfamily¹¹⁰ or be regulated by the TGF- β superfamily^{110,111}. Different branches of the TGF- β superfamily may influence SLRP expression differently. Osteoadherin, also known as osteomodulin (OMD) expression is induced by BMP-2 whereas TGF- β 1 mediates OMD downregulation. The BMP-4 signaling can be inactivated through binding with biglycan. With regard to TGF- β , more complex interactions have been

described. Decorin, biglycan, asporin and fibromodulin are known to bind TGF- β ^{31,32} with activating or inhibitory effects depending on the cell specific context. In cartilage, most evidence points to an inhibition of TGF- β signaling by decorin, whereas biglycan and fibromodulin induces an overactivation of TGF- β signaling pathways.

The case of asporin and TGF- β is particularly interesting and may therefore serve as a perfect example to demonstrate the complexity of interactions between SLRP's and the TGF- β family (as recently reviewed by Lei and colleagues¹¹⁰). There has been increasing evidence for asporin involvement in OA. Association studies, trying to link polymorphisms in the asporin gene with OA susceptibility, have generated inconsistent results. Asporin's pro-osteoarthritic effects mediated via TGF- β are well documented¹¹⁰. Asporin's binding to TGF- β 1 inhibits binding with TGF- β RI/RII receptor complex. In this way, it is able to inhibit canonical SMAD 2/3 pathway downstream of this receptor, thus inhibiting chondrogenesis and other anabolic TGF- β effects. It seems asporin-TGF- β interaction does not inhibit the SMAD 1/5/8 pathway, which is responsible for catabolic events occurring in OA attributed to TGF- β such as MMP-13 induction, subsequent cartilage erosion and osteophyte formation. This suggests asporin might be involved in the shift from mainly anabolic SMAD 2/3 TGF- β signaling towards more catabolic SMAD 1/5/8 signaling observed in OA. Interestingly, TGF- β itself regulates asporin expression through the canonical

pathway. This implies a regulatory feedback loop exists between aspirin and TGF- β ¹¹⁰.

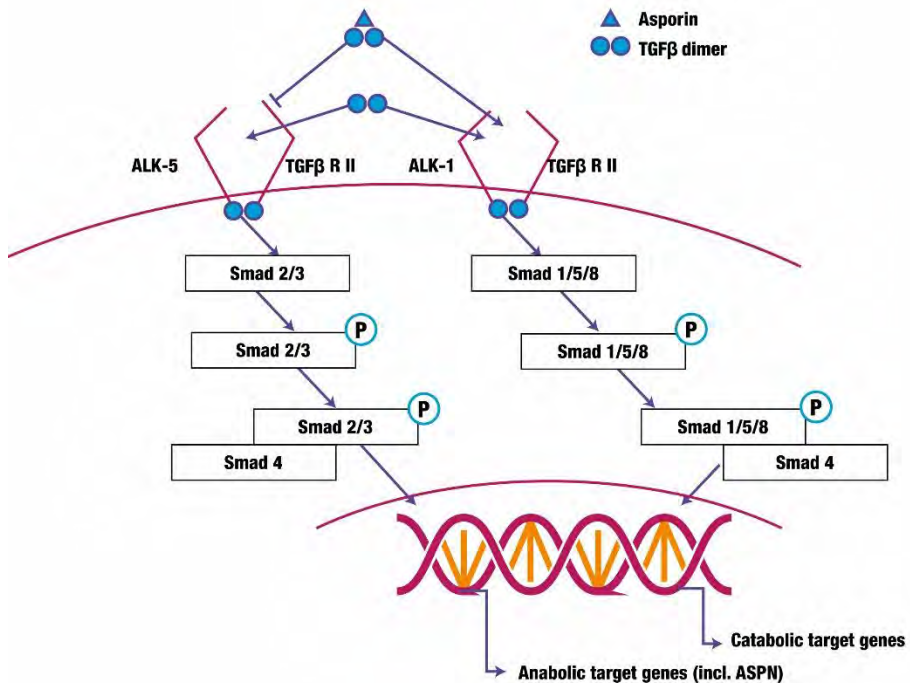


Figure 8: Schematic overview of Aspirin and TGF β interactions. Aspirin-bound TGF- β 1 is inhibited from binding the ALK-5/TGF- β RII complex which signals through SMAD 2/3 and activates transcription of mainly anabolic cartilage genes. Whereas binding to the ALK-1/TGF- β RII complex, signalling through the catabolic SMAD 1/5/8 pathway is not inhibited by Aspirin binding.

Immunomodulatory role of SLRP's

In recent years increasing evidence has indicated a role for soluble SLRP's in regulation of immune responses, mainly in innate immunity. Several SLRP's interact with complement system components, either to activate (e.g. fibromodulin and lumican) or inhibit (e.g. biglycan and decorin) classical complement activation through binding with C1qM^{101,111}. Another way the SLRP's can regulate inflammatory responses is by activating pattern recognition receptors (PRR). Both TLR's and NLR's possess LRR-motifs which allow them to interact with other LRR-containing proteins, such as SLRP's^{112,113}. Lumican can activate TLR4¹¹⁴ and biglycan has been extensively reported to activate both TLR2 and 4. Through this interaction, biglycan induces TNF α and MIP-2 synthesis¹¹⁵. Biglycan interaction induces a clustering of TLR2/4 and purinergic P2X receptors in macrophages which increases pro-IL-1 β synthesis and in addition directly activates the NLRP3 inflammasome which induces an increased release of mature IL-1 β ¹¹⁶. Interestingly, only soluble SLRP's are able to induce these immune responses. Recent evidence suggests soluble forms of SLRP's are released during tissue injury or stress by proteolysis from to ECM. However, activated macrophages have been shown to secrete

soluble biglycan. This might potentiate immune signaling via biglycan as a DAMP¹¹⁵.

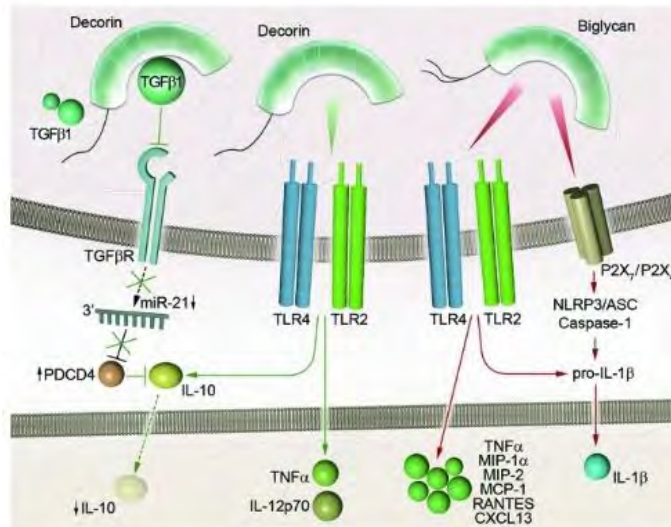


Figure 9: Schematic view of SLRP's interactions with the innate immune system¹¹¹. Decorin and biglycan induce inflammation through activation of TLR2/4 pathways. Activation of the inflammasome through purinergic receptors by biglycan increases IL-1 β levels. IL-10 levels are reduced by decorin-TGF β binding through elevated PDCD4 via miR-21 reduction.

Decorin also functions as a danger signal through TLR2/4 binding and induces PDCD4 expression, an IL-10 inhibitor, thereby dampening anti-inflammatory response¹¹⁷. However decorin can induce a hyper-inflammatory state since it is able to stop normal PDCD4 inhibition, inducing a decline in mi-R21, a PDCD4 inhibitor, through binding and blocking of TGF- β . In this way IL-10 will be inhibited continuously and inflammation cannot be resolved^{117,118}. Although most interactions of SLRP's seem to be with the innate immune system, SLRP's appear to have an influence on adaptive immunity as well. For instance

biglycan, induces CXCL13 expression in macrophages and dendritic cells which mediates B cell recruitment¹¹⁹. Moreover biglycan is a ligand for CD44 and is suggested in CD16- NK cell recruitment¹²⁰.

1.4. Role of heat shock proteins in stromal tissue of the joints

Adapted from:

Heat-shock proteins in stromal joint tissues: innocent bystanders or disease-initiating proteins?

Stijn Lambrecht, Nele Juchtmans and Dirk Elewaut

Rheumatology (2014) 53 (2): 223-232.

The name heat-shock proteins (HSPs) originates from their initial identification as stress-inducible proteins that act as an intracellular defense mechanism against thermal stress. Functionally, stress-inducible proteins can be classified into seven classes, including chaperones, cytoskeletal proteins and metabolic enzymes (for an overview, see Richter et al¹²¹). The predominant class, in terms of expression level, across species is the initially discovered HSPs¹²². In this review we will specifically focus on this class. The primary characterization of HSPs suggested that they were largely regulated at the transcriptional level, however, subsequent mechanistic data have shown that HSPs have multiple levels of regulation, such as phosphorylation, oligomerization and protein interactions¹²³. At the transcriptional level, the primary stress-inducible transcription factor is heat shock factor 1 (HSF1). Many HSPs also contain alternative promoter recognition sites, allowing for transcriptional activation via multiple signaling pathways¹²³. The HSPs show a remarkable sequence homology between species, emphasizing their important biological role throughout evolution. In humans, the HSPs can be further subdivided into five classes: chaperonins (HSP10/HSP60, primarily located in the mitochondria), the HSP70 (HSPA/HSPH) family, the HSP40 (dnaJ) family, the HSP90 (HSPC) family and the small HSPs (HSPb)¹²⁴. HSPs have a long history in the arthritis research field. Ever since the discovery that T cells recognizing HSPs may elicit an arthritic phenotype, a lot of research has been conducted to study the role of HSPs in arthritis and how these can be used as diagnostic and/or therapeutic tools. This initial

discovery described the role of T cells recognizing HSP65 in eliciting arthritis¹²⁵. It has been shown that T cells and antibodies isolated from arthritic animals and arthritis patients recognize different HSPs¹²⁶. Surprisingly, despite the fact that HSPs are a major immune target, administration of purified bacterial HSPs does not induce arthritis¹²⁷. It is now clear that endogenous HSPs are not immunogenic per se, but that the immunodominance of bacterial HSPs may induce cross-reactivity with endogenous HSPs. Moreover, HSPs may associate with danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) or other antigenic peptides and thus alter the immune response to DAMPs and PAMPs, further emphasizing their immunological significance¹²⁸. Recently it has become clear that immune responses to HSPs are very complex and may be arthritogenic as well as protective. This complex behavior may be explained by the presence of different epitopes, the method of immune activation and the administration route. How HSPs interact with the immune system in autoimmune diseases and arthritis has been extensively reviewed elsewhere^{129,130}. In this review we will mainly focus on altered expression of HSPs by stromal joint compartments and how these can affect tissue biology and/or immune activation. An intriguing feature of HSPs is that they are located intracellularly, at the cell surface or even in the extracellular environment. Given the intimate contact between immune and stromal cells in the joint, the presentation of HSPs in the extracellular space by stromal cells may thus modulate the immune system and contribute to joint inflammation (Fig.

1). In contrast to the research focused on regulation of the immune system by HSPs in arthritis, studies on the direct biological function of HSPs in cells from the joint are rather limited, but they provide exciting avenues for future research.

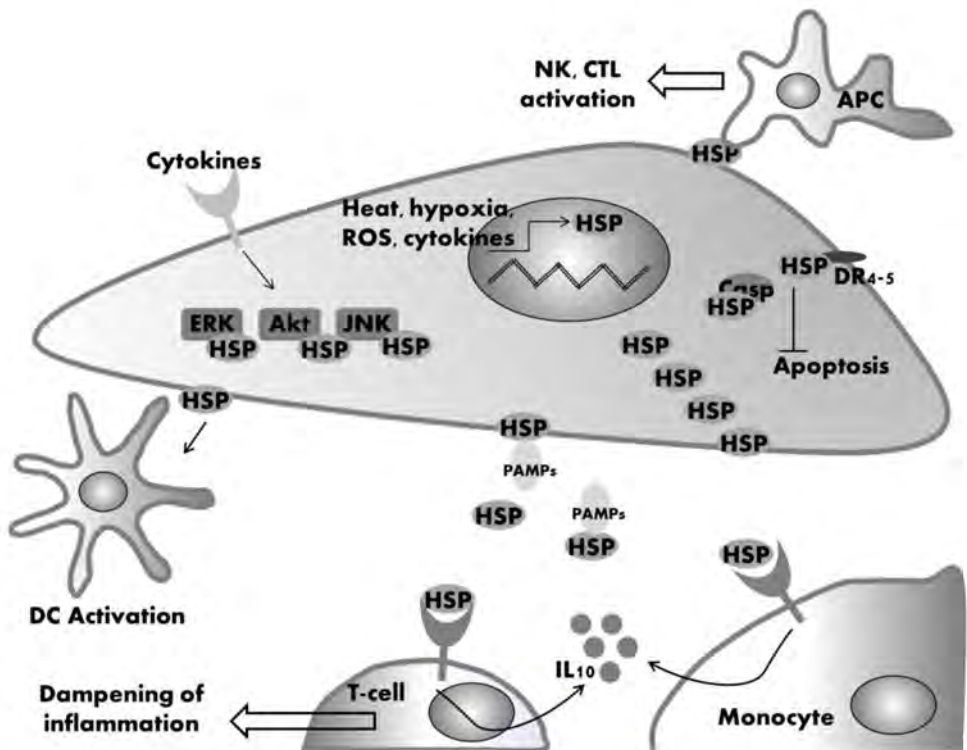


Figure 10: HSPs link stromal and immune cells in joint inflammation. HSPs are predominantly located in the intracellular environment where they primarily act as molecular chaperones. Cellular stress factors (including cytokines, Reactive Oxygen Species, heat, hypoxia) will trigger expression of HSPs, secretion of HSPs to the extracellular environment and/or translocation of HSPs to the cell membrane. In the extracellular environment, the HSPs can elicit an inflammatory as well as a toleration response, involving the innate and the adaptive immune system

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HSPs: a collection of multiple protein families with pleiotropic effects in joint diseases

HSP90 family

The HSP90 protein family consists of 17 gene members divided into four classes based on phylogenetic analysis¹³¹. The HSP90 family contains five members in humans¹²⁴. This family has been implicated in the physiology of joint tissues in the past, even with therapeutic potential. Interestingly, HSP90 expression varies with age. Moreover, the ability of a cell to respond to cellular stress by up-regulating HSP90 (among other HSPs) expression decreases with age¹³². Such a regulatory mechanism may be extremely relevant in degenerative diseases such as OA. Moreover, it is well known that HSP90 protects cells from apoptosis and interacts with a variety of substrates¹³³, including signaling proteins such Akt¹³⁴ and enzymes such as iNOS¹³⁵. In RA fibroblast-like synoviocytes (FLS) it was shown that HSP90 protects synovial cells from apoptosis and thus contributes to synovial overgrowth via interaction with Akt and Erk¹³⁶. Boehm et al.¹³⁷ proposed a role for HSP90 in chondrocyte biology by demonstrating that HSP90 expression regulates MMP13 expression. However, specific blockade of HSP90 β (HSPC3) by small interfering RNA (siRNA) induces MMP13

expression. This is probably due to destabilization of a protein complex that interacts with a specific MMP13 promoter site, thereby inhibiting MMP13 expression¹³⁸. As MMP13 is one of the major proteases involved in cartilage breakdown, targeting these proteins was proposed as a potential cartilage repair strategy. Commonly used chemical blocking strategies, such as geldanamycin, do not necessarily have a strong specificity to one of the HSP90 proteins. Thus it is clear that more specific inhibitors await future development¹³⁹. Kimura et al.¹⁴⁰ described a selective HSP90 inhibitor that specifically targets HSP90 α 1 and more specifically induces HSF1 activation, thereby inducing HSP70 expression in cartilage, a protein known to prevent nitric oxide (NO)-induced apoptosis¹⁴¹, ultimately leading to reduced MMP13 levels. All of this work clearly suggests an important role for HSP90 proteins in chondrocyte biology. However, the *in vivo* impact of HSP90 blocking on cartilage metabolism in OA is still unclear. Such *in vivo* proof of principle was demonstrated in inflammatory arthritis models (CIA and AIA) by a small molecule inhibitor of HSP90¹⁴². The authors demonstrate reduced nuclear factor κ B (NF- κ B) nuclear translocation in synovial fibroblasts and reduced cytokine production upon compound administration.

In conclusion, it is clear that HSP90 directly interferes with important pathological processes in chondrocytes and synovial fibroblasts such as NO synthesis, stabilization of signaling molecules (directly or by mediating other HSP expression) and angiogenesis. However, given the ubiquitous expression of

HSP90 and its pleiotropic interaction profile, it remains very questionable whether the HSP90 targeting strategies will have a sufficient therapeutic index, especially given observations that targeting of HSP90 may induce osteoclastogenesis *in vitro*^{143,144}, which could worsen bone destruction in joint diseases. Thus it is likely that targeting HSP90 in one tissue may have beneficial effects, whereas in others detrimental effects may be observed. Overall, the therapeutic potential of HSP90 is therefore uncertain. Indeed, recent clinical studies in multiple myeloma with HSP90 inhibitors report adverse events such as thrombocytopenia, neutropenia and anemia¹⁴⁵.

HSP90 proteins may also play an important role in the interaction between stromal cells and the immune system. Overexpression and surface presentation of gp96 by stromal tissues under stress conditions such as inflammation may contribute to the chronicity of inflammation in arthritis. Gp96 (or grp94) was shown to be elevated in RA synovial tissue. Moreover, the authors reported gp96 as a TLR2 ligand that activates macrophages and identified the protein as a potential contributor to chronic inflammation in RA^{146,147}. It was shown *in vivo* that cell surface expression of gp96 elicits a proinflammatory lupus-like phenotype characterized by chronic stimulation of dendritic cells (DCs), which may represent an alternative pathway to initiate spontaneous autoimmune disease¹⁴⁸. Additionally, fibroblasts overexpressing gp96 elicit a T cell activation involving antigen-presenting cells (APCs)¹⁴⁹. Such interactions may be of major importance in arthritis

pathogenesis given the close proximity of stromal and immune cells in the joint.

HSP70 family

The HSP70 family is one of the most highly conserved chaperone families. Typically HSP70s never function alone. They require a co-factor such as J protein (HSP40 family) as well as nucleotide exchange factors. These co-factors act together as an HSP70 machine to bind client proteins and nucleotides in an adenosine triphosphate (ATP)-dependent manner¹⁵⁰. HSP70 has been mainly investigated in the inflammatory arthritis context, based on initial observations that bacterial HSP65 protected rats from adjuvant arthritis¹²⁵. As stated above, in this review we do not focus on how HSPs may modulate the immune system and induce tolerance (see the review by Borges et al.¹⁵¹). HSP70 has primarily been investigated in synovial tissue. One of the landmark articles in this field described the enhanced expression of HSP70 in RA synovial tissue compared with OA tissue. In addition, the authors described the induction of HSP70 upon IL-1 β and TNF- α stimulation but also under shear stress in synovial fibroblast-like cells¹⁵². This was later confirmed by Kang et al.¹⁵³, who additionally showed that down-regulation of HSP70 may protect synoviocytes from apoptosis. Furthermore, it was reported that HSP70 synovial fluid levels are elevated in RA patients. Indeed, whereas HSPs are generally accepted to act as an intracellular chaperone, the authors showed the release of HSP70 under stress conditions from viable cells¹⁵⁴. Of great interest, in RA patients surface HSP70

expression was observed in a high percentage of synovial fluid cells, in particular DCs. Based on the known interactions between HSP70 and MHC class II shared epitopes, this may reflect an initiating mechanism in the activation of autoreactive T cells¹⁵⁵. In contrast to the conventional role of HSPs as intracellular chaperones, Asea et al.¹⁵⁶ and others have also described a cytokine role for HSP70. However, these reports have been rather controversial. Later on, subsequent studies^{157,158} have shown that lipopolysaccharide (LPS) contamination of HSP preparations may be responsible for these results. Thus caution is needed when interpreting results of data based on recombinant HSP preparations without stringent endotoxin control. Detanico et al.¹⁵⁹ showed reduced TNF- α production by monocytes isolated from synovial fluid upon HSP70 stimulation. Interestingly, Luo et al.¹⁶⁰ confirmed a similar role for extracellular HSP70 in suppressing cytokine release from tissue-derived FLS cultures upon TNF- α stimulation by inhibiting p38, extracellular regulated kinase (ERK) and Jun-N-terminal kinase (JNK) phosphorylation and clearly confirmed that LPS was not involved. These data provided evidence that extracellular HSPs have a direct effect on fibroblast-like synoviocytes. However, the *in vivo* relevance of these findings still needs to be demonstrated.

The HSP70 family contains several more members, including HSP72. Administration of HSP72 in mice at the onset of arthritis reduced synovial inflammation by reduced NF- κ B activation in the synovial tissue¹⁶¹. A particular member of the HSP70 family

is GRP78. The protein is expressed at the cell surface and can be detected in serum and synovial fluid¹⁶². Interestingly, the expression of GRP78 is elevated in the synovial lining and sublining of RA patients¹⁶³. A direct pathogenic role on the synovium GRP78 (BiP, HSPA5) was recently demonstrated¹⁶⁴. The authors confirmed that GRP78 levels are elevated in RA synovium and that *in vitro* down-regulation of GRP78 abolished TNF-induced synoviocyte proliferation and inhibited angiogenesis. *In vivo* data confirmed that GRP78 haploinsufficient mice compared with wild-type control mice exhibit reduced arthritic symptoms, including synovial proliferation and angiogenesis. Induction of GRP78 alone, however, is not sufficient to induce arthritis and thus GRP78 has been suggested to act as an important amplifying factor in synovial hyperplasia and arthritis¹⁶⁴. Whereas GRP78 has a pathogenic role in the synovium, it has previously been shown that exogenous extracellular GRP78 helps to restore immune system homeostasis and thus resolve acute inflammation, mainly by acting on the myeloid lineage¹³⁰. Overexpression of GRP78 was shown to prolong lifespan and increase the repair capacity of chondrocytes¹⁶⁵, and it might play a crucial role in mineralization events during bone formation¹⁶⁶, thus further demonstrating the pleiotropic effects of HSPs on different tissues. At least in cancer, the subcellular location of GRP78 seems to determine the pro- or anti-tumor activity. Based on the parallels in the tumor environment and the synovial pannus, one can hypothesize that similar phenomena may occur in the

joint and may thus explain the contradictory role of GRP78 in arthritis¹³⁰.

In conclusion, along with the well-documented role of HSP70 proteins in dampening inflammation by its direct tolerogenic effect on immune cells (primarily DCs and monocytes), HSP70 proteins also have a direct effect on cell metabolism of articular cell types such as FLS and chondrocytes.

HSP40 family

The dnaJ, or HSP40 family, is functionally related to the HSP70 family since the dnaJ proteins are necessary for HSP70 chaperone function. The dnaJ proteins have an HSP70 binding domain and activate the ATPase activity required for chaperone function. It is probably the largest HSP family in humans, although very little is known in arthritis and joint diseases. The dnaJ family was first introduced by the discovery that RA patients show strong immune responses to bacterial dnaJ while normal subjects do not¹⁶⁷. Based on the sequence homology between peptide sequences encoded by HLA genes and dnaJ genes, it has been proposed that in arthritis an interplay between HLA and dnaJ-derived peptides maintains and stimulates T cells, which participate in autoimmune inflammation¹⁶⁸. Nowadays it is known that differential recognition of epitopes from human and bacterial dnaJ proteins may be a natural mechanism for amplifying and subsequently down-regulating inflammation¹⁶⁹. Further research has indicated that tolerization with peptides derived from the dnaJ

family is feasible¹⁷⁰. Increased expression in synovial tissues of arthritis patients of dnaJ proteins has been reported by several authors and may further contribute to the regulation of immune responses^{169,171}. Interestingly, HSP40 (dnaJB1) has been reported to be a mechanosensitive gene¹⁷² that may be extremely relevant in linking immune activation and physical stress in the joint.

HSP60 family

Similar to the dnaJ family, research on the HSP60 family in the arthritis field has primarily focused on studying autoimmune responses to self and bacterial HSP60s (reviewed in Vercoolen et al.¹⁷³). It is well known that T cell-recognizing self HSP60 plays a role in disease protection by inducing a tolerogenic effect in arthritis¹⁷⁴. However, very little is known about how these proteins function in stromal tissues of the joint and how changing expression levels in stromal tissues may influence immune responses. HSP60 has been reported to regulate osteoblast survival¹⁷⁵ and bone marrow mesenchymal cell apoptosis¹⁷⁶. Interestingly, HSP60 induces pro-inflammatory cytokine secretion through mitogen-activated protein kinase (MAPK) activation in other mesenchymal stem cell (MSC)-derived cell types, such as adipocytes¹⁷⁷. This might be important in the context of the elevated levels of HSP60 in the synovium¹⁷⁴, suggesting a pro-inflammatory role of HSP60 in the synovium.

Small HSPs

A particular class within the HSPs are the small HSPs (sHSPs). sHSPs are a family of proteins with molecular weights <30 kDa¹⁷⁸. The proteins are structurally characterized by a conserved α -crystalline domain that is flanked by non-conserved sequences essential for chaperone function¹⁷⁹. The human genome encodes 10 members of the sHSP family. Some of these are expressed ubiquitously (such as HSPb1, HSPb5, HSPb6 and HSPb8), while others have a more restricted expression pattern¹⁸⁰. From a structural point of view, the sHSP family is particularly interesting as they show several phosphorylation sites and tend to form oligomeric structures¹⁸¹. The phosphorylation status of sHSPs may alter their subcellular localization and function. For example, in osteoblasts the unphosphorylated HSPb1 acts as a positive regulator of bone calcification, in contrast to phosphorylated HSPb1¹⁸². Typically sHSPs, similar to other HSPs, primarily function to protect cells against stress factors. Unlike the large HSPs, the sHSPs do not consume ATP to refold proteins. The expression of sHSPs may be induced by various stresses, such as heat shock, oxidative stress and chemical stresses. Besides, sHSPs show a constitutive expression in particular tissues. In these tissues these molecular chaperones are implicated in many different cellular processes (reviewed in Haslbeck et al.¹⁸¹). This is primarily demonstrated by several congenital diseases that are associated with mutations in these proteins. For example, mutations in the HSPb1 and HSPb8 genes are associated with

Charcot–Marie–Tooth disease and distal motor neuropathy^{183,184}, while mutations in the HSPb5 protein have been associated with myopathy¹²⁶ and cataracts¹⁸⁵.

HSPb1, together with large HSPs, was previously shown to be dysregulated in RA synovial tissue¹⁸⁶. Our own group previously demonstrated that HSPb1 as well as HSPb5 are down-regulated in OA chondrocytes and that the expression is regulated by proinflammatory cytokines^{187,188}. Moreover, it was clearly shown that dysregulation of both HSPb5 and HSPb1 have a major impact on the differentiation status as well as the cytokine secretion of chondrocytes. Indeed, HSPb1 directly influences the IL-1 β -induced gene expression in human articular chondrocytes¹⁸⁹. A similar relationship between HSPb1 and TNF- α was recently shown in osteoblasts¹⁹⁰. Our observations of lowered expression of HSPb1 in OA cartilage and the observation that suppressed HSPb1 expression results in a decreased responsiveness towards IL-1 β may contribute to the well-known reduced responsiveness of OA cartilage¹⁹¹ to IL-1 β . Such a mechanism may represent another way in which HSPs regulate cell/tissue defense under stress conditions such as OA.

Anti-inflammatory properties for HSPb5 were described through an extracellular mechanism. Indeed, sHSPs are primarily found in the nucleus and cytosol, similar to other chaperones. However, it has been described that HSPb5 may be secreted from cells via exosomes¹⁹², opening new avenues in our understanding of sHSPs. Indeed, Rothbard et al.¹⁹³ recently demonstrated that systemic administration of HSPb5 has a

therapeutic effect in autoimmune disease [the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis] by binding proinflammatory proteins, in particular acute-phase proteins such as complement factors. Given the increasing evidence for a role of complement factors in degenerative joint diseases⁹⁷, studying the interactions between complement and sHSPs may be an interesting avenue for future research, especially given the potential role of sHSP levels in joint diseases. Moreover, the authors reported temperature-dependent binding capacity, which may be extremely relevant at sites of inflammation. Apart from protein–protein interactions of sHSPs with inflammatory proteins, sHSPs such as HSPb8 and HSPb4 may also act as Toll-like receptor ligands¹⁹⁴. Here, the authors propose HSPb8/TLR4 signaling as a potential amplification loop in RA pathogenesis¹⁹⁴. In this context, Toll-like receptor (TLR) signaling is well known to be involved in the onset and pathogenesis of experimental arthritis^{195,196}.

Other HSPs

Several other proteins act as heat-inducible molecular chaperones but are not classified in any particular HSP families. For example, RA-related antigen 47 kDa (RA-A47; HSP47) acts as a collagen-specific molecular chaperone assisting in the maturation of collagens^{197,198}, a process that is crucial for normal cartilage development and endochondral bone formation¹⁹⁹. Indeed, a missense mutation in the gene encoding HSP47 is associated with severe osteogenesis imperfecta²⁰⁰. HSP47 has been identified as an antigen expressed by human

chondrocytes showing immunoreactivity with sera from RA patients²⁰¹. Interestingly, TNF- α and other proinflammatory cytokines caused down-regulation of HSP47 expression in chondrocytes and an altered subcellular localization (i.e. cell surface appearance), which may represent the mechanism for the recognition of RA-A47 as an autoantigen during RA²⁰².

HSP-family member	Biological effect
HSP90	
HSP90 (HSPC1 and HSPC3) TRAP1 gp96	Pleiotropic effects. Targeting HSP90 suppresses inflammation [24], reduces expression of matrix degrading enzymes [22] but also induces osteoclastogenesis [25, 26]. Promotes chondrocyte survival under hypoxic conditions [107]. Activates macrophages, suspected to contribute to chronicity of inflammation [28, 29]
HSP70	
HSP70 HSP72 GRP78	Extracellular HSP70 suppresses cytokine secretion from FLS[42]; involved in tolerance and autoimmune processes [33, 37]. Reduces synovial inflammation in animal models [43]. Reduces synovial proliferation [46], increases survival of chondrocytes [47] and promotes mineralization [48].
sHSP	
HSPb1 HSPb5 HSPB8 Other	Involved in cytokine expression in cartilage [71] Involved in regulation of cartilage metabolism [72] TLR ligand, may amplify inflammation responses [79]
HSP47	Involved in cartilage and bone development [84], may act as an auto-antigen in RA[86]

Clinical applications

As mentioned earlier, the primary research on HSPs has been done on the immunogenic role of HSP fragments and how these can modulate the immune system and contribute to inflammation. The working mechanism is based on the findings that HSPs may act as bystander antigens that can induce regulatory immune responses. A lot of pre-clinical work has been performed in animal models to induce tolerization, including work on GRP78²⁰³, HSP60²⁰⁴ and HSP10²⁰⁵. This has resulted in a phase II clinical trial with positive results in terms of the impact on inflammation¹⁷⁰. In another study the chaperonin HSP10, a reported inhibitor of TLR signaling, was administered and resulted in short-term suppression of symptoms in RA²⁰⁵.

In the cancer field a lot of research effort is put into developing HSP inhibitors. These inhibitors block the anti-apoptotic function of HSPs in tumor cells, thereby making the tumor more sensitive to other anti-cancer agents. Since inflammatory synovia are characterized by high levels of HSPs, such an approach may be useful to dampen synovial proliferation. However, as described earlier, HSPs may have protective effects under some conditions, or in some tissues promoting apoptosis may not be desirable (e.g. chondrocytes). Moreover, their ubiquitous expression and broad interaction profile hamper the development of therapeutic strategies that target HSPs. Limiting the side effects of such therapies remains a challenging task. Further insights into the mechanisms involved in HSP

regulation of joint homeostasis are needed to pave the way for future therapeutic applications.

From a diagnostic point of view, altered levels of HSPs¹⁸⁶ or antibodies to HSPs^{201,206-211} occur in several inflammatory joint diseases. Most of these studies compare RA with healthy subjects or non-inflammatory controls or report the presence of autoantibodies in other inflammatory joint diseases (AS, SLE, SS). Although this is interesting from a scientific point of view, it is less relevant in a clinical setting. Real diagnostic tests should be able to discriminate between inflammatory pathologies and be predictive for future disease activity or treatment response. For example, anti-GRP78 (anti-BiP) antibodies have been shown to be elevated in RA patients compared with other inflammatory diseases, healthy controls and non-inflammatory joint diseases^{163,212}. Interestingly, these antibodies appear several years before initial symptom presentation²¹². Currently, however, little or no information is available on the added value of these biomarkers compared with current clinical standards such as RF and anti-CCP tests. Such information is key to evaluating the clinical usefulness of these biomarkers to diagnose disease.

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2. Research objective

Osteoarthritis (OA) is the most prevalent musculoskeletal disease with a large socio-economic burden, as it is a major cause of disability. Currently, treatment options are still focussed mainly on pain relief by NSAIDs, opioids and corticosteroids. However these drugs do not mitigate disease progression. Chondroprotective drugs (e.g. hyaluronic acid, chondroitin sulphates) can also be used but eventually joint replacement is considered the end stage treatment option. The search for suitable targets and the development of disease modifying drugs has been the holy grail in OA research for many decades now. Despite increased understanding in the disease pathogenesis in the latest decennia, large parts of the disease mechanism remain unknown and no disease modifying drug has been developed yet. Overall the aim of this research project is twofold. Firstly, we aim to add evidence to the concept of OA as whole joint failure. On the other hand, we wish to investigate to what extent stress related protein families are differentially regulated in OA stromal joint tissue. Furthermore we want to address whether these families may provide possibilities for disease modifying therapies for OA.

The paradigm of OA as a whole joint organ failure has emerged in recent years¹. Apart from chondrocyte-populated articular cartilage which is a major player in OA mechanism, subchondral bone, synovium and meniscus have also been recognized as players involved in OA development¹⁻³. The acetabular labrum is a meniscus-like fibrocartilagenous structure in the hip joint. However, the role of the acetabular

labrum in OA is unclear. In chapter 1 we aim to further contribute to the whole joint concept by investigating if and how the metabolism of fibrochondrocytes from acetabular labrum is dysregulated in OA.

The joints, and especially weight bearing joints, are continually subjected to several types of stresses (e.g. shear, hydrostatic, biomechanical and oxidative stress). These stress events can trigger the activation of proinflammatory and catabolic signalling pathways, resulting in joint degradation⁴⁻¹⁰. Therefore, we aimed to investigate the potential role of a series of poorly studied stress related proteins in OA. We therefore focussed on two protein families, the short leucine rich repeat proteoglycans (SLRP) (Chapter 3.1) and the small heat shock proteins (sHsp) (Chapter 3.2).

The SLRP can function as DAMP and interact with the immune system¹¹⁻¹⁴, moreover they are an essential part of the normal cartilage ECM¹⁵. This makes them potential research targets in the context of OA and they have been investigated previously in OA cartilage¹⁶⁻¹⁸, however no data is available concerning their role in supporting fibrocartilagenous structures like the acetabular labrum. In light of OA as whole joint organ failure we wished to extend the knowledge on SLRP into this tissue and address to which extent SLRP are distinctly regulated in fibrochondrocytes compared to chondrocytes. Additionally we wished to examine the functional implication of SLRP dysregulation in OA fibrocartilage. Apart from the SLRP family,

we aimed to map the transcriptional profile of degenerated acetabular labrum compared to non-damaged tissue.

The sHsp are part of a larger family of heat shock proteins (HSP)^{19,20}. Some of the larger HSP have been investigated in rheumatic diseases, though they are known mostly for their role in the immune system and in the context of inflammatory arthritis²¹⁻²⁴. Even though sHsp have been implicated in numerous degenerative human diseases such as neurodegenerative disorders (e.g. Alzheimer's disease, Parkinson's disease) and cardiovascular disease, the role of sHsp in arthritis, and especially OA, remains under investigated²⁵⁻²⁹. Based on initial chondrocyte proteome studies^{30,31} performed by our research group, we aimed to further investigate the regulation and potential role of sHsp in OA development and progression by performing *in vitro* assays on human articular chondrocytes and *in vivo* studies in experimental OA models.

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3. Results

Chapter 3.1:

Distinct dysregulation of the small leucine-rich repeat protein (SLRP) family in osteoarthritic labrum compared to articular cartilage

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DE and SL shared the supervision of this work.

Juchtmans N *et al.* Distinct dysregulation of the small leucine-rich repeat protein family in osteoarthritic acetabular labrum compared to articular cartilage. *Arthritis Rheumatol.* 2015 Feb;**67**(2):435-41.

Abstract

Objective

Articular cartilage is well studied in osteoarthritis (OA). However, the role of supporting structures, such as the acetabular labrum, a sealing structure surrounding the hip joint, has been investigated much less. We recently showed that fibrochondrocytic labrum cells are metabolically active. This study was undertaken to investigate hip OA-associated changes in human acetabular labrum cells.

Methods

Microarray analysis was performed to compare OA labrum cells to healthy labrum cells cultured in a 3-dimensional alginate bead system. Data were analyzed by cluster analysis using gene set enrichment analysis software and by gene list analysis using PANTHER gene family tools. Selected candidates were validated by quantitative polymerase chain reaction analysis on labrum and cartilage samples and by immunohistochemistry. The functional impacts of the genes identified were investigated by in vitro stimulation experiments in labrum cells.

Results

Pathway analysis revealed increased cytokine and chemokine signaling in OA labrum cells, whereas reduced extracellular matrix interactions and transforming growth factor β signaling were observed. Several genes were significantly differentially expressed in OA compared to healthy labrum. We specifically focused on 3 small leucine-rich repeat proteins (SLRPs),

osteomodulin, osteoglycin, and asporin, that appeared to be distinctly regulated in OA labrum compared to OA cartilage. SLRPs were strongly down-regulated in OA labrum but up-regulated in OA articular chondrocytes. Moreover, in vitro stimulation with osteomodulin increased aggrecan expression in OA labrum cells.

Conclusion

OA labrum fibrochondrocytes have several features similar to OA chondrocytes. However, SLRP expression seems to be differentially influenced by degeneration in OA labrum compared to cartilage, suggesting a specific role for this supporting structure in OA. The functional impact of SLRPs on labrum cells makes them interesting targets for further studies in hip OA.

Osteoarthritis (OA) is characterized by a gradual progression of extracellular matrix (ECM) degradation resulting in structural and functional failure of synovial joints. In degenerative joint diseases, the balance between anabolic and catabolic responses of the chondrocyte is disturbed, causing a shift toward degeneration of the cartilage ECM ([1]). Although OA has traditionally been viewed as an articular cartilage disease, the current paradigm is that OA is a whole-joint disease involving not only the articular cartilage but also synovium, subchondral bone, or menisci ([2]). Whereas most studies to date have focused on the articular cartilage in OA, much less is known about supporting structures such as menisci and the acetabular labrum.

The biomechanical role of the labrum, a fibrocartilagenous structure sealing the hip joint, has been investigated ([3]), but we recently demonstrated that labrum cells are metabolically active fibrochondrocytes that produce and react to proinflammatory stimuli ([4]). Based on the knowledge that changes in chondrocyte metabolism drive cartilage degeneration, we conducted an exploratory genome-wide gene expression study to investigate gene expression changes associated with labrum degeneration in hip OA. Our goal was to investigate potential gene expression differences and analyze their functional importance in hip OA pathology. Several pathways were strongly activated during labrum degeneration. Of particular interest were members of the small leucine-rich repeat protein (SLRP) family that were found to be

functionally important for matrix synthesis by labrum fibrochondrocytes.

MATERIALS AND METHODS

Cell isolation and alginate culture

Acetabular labrum cells were obtained from control subjects without OA (n=3; with a mean \pm SD age of 28.3 ± 9.0 years) and OA patients (3 men and 2 women with a mean \pm SD age of 59.6 ± 7.8 years). The Ghent University Hospital Ethics Committee approved this study, and informed consent was obtained from all patients. Acetabular labrum cells were isolated as previously described ([4]). Briefly, labrum samples from OA patients were collected within 12 hours after total hip replacement surgery. Donor samples were obtained within 12 hours postmortem. Samples were diced into small fragments, and cells were isolated by enzymatic digestion with collagenase (Sigma-Aldrich) for 6 hours followed by 2 hours with Pronase E (Sigma-Aldrich). Trypan blue exclusion indicated that >90% of the cells were viable. Chondrocytes from healthy cartilage (n=3) and from degenerated cartilage from 4 men and 2 women with OA, with a mean \pm SD age of 68.2 ± 11.2 years, were isolated as previously described by sequential enzymatic digestion (with hyaluronidase, Pronase, and collagenase) ([4]). The articular chondrocytes were obtained from the knee, because it is extremely hard to obtain hip-derived chondrocytes, due to local regulatory issues in our tertiary center. Both cell types were immediately cultured in a 3-dimensional alginate bead system (~50,000 cells/bead) in

Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 units/ml penicillin, 10 mg/ml streptomycin, and 0.002M L-glutamine. Medium was replaced twice weekly. After 7 days in culture, a stable metabolism with restored ECM production was reached ([4]). After the culture period, cells were isolated and stored at -80°C until prepared for RNA extraction.

RNA extraction and microarray analysis

RNA was extracted using TRIzol (Invitrogen) and an RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA samples were submitted to the Nucleomics Core Facility (VIB). All samples fulfilled the preanalytic RNA quality control criteria (RNA integrity number >8 ; A260/A280 ratio >1.8). Samples were analyzed using the Affymetrix Human Gene 1.0 ST Array. Quality control analysis was performed at the VIB microarray facility, by displaying the distribution of the perfect match values for each hybridization to detect arrays with higher or lower overall intensities and by analyzing the number of present calls and average background and scaling factors. All arrays fulfilled the quality control criteria. Cluster analysis was performed using gene set enrichment analysis (GSEA) software version 2.013 (Broad Institute of MIT and Harvard) ([5]). GSEA (<http://www.broadinstitute.org/gsea>) and PANTHER 8.2 ([6]) (<http://www.pantherdb.org>), a gene family database, were used for gene list analysis. The data discussed in this publication have been deposited in the NCBI GEO database and are

accessible through GEO Series accession no. GSE60762 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60762>).

Quantitative polymerase chain reaction (qPCR) analysis

RNA was extracted from additional samples, unrelated to the microarray analysis samples (obtained from 4 subjects without OA with a mean \pm SD age of 31.5 ± 9.7 years and from 3 men and 5 women with OA with a mean \pm SD age of 63.4 ± 12.8 years) as described above. Genomic DNA removal and complementary DNA (cDNA) synthesis were performed using a QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative PCR analysis was performed using a LightCycler 480 real-time PCR system (Roche), and the data were analyzed using the $\Delta\Delta C_t$ method. Reactions were performed in duplicate. QuantiTect primer assay numbers are available upon request from the corresponding author. All primer sets were validated in-house for specificity, by melting curve analysis and efficiency.

Immunohistologic analysis

EDTA-decalcified paraffin sections of labrum tissue were used for immunostaining. Antigen retrieval was performed using protease type XIV, followed by 0.5% Triton permeabilization. Sections were blocked for 1 hour with 5% normal goat serum in 3% bovine serum albumin solution and incubated overnight at

4°C with anti-asperin antibody (ab58741), anti-osteomodulin antibody (ab154249), or anti-osteoglycin antibody (ab110558) (Abcam). Alexa 488-conjugated or Alexa 647-conjugated goat anti-rabbit IgG antibody (diluted 1:200; Invitrogen) was incubated for 1 hour, and sections were mounted with ProLong Gold DAPI (Molecular Probes). Sections were washed 3 times between incubation steps. Isotype controls were prepared and analyzed as negative controls. Staining was examined with a Leica TCS SP5 widefield microscope.

In vitro functional studies

Labrum samples were obtained from 6 patients with hip OA (2 men and 4 women with a mean \pm SD age of 63.6 ± 6.7 years) within 12 hours after total hip replacement surgery. Cells were isolated and cultured as described above. After 8 days of culture, alginate beads were transferred to 24-well plates (10 beads/well) in serum-free medium containing ITS+1 (insulin-transferrin-sodium selenite; Sigma-Aldrich). After 1 day, beads were stimulated with recombinant osteomodulin (1 ng/ml or 10 ng/ml) or vehicle for 24 hours followed by RNA extraction, cDNA synthesis, and qPCR analysis, performed as described above.

Statistical analysis

Statistical analysis was performed using SPSS 21. Gene expression data were tested using the nonparametric Mann-Whitney U test. Stimulation experiments were analyzed by

nonparametric Wilcoxon's matched pairs signed rank test. P values less than 0.05 were considered significant.

RESULTS

Genome-wide expression assay and pathway analysis indicate a specific expression pattern and signaling in degenerated labrum

The results of a previous study by our group indicated a labrum-specific cell metabolism compared to menisci and articular cartilage ([4]). To investigate gene expression changes associated with labrum degeneration, gene expression levels in cells isolated from 5 OA patients were compared to those in cells from 3 control subjects, using the Affymetrix Human Gene 1.0 ST Array. Quality control analysis was performed, and all arrays fulfilled the quality control criteria. Hierarchical cluster analysis performed on these data using GSEA software version 2.013 indicated 2 clear clusters, namely, the OA samples in one group and the healthy samples in a separate group, when considering the top 100 gene hits. GSEA pathway analysis showed several pathways to be significantly enriched in either healthy or OA labrum cells (Table 1). We estimated the relevance of the pathways identified by using an enrichment score, which reflects the degree of enrichment of a specific pathway, and by taking into account the nominal P value (estimating the statistical significance with a cutoff of 0.05) as well as the false discovery rate.

Table 1. Top 10 significantly differentially down-regulated and up-regulated pathways in OA versus healthy labrum cells, as determined by GSEA pathway analysis*

Pathway	Enriched condition	Enrichment score	Nominal <i>P</i>	FDR
Down-regulated				
KEGG_ECM receptor interaction†	Healthy	-0.572	<0.001	0.018
KEGG_Ribosome	Healthy	-0.515	0.002	0.17
KEGG_Tyrosine metabolism	Healthy	-0.579	0.013	0.14
KEGG_TGFβ signaling pathway†	Healthy	-0.481	0.012	0.288
KEGG_Fatty acid metabolism	Healthy	-0.538	0.015	0.255
KEGG_Cell cycle	Healthy	-0.455	0.005	0.245
KEGG_DNA replication	Healthy	-0.537	0.037	0.217
KEGG_PPAR signaling pathway	Healthy	-0.461	0.029	0.301
KEGG_Dilated cardiomyopathy	Healthy	-0.434	0.023	0.309
KEGG_Oocyte meiosis	Healthy	-0.420	0.013	0.273
Up-regulated				
KEGG_NOD-like receptor signaling pathway	OA	0.776	<0.001	<0.001
KEGG_Cytosolic DNA sensing pathway	OA	0.745	<0.001	<0.001
KEGG_Cytokine-cytokine receptor interaction†	OA	0.582	<0.001	<0.001
KEGG_Intestinal immune network for IgA production	OA	0.748	<0.001	<0.001
KEGG_Chemokine signaling pathway†	OA	0.601	<0.001	<0.001
KEGG_Graft-versus-host disease	OA	0.800	<0.001	<0.001
KEGG_Type 1 diabetes mellitus	OA	0.76	<0.001	<0.001
KEGG_Viral myocarditis	OA	0.656	<0.001	<0.001
KEGG_Allograft rejection	OA	0.758	<0.001	<0.001
KEGG_Hematopoietic cell lineage	OA	0.619	<0.001	<0.001

* The enrichment score reflects the degree of overrepresentation, the nominal *P* value estimates the statistical significance of the given enrichment score, and the false discovery rate (FDR) estimates the probability of a false-positive finding. OA= osteoarthritis; GSEA = gene set enrichment analysis; KEGG = Kyoto Encyclopedia of Genes and Genomes; ECM = extracellular matrix; TGFβ = transforming growth factor β; PPAR = peroxisome proliferator-activated receptor.
† Pathways that were further investigated.

Four pathways were of special interest. Cytokine receptor interaction and chemokine signaling were enriched in OA cells. In contrast, transforming growth factor β (TGFβ) signaling and ECM receptor interaction, interactions between ECM components and transmembrane receptors such as integrins and proteoglycans, were enriched in healthy cells (Figure 1A). The enriched ECM receptor signaling is consistent with known changes in ECM, such as ECM degradation in OA samples. Interestingly, the genes that contributed most to TGFβ enrichment include thrombospondins, TGFβ3, TGFβ receptor type II (TGFβRII), and Smad6, a specific inhibitor of Smad1/5/8 signaling, which was further confirmed by qPCR (Figure 1B).

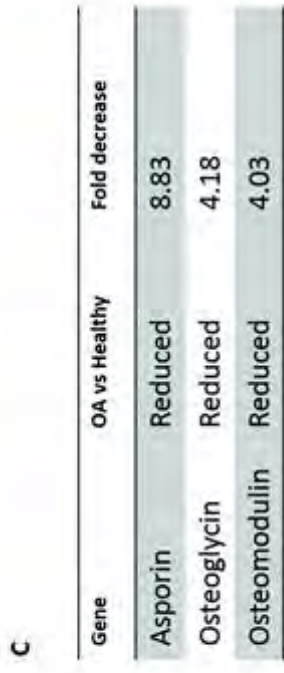
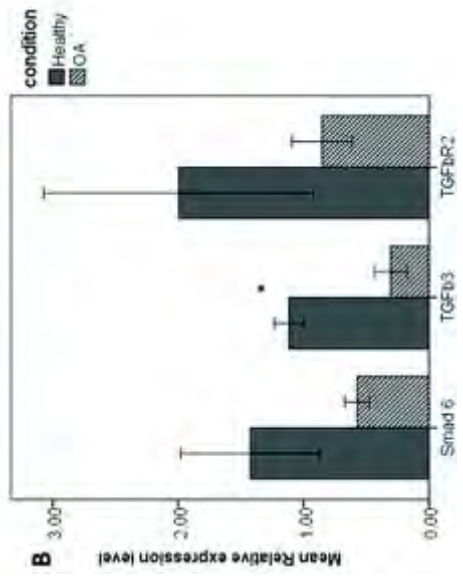
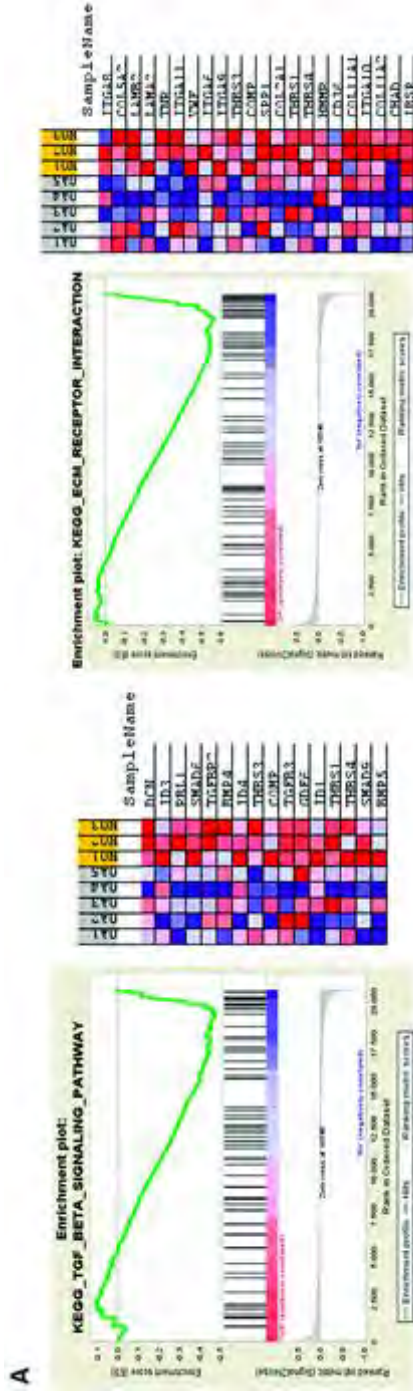


Figure 1. Distorted transforming growth factor β (TGF β) pathways in osteoarthritic (OA) labrum. A, Enrichment plots determined by gene set enrichment analysis (GSEA) software, indicating enrichment of the TGF β signaling pathway ($P < 0.012$; false discovery rate [FDR] 0.288) (left) and extracellular matrix (ECM) receptor interaction pathway ($P < 0.001$; FDR 0.018) (right) in OA labrum compared to healthy labrum. Lower plot visualises signal/noise ratio. The enrichment score reflects the degree of overrepresentation, the nominal P value estimates the statistical significance of the given enrichment score, and the FDR estimates the probability of a false-positive finding. Upper portion of the plot depicts the running ES for the gene set walking down the ranked list. The peak value is the ES for the gene set. The middle plot indicates where the overrepresented genes appear in the ranked list. The bottom portion indicates the signal to noise ratio. Heatmaps were generated by GSEA software and indicate the genes that contributed most to the enrichment results. Red indicates high expression; blue indicates low expression. KEGG = Kyoto Encyclopedia of Genes and Genomes. B, Quantitative polymerase chain reaction analysis of additional labrum samples. Reduced expression of core contributors in OA labrum cells was confirmed ($P = 0.015$ for TGF β 3; $P = 0.065$ for Smad6). Values are the mean relative expression as calculated by the $\Delta\Delta C_t$ method, normalized to housekeeping genes and compared to the reference sample. Bars show the mean \pm SEM ($n = 6$ OA samples and 3 healthy samples). * = $P < 0.05$. TGF β R2 = TGF β receptor type II. C, Reduced expression of multiple small leucine-rich repeat proteins in OA labrum compared to healthy labrum, as determined by microarray analysis.

Functional reduction in SLRP expression in OA labrum

Closer inspection of the array revealed 3 members of the SLRP family, osteomodulin, osteoglycin, and asporin, to be severely down-regulated in OA compared to healthy labrum. Since some SLRPs are known to bind TGF β and influence its signaling, they were further investigated. Osteomodulin, osteoglycin, and asporin were confirmed by qPCR assays on additional samples to be strongly down-regulated in OA compared to healthy labrum-derived fibrochondrocytes. The observed 10–24-fold down-regulation was statistically significant for each of them ($P = 0.016$ for osteomodulin, $P = 0.011$ for osteoglycin, and $P = 0.007$ for asporin) (Figure 2A). This reduction was shown to be driven by interleukin-1 β (IL-1 β) (Nakajima M, et al: unpublished observations). However, in striking contrast, osteomodulin ($P =$

0.032), osteoglycin ($P = 0.016$), and asporin ($P = 0.063$) expression levels were up-regulated in OA compared to healthy knee-derived chondrocytes (Figure 2A).

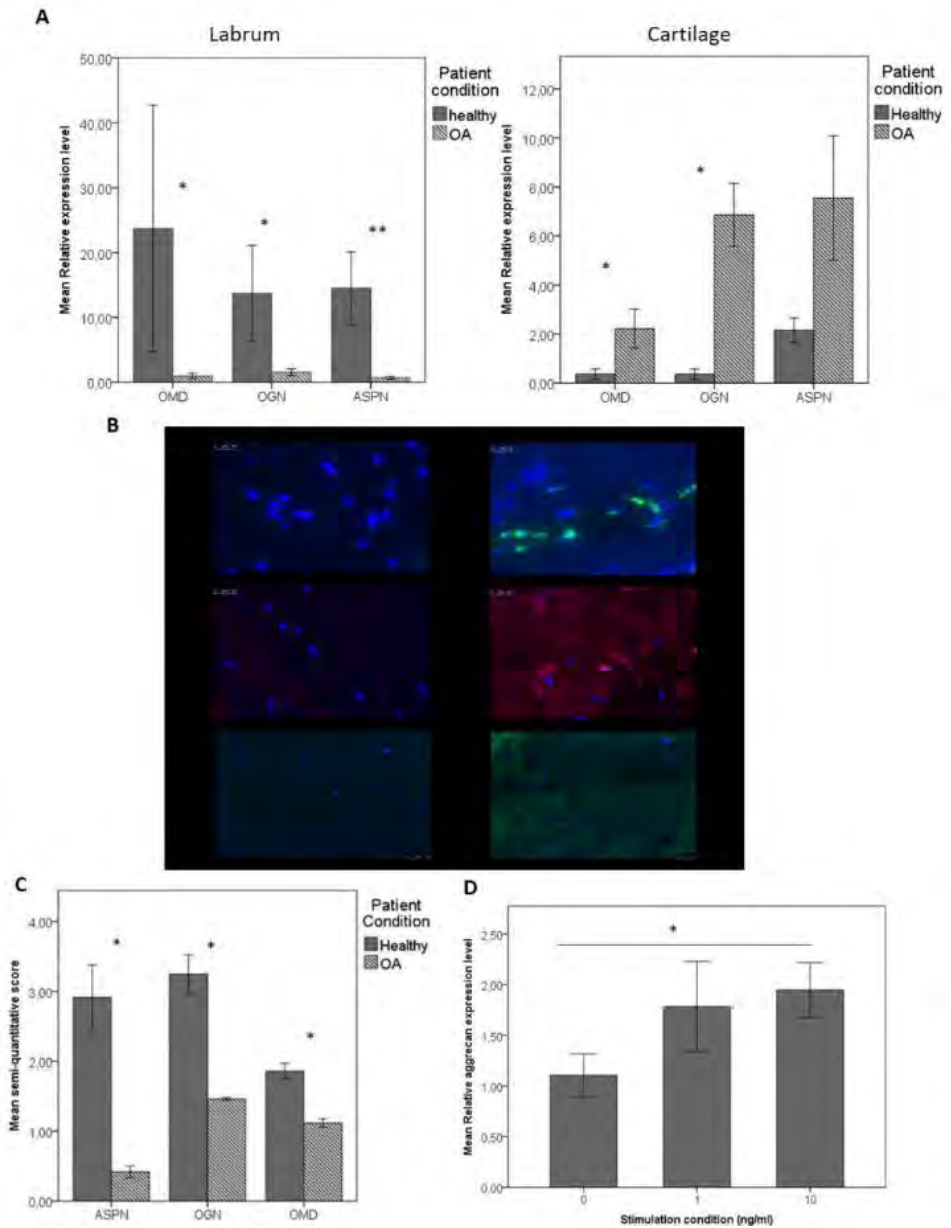


Figure 2. Dysregulation of small leucine-rich repeat proteins (SLRPs) in osteoarthritic (OA) labrum. A, Significant down-regulation of all 3 SLRPs (osteomodulin [OMD], osteoglycin [OGN], and asporin [APSN]) in OA labrum-derived fibrochondrocytes (n = 8) compared to healthy labrum-derived cells (n = 4) (left) and up-regulation of SLRPs in OA knee-derived chondrocytes (n = 5) compared to healthy knee-derived chondrocytes (n = 4) (right), as determined by quantitative polymerase chain reaction. B, Representative images of immunohistochemical staining for asporin, osteoglycin, and osteomodulin. Protein abundance was reduced in OA labrum compared to healthy labrum. Original magnification $\times 40$. C, Scoring of stained tissue. Samples were scored independently by 3 investigators in a blinded manner, on a scale from 0 to 4 for immunofluorescent intensity. D, Increased aggrecan expression in labrum cells (n = 6) stimulated with osteomodulin (1 ng/ml or 10 ng/ml). In A, C, and D, bars show the mean \pm SEM. Values are the mean relative expression as calculated by the $\Delta\Delta C_t$ method, normalized to housekeeping genes and compared to the reference sample. * = $P < 0.05$; ** = $P < 0.01$.

Furthermore, immunohistochemical staining confirmed that the down-regulated expression in OA labrum cells was associated with a reduced protein abundance of osteomodulin, osteoglycin, and asporin (in 3–4 OA samples and 2 healthy control samples) (Figures 2B and C). To investigate the functional impact of these SLRPs in labrum, qPCR was used to analyze the expression profile of labrum cells obtained from 6 patients with OA and separately stimulated with osteomodulin (1 ng/ml or 10 ng/ml) or vehicle. This analysis indicated a dose-dependent increase in aggrecan expression (Figure 2D).

Discussion

This study compared gene expression patterns in healthy and degenerated labrum cells, and the results clearly indicate a distinct gene expression pattern in degenerated compared to healthy labrum cells. Due to the extensive ECM in which chondrocytes are embedded, the number of cells that can be isolated is too limited to allow extraction of the high-quality RNA necessary for microarray studies. However, the alginate system

used to culture the isolated chondrocytes is known to maintain a stable chondrocyte phenotype ([7]). A clearly distinct gene expression pattern was found. The enrichment in cytokine and chemokine signaling indicates the presence of a proinflammatory milieu in degenerated OA labrum, which is consistent with the proinflammatory environment that has been shown to be present in OA cartilage ([8]). Moreover, pathway analysis indicated that ECM receptor binding was reduced in OA labrum cells, which is also consistent with the available data that indicate a disturbed ECM homeostasis in articular cartilage during OA ([1, 8]). These results indicate that some of the processes that occur in degenerated OA chondrocytes are similar to those in OA labrum fibrochondrocytes.

Similar to articular cartilage, the TGF β signaling pathway is disturbed in degenerated OA labrum cells. The role of TGF β in OA, however, is not straightforward ([9]). Canonical TGF β signaling via Smad2/3 pathways plays a role in ECM synthesis. TGF β has also been shown to be involved in osteophyte formation, chondrocyte differentiation, and hypertrophy ([9, 10]). Traditionally, the Smad1/5/8 pathway has been viewed as bone morphogenetic protein-dependent signaling, but recently it has been pointed out that TGF β is able to signal via both the Smad2/3 and Smad1/5/8 pathways in chondrocytes ([11]).

Interestingly, in this study, down-regulated expression of Smad6, the specific Smad1/5/8 inhibitor, was observed, and it was confirmed in labrum cells. Since Smad6 is a specific inhibitor of the Smad1/5/8 pathway and has no inhibitory influence on the Smad2/3 pathway induced by TGF β ([12]), the

reduced expression of Smad6 in OA labrum cells points to a shift in TGF β signaling from Smad2/3 in healthy labrum cells toward the catabolic, hypertrophic Smad1/5/8 in OA labrum cells. Furthermore, apart from the shift in TGF β signaling, a reduced sensitivity of the labrum fibrochondrocyte to canonical TGF β signaling is confirmed by the reduced expression of TGF β RII ([10]). It has been observed that in chondrocytes, proinflammatory cytokines such as IL-1 β can induce this shift in TGF β signaling via reduced TGF β RII expression ([10]). Given the proinflammatory environment that labrum cells are exposed to during OA, IL-1 β signaling may be involved in the TGF β disturbance observed in labrum cells. Other core contributors, such as TGF β 3, which is known to stimulate ECM synthesis, and thrombospondin 1, which activates latent TGF β ([13]), were down-regulated in OA labrum, further pointing to a reduced availability of active anabolic TGF β ligands.

The labrum ECM consists mostly of a collagenous component (mainly type I collagen) and proteoglycans (mainly aggrecan, but also SLRPs). SLRPs are known to interact with collagen fibrils, thereby partially protecting them against degradation by steric hindrance from collagenases ([14]). Furthermore, some of the SLRPs, including osteomodulin, can bind integrins ([15]), and most of them interact with growth factors, such as TGF β ([16]). As such, the SLRPs are not only a structural component of the ECM but also function in cell membrane interactions, modulating signaling pathways. In our experiments, no direct involvement of osteomodulin in the regulation of TGF β signaling could be shown (Juchtmans N, et al: unpublished observations);

however, osteomodulin seemed to be a downstream regulator of altered TGF β signaling. These findings are consistent with those of previous studies indicating induced osteomodulin expression by TGF β signaling in odontoblasts and pulpal fibroblasts ([17]). In the current study, osteomodulin, osteoglycin, and asporin, 3 chromosomally clustered SLRPs ([18]), were shown to be strongly reduced in OA labrum cells. In contrast, SLRP expression was increased in OA chondrocytes (Figure 2). This clearly indicates a distinct SLRP response in degenerated labrum fibrochondrocytes compared to cartilage chondrocytes. Moreover, the induction of aggrecan expression by in vitro stimulation of labrum cells with osteomodulin suggests that the observed reduction in SLRP abundance is functionally implicated in the observed disturbed synthesis of ECM proteins, namely, aggrecan, by OA labrum fibrochondrocytes. This indicates a novel, possibly anticatabolic, role for osteomodulin in labrum cell ECM homeostasis.

Taken together, our results indicate that in addition to chondrocytes of the connective tissue, fibrochondrocytes of the supporting labrum structure are actively involved in degenerative OA. Although similarities with OA cartilage, such as enrichment of cytokine signaling, were found, some features, such as osteomodulin, osteoglycin, and asporin expression, were found to be oppositely influenced in labrum compared to cartilage, suggesting a specific role for these supporting structures in degenerative OA. Additionally, the reduction was shown to be of functional importance in labrum, pointing to a novel set of regulators implicated in the pathogenesis of hip OA.

Authors contributions

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

Study conception and design. Juchtmans, Dhollander, Lambrecht, Elewaut.

Acquisition of data. Juchtmans, Dhollander, Coudenys, Audenaert, Pattyn.

Analysis and interpretation of data. Juchtmans, Dhollander, Coudenys, Lambrecht, Elewaut.

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Chapter 3.2:

Downregulation of small heat shock proteins in articular cartilage: a novel protective mechanism against osteoarthritis

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DE and SL shared the supervision of this work

Submitted to Annals of the Rheumatic diseases

ABSTRACT

Objectives: To investigate the role of small heat shock proteins (sHsp) in cartilage and fibrocartilage from osteoarthritis and to assess their *in vivo* role in joint degradation and remodeling.

Methods: Human (fibro)chondrocytes were isolated for mRNA expression analysis of sHsp in steady state and upon exposure to IL-1 β . Mice deficient in both HspB2 and HspB5 were compared to controls for cartilage degradation and osteophyte formation in two different models: collagen antibody induced arthritis (CAIA) and destabilization of medial meniscus (DMM).

Results: We found HspB1, HspB2, HspB5 and HspB6, but not Hsp8, to be downregulated in OA chondrocytes and fibrochondrocytes, which could be mimicked by exposure to IL-1 β . In the absence of HspB2/HspB5, a remarkable *in vivo* protection against cartilage degradation and joint remodeling was observed in CAIA and DMM.

Conclusion: Our data are consistent with a model whereby reduction of a particular pattern of sHsp protects against OA development by modulating cartilage and fibrocartilage homeostasis.

Keywords: Osteoarthritis, Cartilage degradation, Small heat shock proteins

Osteoarthritis (OA), is the most prevalent musculoskeletal disease,^{1,2}. However, effective disease modifying drugs are currently lacking. Heat shock proteins (Hsp), originally identified as stress-inducible proteins, have been suggested to play a role apart from stress defense.³⁻⁵. Little is known on the role of these proteins in joint supporting structures such as cartilage and fibrocartilage. Initial studies concerning the small heat shock proteins (sHsp) and their involvement in cellular pathways suggests they might have a role in arthritis and other degenerative diseases.⁶⁻⁹ In this study we aimed to investigate their role in joint homeostasis *in vitro* and *in vivo*. Our data highlight a particular pattern of sHsp to be deregulated in OA cartilage and fibrocartilage. We also show this downregulation protects against further development of OA.

METHODS

Cell cultures

Human chondrocytes [(68.2 (11.2) years; n=6)] and fibrochondrocytes [(59.6 (7.8) years)] were isolated by sequential enzymatic digestion, as previously described.^{8,10} Chondrocytes were isolated from undamaged and OA degenerated articular cartilage from knee tissue, fibrochondrocytes were obtained from OA degenerated labrum (n=5) or healthy tissue (n=4). Isolation occurred within 12 hours after joint replacement surgery or postmortem. Cells (50 000 cells/bead) were cultured in a 3D alginate beads system in Dulbecco's Modified Eagle's

Medium supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin and 0.002M L- glutamine. The alginate beads system is created by dropping cell suspension containing 2% alginate in a 102mM CaCl₂ solution. The Ghent University Hospital Ethics Committee approved these studies. Informed consent was obtained from all patients. After culture period, or stimulation experiments, cells were isolated and stored at -80°C until prepared for RNA extraction.

RNA extraction and qPCR

RNA was extracted using Trizol (Invitrogen) followed by RNeasy mini-kit (Qiagen). Genomic DNA removal and cDNA synthesis was performed using the Quantitect reverse transcription kit (Qiagen) according to manufacturer's instructions. qPCR analysis was performed using the LightCycler 480 Real-Time-PCR system (Roche) and analyzed using the $\Delta\Delta C_t$ method. Reactions were performed in duplicate. Primer assay numbers or sequences are available upon request from the corresponding author. All primersets were validated in house for specificity, by melting curve analysis, and efficiency.

Mice models

Double knock out mice were obtained thanks to, Prof Wawrousek from the National Eye Institute, NIH, Maryland, USA.¹¹ For induction of these arthritis models HspB2/HspB5 wild type and knockout mice were anesthetized with 100 μ l/mice intraperitoneal injection of ketamine (10ng/ml) and xylazine (10mg/ml). Destabilization of the medial meniscus (DMM) was

performed in 8 week old mice, as previously described¹². Briefly, the medial menisco tibial ligament was transected, for sham surgery the ligament was exposed and touched. Buprenorphine (0.05mg/ml; 50µl/mice) was injected intramuscular after surgery. Serum samples were obtained at several time points. Mice were sacrificed 20 weeks after surgery. Hind paws were isolated for histology. Collagen antibody induced arthritis (CAIA) was induced as previously described.¹³ Briefly, mice were IV injected with 120µl antibody cocktail under general anesthesia, after 3 days 150µl LPS was injected IP. Serum samples were collected at several time points. Mice were sacrificed after 18 days. Paws were isolated for histology. CTX-II elisa (IDS) was performed in duplo on serum samples according to manufacturer's instructions. CAIA is typically used as an animal model for rheumatoid arthritis. However when the duration of the model is extended by allowing a longer recovery phase after inflammation, a remodeling component can be observed which makes this model more broadly applicable.

Histology

Paws and knee joints were fixed in 4% formaldehyde, decalcified in 0.5M ethylenediamine tetraacetic acid at 4°. Paraffin sections were stained with safranin-O. Paws were evaluated independently by three blinded assessors, and scored on three parameters, proteoglycan loss, cartilage degradation and new bone formation using a semi-quantitative grading system.

Statistical Analysis

Statistical analysis was performed using SPSS 21. Independent samples were tested using non-parametric Mann-Whitney U test or independent t test. Paired samples were analyzed by non-parametric Wilcoxon signed ranked test. p-values less than 0.05 were considered significant.

RESULTS

HspB1, HspB2, HspB5, HspB6 though not HspB8 are dysregulated in osteoarthritic joint cells

qPCR expression analysis indicates significant lower expression of HspB1, HspB2, HspB5 and HspB6 but not HspB8 in OA damaged cartilage compared to chondrocytes from undamaged cartilage zones in the same patients (Fig 1 upper left) or intact cartilage from healthy donors (data not shown). Moreover IL-1 β , a key proinflammatory cytokine in osteoarthritis, induces a significant downregulation of these four sHsp (HspB1, HspB2, HspB5 and HspB6), but not HspB8 in chondrocytes (Fig 1, bottom left).

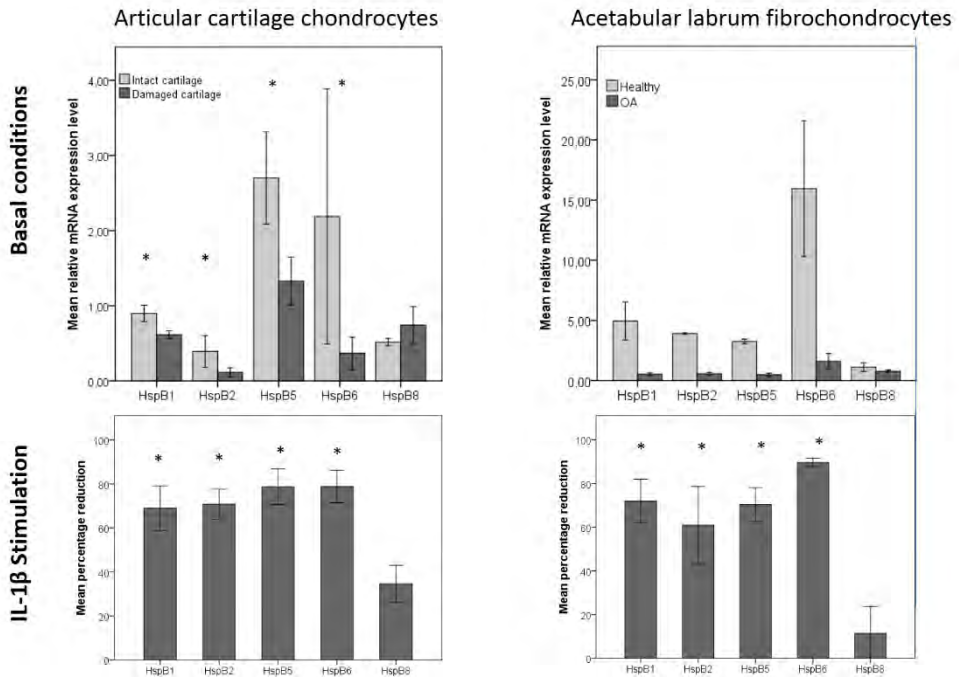


Figure 1: Downregulation of sHspB1, B2, B5, B6 but not B8 in human OA chondrocytes. Bar charts + SEM. $*=p<0,05$. mRNA levels have been calculated as the relative expression compared to three different household genes. **A:** Upper: comparing the expression levels of HspB1, HspB2, HspB5, HspB6 and HspB8 in chondrocytes isolated from visually intact zones and damaged zones of the same knee joint from 4-6 different patients. In all patients expression levels of HspB1 ($p=0,039$), HspB2 ($p=0,043$), HspB5 ($p=0,043$), and HspB6 ($p=0,043$), were significantly lower in damaged compared to intact cartilage samples. HspB8 expression was not altered. Lower: mRNA levels of HspB1 ($p=0,039$), HspB2 ($p=0,043$), HspB5 ($p=0,018$), HspB6 ($p=0,018$), but not significantly for HspB8 ($p=0,068$), were reduced significantly in IL-1 β stimulated chondrocytes compared to unstimulated chondrocytes. p values were calculated by Wilcoxon signed ranked test. **B:** Upper: comparing the expression levels of HspB1, HspB2, HspB5, HspB6 and HspB8 in fibrochondrocytes isolated from acetabular labrum form OA patients ($n=7$) and healthy donors ($n=5$). Expression levels of HspB1 ($p=0.053$), HspB2 ($p=0.053$), HspB5(0.053) and HspB6(0.053) were lower in OA compared to healthy samples. No difference in expression levels for HspB8 could be observed. p values were calculated by Mann Whitney U test. Lower: mRNA levels of HspB1($p=0.031$), HspB2 ($p=0.031$), HspB5($p=0.031$), and HspB6($p=0.031$), were significantly reduced in IL-1 β stimulated fibrochondrocytes compared to unstimulated fibrochondrocytes. HspB8 expression remained unaltered. p values were calculated by Wilcoxon signed ranked test.

Interestingly, qPCR analysis on acetabular labrum fibrochondrocytes isolated from healthy and OA patients demonstrates the same sHsp expression pattern (HspB1, HspB2, HspB5, HspB6 but not HspB8) as well as an IL-1 β dependent downregulation of these sHsp in fibrochondrocytes (Fig 1, right panel)

Absence of HspB2 and HspB5 protects against joint destruction in experimental OA models

To investigate the potential influence of the observed reduced sHsp expression in OA-patients, double knockout mice deficient for HspB2 and HspB5 were used as a model for partial sHsp reduction, in two animal models. When subjected to the inflammatory collagen antibody induced arthritis (CAIA) no differences could be observed in clinical arthritis score (Fig 2a) between double knockout (HspB2/B5^{-/-}) and wild type (HspB2/B5^{+/+}) littermate controls which suggests HspB2 and HspB5 have no effect on inflammation in this arthritis model, as also indicated by equal serum levels of IL-6 and MMP-3 (data not shown). At endpoint no difference in histological inflammation was observed. Interestingly, histological analysis showed significantly ($p= 0.002$) less amounts of proteoglycan depletion (Fig 2c,d). Moreover, significant reduced serum levels of collagen type II fragments (CTX-II) ($p=0.008$) (Fig 2b) were observed in double knockout mice, indicating these mice are protected against inflammation induced cartilage damage.

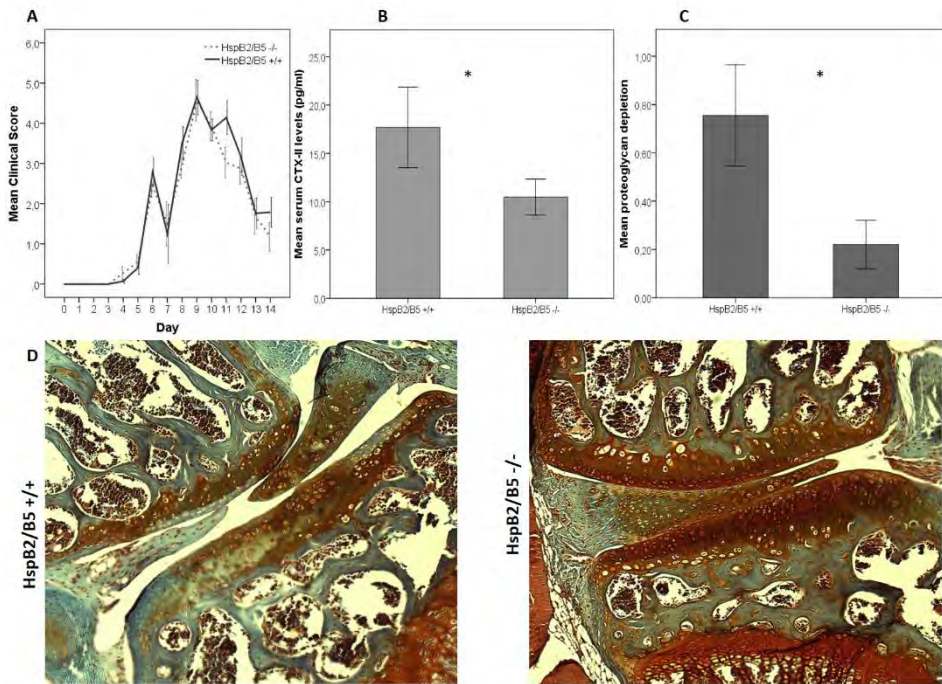


Figure 2: HspB2/B5^{-/-} are protected against CAIA. **A:** No difference in clinical arthritis score (left) was observed in HspB2/HspB5^{-/-} (n=20) compared to wild type littermate controls (n=21). Mice were scored daily, independently by two blinded persons. **B:** CTX-II serum levels at endpoint (18 days after arthritis induction) were lower in HspB2/HspB5^{-/-} mice versus controls (p=0.008). **C:** Histological scoring (0= no discoloring; 0,5= single site discoloring; 1= clear discoloring at 2 sites; 2= broad discoloring at several sites) indicates a reduced degree of proteoglycan depletion (p=0.002) in HspB2/HspB5^{-/-} compared to controls. **D:** Representative images of proteoglycan destruction as observed in Safranin O stained slices of WT (left) and HspB2/HspB5^{-/-} (right) mice. Safranin O stained slices were scored blinded by three independent researchers. p values were calculated by independent sample t test.

To confirm the role of sHsp in cartilage degradation, double knockout mice were subjected to DMM, a non-inflammatory, trauma induced OA model. Histologically, both HspB2/B5^{+/+} and HspB2/B5^{-/-} mice develop a severe degree of OA induced cartilage degradation at endpoint, whereas no damage could be observed in sham operated mice. Interestingly, osteophyte

formation, occurring in later stages of the OA pathology, was markedly reduced in the absence of HspB2/B5, as shown by histological safranin O staining images (Fig 3a).

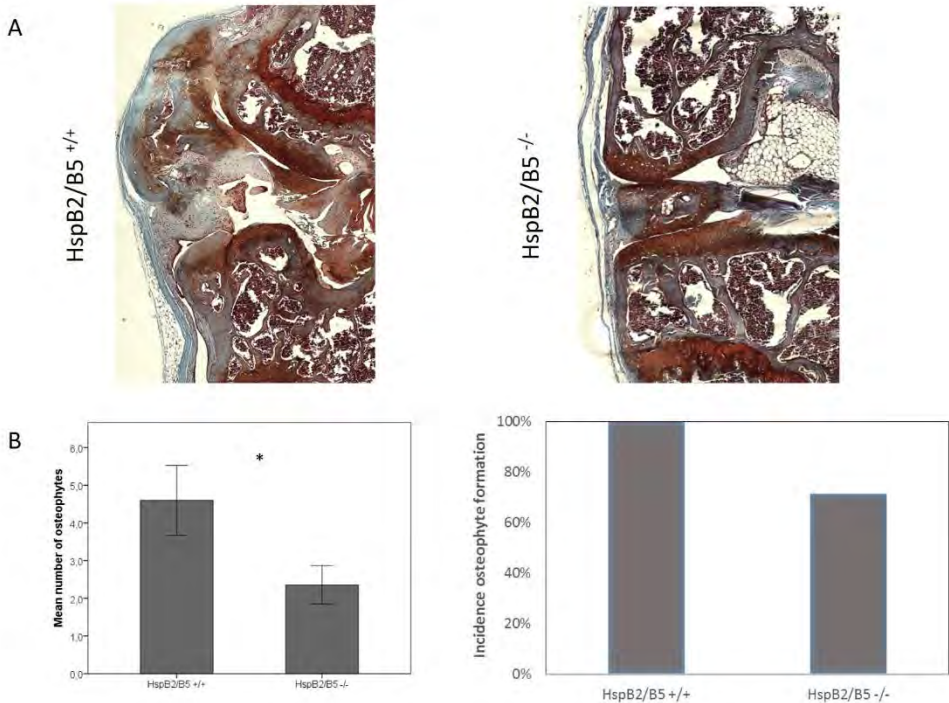


Figure 3: Reduced osteophyte formation in the absence of HspB2 and B5. A: Representative images of osteophyte bone new formation destruction in DMM model using Safranin O stained slices of HspB2/B5^{+/+} (left) and HspB2/HspB5^{-/-} (right) mice **B:** Histological scoring indicates a significant reduced number of osteophytes (p=0.048) in HspB2/HspB5 double knock out mice (n=7) compared to wild type littermate controls (n=6) (left) as well as reduced incidence of bone new formation (right) in HspB2/HspB5 double knock out mice compared to wild type littermate controls (lower) Safranin O stained slices were scored blinded by 3 independent researchers based upon the Glasson scoring system. p values were calculated by Mann Whitney U test

Further quantification indicated a significant reduction in the number of osteophytes (p= 0.048) (Fig 3b) as well as a reduced incidence of new bone formation in HspB2/B5 knockout mice

compared to wild type littermate controls (Fig 3c). These findings indicate that HspB2/B5^{-/-} mice are protected from experimental OA.

DISCUSSION

The small heat-shock proteins (sHsp), consist of 10 members several of which are ubiquitously expressed.^{14,15} Even though some sHsp have been implicated in human disease, their *in vivo* role in arthritis and joint degradation remained to be investigated^{3,16-18}. Our goal was to unravel their regulation during OA and their potential role in joint degradation. We found a particular panel of sHsp to be downregulated in OA cartilage and fibrocartilage and most importantly that deficiency in HSPB2/B5 is associated with a markedly reduced cartilage degradation *in vivo*. This suggest these sHSP are part of a negative regulatory mechanism prohibiting damage and ultimate joint failure.

We investigated the expression pattern of the most prominent members of the sHsp family (HspB1, HspB2, HspB5, HspB6 and HspB8) in (fibro)cartilage.^{14,15,19} The distinct expression profile between HSPb8 and HspB1, HspB2, HspB5 and HspB6, suggests that the latter group plays a similar role in the cellular metabolism and may share a common transcription factor. Bioinformatics as well as *in vitro* experiments point to C/EBP β as a common promoting transcription factor for these sHsp (see supplemental figure 1). *In vitro* experiments with C/EBP β inhibitor molecule, however, indicated C/EBP β is not

indispensable for their regulation (suppl Fig 1), which suggests co-regulatory pathways may exist.

To investigate a possible role for the reduced sHsp abundance in OA, double knockout mice deficient for HspB2 and HspB5 were used as an *in vivo* model.¹¹ These mice show a strikingly protective phenotype when subjected to arthritis mice models. The HspB2/B5^{-/-} mice were markedly protected from cartilage degradation in inflammatory arthritis. Moreover in DMM, an aggressive, end-stage trauma-induced OA model, a reduced new bone formation by osteophytes was apparent in the absence of HspB2 and HspB5. A likely explanation for the observed reduction in bone remodeling is that cartilage degradation is delayed in these double deficient mice, as observed in the CAIA model, which would result in a more extensive bone remodeling at the end of the DMM model in the wild type mice because of the earlier onset of joint destruction. Alternatively, the sHSP have no effect on cartilage degradation and proteoglycan depletion in DMM but reduces bone new formation by directly interfering with osteophyte formation and pathways of bone new formation, independently of cartilage damage. Indeed, our group showed earlier that BMP-2 expression, which is strongly implicated in OA osteophyte formation²⁰ and terminal chondrocyte differentiation, was downregulated in HspB5 deficient chondrocytes.⁷ Further experiments studying the progress of proteoglycan depletion and cartilage degradation in these mice during DMM will be performed to address these questions.

Altogether our results point to a specific sHsp group (HspB1, HspB2, HspB5 and HspB6) that is consistently downregulated in the OA cartilage and fibrocartilage. Furthermore, the findings suggest this dysregulation is part of a novel protective feedback mechanism against cartilage damage and joint failure.

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Supplementary Data

METHODS

Bioinformatics

For identification of transcription factors a combination of different bioinformatics tools were used. TFdiff ENCODE (<http://bioit2.irc.ugent.be/tfdiff/encode/>) takes as input a set of putative functionally related genes and identifies the transcription factors with the highest regulatory potential for this set of genes based on both over-representation and association of ENCODE TF ChIP-Seq peak regions linked to their target genes. TFdiff ENCODE Single Gene Regulator Analysis (<http://bioit2.irc.ugent.be/tyrant/>) identifies the transcription factors having the highest regulatory potential for a gene of interest and PhysBinder (<http://bioit.dnbr.ugent.be/physbinder/>) predicts transcription factors binding sites based on motif sequence as well as biophysical DNA properties and DNA-protein interactions²¹.

Cell cultures and expression analysis

Human synovial fibroblasts were isolated by enzymatic digestion (dispase 2mg/ml) of synovial tissue in Dulbecco's modified eagle's medium supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin. 24 hours before RNA isolations cells were stimulated with 0.1 ng/ml IL-1 β or vehicle. For C/EBP β inhibition experiments synovial fibroblasts were treated for 1 hour with p38 inhibitor

SB203580 or vehicle, followed by 4 hours stimulation with or without 0.1ng/ml IL-1 β . Afterwards RNA isolation was performed by the RNeasy mini-kit (Qiagen), according to manufacturer's instructions. Genomic DNA removal and cDNA synthesis was performed using the Quantitect reverse transcription kit (Qiagen) according to manufacturer's instructions. qPCR analysis was performed using the LightCycler 480 Real-Time-PCR system (Roche) and analyzed using the $\Delta\Delta$ Ct method. Reactions were performed in duplicate. Primer assays assay numbers or sequences are available upon request from the corresponding author. All primer sets were validated in house for specificity, by melting curve analysis, and efficiency.

RESULTS

C/EBP β as common transcription factor for sHsp panel

In order to provide insights in the potential molecular mechanisms involved in the role of sHSPs, common transcription factors were screened by TfDiff Encode. The analysis was performed against a background of 30 unrelated genes and a background of unrelated sHsp family members. This TfDiff Encode output was entered in TfDiff Encode Single Gene Regulator Analysis to identify which of the common transcription factors regulates these sHsp. CCAAT/enhancer-binding protein beta (C/EBP β) was predicted to regulate all four sHsp, furthermore apart from HspB4 (Cryaa), which as expression restricted to the eye lens, none of the other sHsp

family members were predicted to be regulated by C/EBP β . In order to get further support for the role of C/EBP β in the regulation of the sHsp, we also used a different method relying on a completely different algorithm for the prediction of transcription factor binding sites. In contrast to other state-of-the-art methods, PhysBinder makes use of both direct (the sequence) and several indirect readout features of protein-DNA complexes (biophysical properties such as bendability or the solvent-excluded surface of the DNA). PhysBinder has been shown to make predictions with both high specificity and sensitivity(21). The PhysBinder algorithm predicted 3, 2, 4 and 1 C/EBP β binding places for respectively HspB1, HspB2, HspB5, HspB6 (Fig 4a). These results suggests C/EBP β as a common regulatory transcription factor for this specific sHsp panel. Additionally, qPCR analysis of unstimulated as well as Il-1 β stimulated chondrocytes revealed a significant positive correlation of C/EBP β expression levels with expression levels of HspB1, HspB2, HspB5, HspB6 but not HspB8 in both stimulated and unstimulated conditions (Table 1), further supporting a role for C/EBP β as a promoting transcription factor for this sHsp panel.

Table 1: Pearson correlation coefficients show a positive correlation between C/EBP β and sHsp expression. Statistical significance is indicated in the table. mRNA levels have been calculated as the relative expression compared to three different household genes

		HspB1	HspB2	HspB5	HspB6	HspB8
<i>Unstimulated</i>	Correlation index	0.863	0.760	0.889	0.567	-0.139
	p-value	0.006	0.029	0.003	0.142	0.742
<i>IL-1β stimulated</i>	Correlation index	0.902	0.956	0.948	0.882	0.434
	p-value	0.005	0.001	0.001	0.009	0.331

Incubation of human fibroblasts with a p38 inhibitor, which also inhibits C/EBP β , strongly reduced IL-1 β induced IL-6 expression (Fig 4c), however no clear effect of C/EBP β inhibition on IL-1 β induced downregulation of sHsp expression could be observed in IL-1 β stimulated fibroblast which received inhibitor prior to IL-1 β stimulation (Fig 4b), suggesting C/EBP β is not indispensable for the regulation of this sHsp panel.

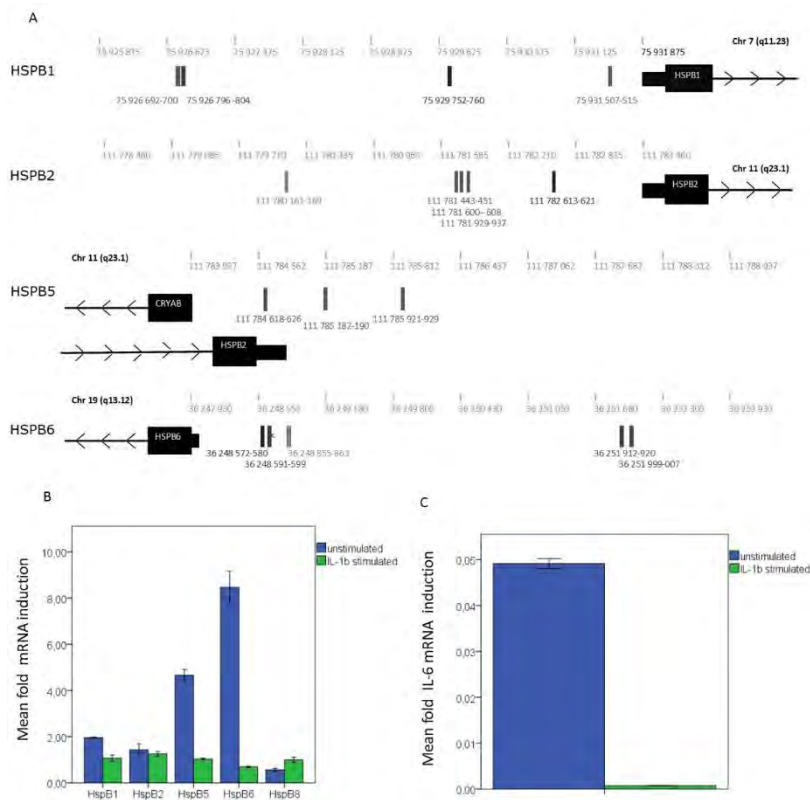


Figure: A: PhysBinder bioinformatic algorithm revealed 3,2,4 and 1 C/EBP β binding place in the promoter region of respectively HspB1, HspB2, HspB5, HspB6. **B:** no effect of C/EBP β inhibition could be observed on IL-1 β induced downregulation of HspB1, HspB2, HspB5, HspB6 in synovial fibroblasts. **C:** Whereas IL-6 induction is strongly reduced by C/EBP β inhibition .

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4. General discussion and summary

OA is an important musculoskeletal disease and even though it is known to be a multifactorial disease, crucial events in the pathogenesis still remain elusive. Increasing the insight in OA pathogenesis and mechanisms driving its progression is crucial to find new targeting strategies to develop disease modifying drugs. Since the introduction of the concept of OA as a whole joint organ failure¹, increasing effort has been made to unravel the contribution of joint compartments other than cartilage to OA degradation and its interactions with cartilage²⁻⁴. In this research we contribute to the concept of whole joint organ failure by providing evidence for a degeneration-specific gene expression pattern in OA labrum fibrochondrocytes which is similar in some but distinct in many other aspects from OA chondrocytes isolated from the articular cartilage. We have demonstrated that during OA development, catabolic pathways are activated in acetabular labrum. These may contribute to degradation of the normal macromolecular structure of the fibrocartilage, ultimately leading to loss of normal 'supporting' function of the labrum and contributing to joint failure⁵⁻⁷. In our further research we have thus focussed on both chondrocytes and fibrochondrocytes as primary stromal joint cell types.

Weight bearing joints are subjected to large amounts of different types of stress (biomechanical, shear, oxidative etc.). In healthy joints however, this does not lead to joint degradation. We hypothesize that in an OA joint, stress related protein families may be dysregulated and contribute to OA progression. To address the role of stress related proteins in osteoarthritis we have focussed on two families: the short leucine rich repeat

proteoglycans (SLRP) and the small heat shock proteins (sHsp). We show both families are distinctly regulated in degenerated connective tissue compared to healthy joint tissue. Moreover we provide evidence that both families act as novel regulators of ECM degradation since both protein families clearly impact joint metabolism and remodelling.

4.1. sHsp downregulation, a rescue mechanism for OA joint remodelling

sHsp are involved not only in cell stress but in several stress unrelated cellular events⁸⁻¹⁰. Larger Hsp have been implicated in arthritis but research into sHsp involvement in stromal OA phenotype was lacking¹¹⁻¹⁴. Our research indicates a distinct group of four sHsp (HspB1, HspB2, HspB5, HspB6 but not HspB8) is consistently downregulated in different cell types isolated from (fibro)cartilaginous tissue of OA patients. This expression profile suggests that these sHsp may be involved in OA progression in stromal cells. Indeed, our findings show double knock out mice, deficient in both HspB2 and HspB5¹⁵, were markedly protected from cartilage damage during the early phases of joint degradation/remodelling as well as from new bone formation by osteophytes in an end stage trauma induced OA model when compared with littermate controls. These findings are particularly interesting, since most reports on sHsp in human disease (e.g. neurodegenerative diseases) have pointed out protective effects for this protein family¹⁶⁻¹⁸. In these diseases, boosting the expression levels of sHsp is considered as a potential endogenous rescue mechanism to prevent disease

progression¹⁷. The protective effects for sHsp in neurodegenerative disease have mainly been attributed to their chaperone activities and prevention of aggregate accumulation¹⁸⁻²⁰. Recently, it was shown by Rothbard et al²¹. that HspB5, can be administered therapeutically in experimental autoimmune encephalomyelitis (EAE), a well-known animal model for multiple sclerosis²¹. Our results, however, indicate a specific downregulation of HspB1, HspB2, HspB5 and HspB6 in OA stromal joint cells, which is not observed in neurodegenerative tissue cells. Furthermore, *in vivo* results in sHsp deficient mice suggest this downregulation may be part of a feedback mechanism attempting to dampen progression of OA induced cartilage damage and ultimate joint failure.

Whether this is a chaperone dependent or independent effect remains to be investigated. However, since HspB8, a sHsp with high chaperone efficacy^{20,22}, is not downregulated in OA joint tissue nor in IL-1 β stimulated fibro(chondrocytes) it may be anticipated that other cellular functions may be responsible for the observed phenotype. The unravelling of upstream pathways responsible for the downregulation of specifically HspB1, HspB2, HspB5 and HspB6 though not HspB8 may further aid in understanding which potential mechanisms may be involved in the protective downregulation of sHsp. Our initial *in silico* findings point to CCAAT/Enhancer-binding protein (C/EBP β) as a potential transcription factor specifically for these four sHsp (HspB1, HspB2, HspB5 and HspB6). However, the pilot inhibitor studies we performed indicate C/EBP β is not

indispensable for sHsp regulation, leading to the hypothesis that other co-regulatory pathways might be involved.

The striking protective phenotype in the HspB2/HspB5 double knock out mice also provides leads to guide further research into the mechanism of the observed rescue mechanism. Especially the reduced osteophyte formation in HspB2/HspB5 double knock out mice in the DMM model merits further discussion. Even though osteophyte formation is a hallmark of OA, it has also been considered as a rescue mechanism, since osteophytes can stabilise the OA-damaged joint. On the other hand, it can increase immobility and pain levels, leading to a reduced quality of life for OA patients²³. It remains to be determined whether the observed impaired osteophyte formation is simply secondary to the protective effect on cartilage or whether, in addition, sHsp have an additional role in the formation of osteophytes. Is cartilage degradation retarded in sHsp knock out mice during the DMM model thereby delaying the need for joint stabilisation and osteophyte formation or are the sHsp directly involved in new bone formation pathways?

4.2. SLRP expression contributes to ECM

synthesis

SLRPs are part of the very heterogeneous collection of DAMPs and are therefore generally considered as stress related proteins. They have been implied in innate immunity through activation of TLR and NLR signalling as well as interaction with the complement cascade^{24,25} and are extremely well suited for

protein-protein interactions²⁶. Moreover an OA-like phenotype is observed in several SLRP knock out mice²⁷⁻³⁰ which indicates these proteoglycans may be important in ECM homeostasis. We therefore aimed to investigate this family in OA-affected joint tissue. The role of SLRP in cartilage is well known and some SLRP are upregulated in OA chondrocytes compared to healthy chondrocytes³⁰⁻³². We aimed to investigate the role of this protein family in fibrocartilage as well. Surprisingly, our findings revealed a strong downregulation of three SLRP: osteomodulin (OMD), osteoglycin (OGN) and asporin (ASPN) in fibrochondrocytes, in contrast to an upregulation in OA chondrocytes. Moreover IL-1 β was able to strongly induce this SLRP downregulation. Our findings point to a distinct SLRP regulation in fibrochondrocytes compared to chondrocytes. Although fibrocartilage and cartilage are distinct joint structures, we observed similar events are ongoing in both tissues in OA. Both tissues are characterized during OA by a disturbed TGF β signalling, which shifts towards catabolic downstream events, as well as an enriched cytokine and chemokine signalling, creating a more pro-inflammatory environment. This observation makes the distinct regulation of SLRP between OA fibrochondrocytes and chondrocytes of particular interest. In order to further investigate the functional significance of the distinct SLRP regulation, we performed *in vitro* stimulation of fibrochondrocytes with full length recombinant OMD. OMD was able to dose dependently induce aggrecan synthesis, which indicated a pro-anabolic effect of OMD in fibrochondrocytes, stimulating ECM synthesis through

induction of aggrecan production. As such, SLRP may take part in a protective mechanism preventing further damage. Currently the functional impact of OMD on articular cartilage chondrocytes has not been addressed yet, assuming OMD would have the same pro-anabolic effects in chondrocytes as well, this would suggest the upregulation of SLRP in OA cartilage might be yet another OA-induced protective mechanism, apart from the above mentioned sHsp downregulation. Furthermore we clearly showed the acetabular labrum is actively involved in OA as evidenced by increased activation of cytokine and ECM degradation pathways. These results further contribute to whole joint organ concept and provide additional evidence for the contribution of supporting joint structures, like the acetabular labrum, to OA development and progression.

In conclusion, we provide two mechanisms of endogenous protective mechanisms in (fibro)cartilage degradation. Research into the upstream regulators and downstream mechanisms in the role of both the SLRP and sHsp may provide novel targets that can be modulated to boost endogenously existing protecting mechanisms that ultimately may aid to dampen OA associated joint degradation.

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5. Samenvatting

Osteoarthritis is een belangrijke speler op het veld van verlies van mobiliteit en levenskwaliteit. In een verouderende maatschappij zal osteoarthritis een steeds grotere rol gaan spelen. Desalniettemin zijn de behandelingsopties nog steeds erg beperkt, daar waar voor andere reumatologische aandoeningen zoals reumatoïde artritis (RA) en spondylarthritis (SpA) de laatste decennia grote sprongen voorwaarts gemaakt zijn in behandelingsmethoden, zeker met de komst van biologicals die ingrijpen op het ziekteverloop zelf. Voor OA blijven we beperkt tot pijnbestrijding en chondroprotectieve medicatie zoals hyaluronzuur en chondroïtinesulfaat, maar uiteindelijk zal orthopedische chirurgie vaak noodzakelijk zijn als laatste redmiddel. Het blijft wachten op een eerste effectieve behandelingsmethode die het ziekteverloop een halt kan toe roepen. Een belangrijke struikelblok hierbij is dan ook een gebrek aan inzicht in het ziekteproces van OA. Slechts in de laatste twee decennia is er een shift te merken in onderzoek naar OA van een visie op OA als zuivere slijtage van kraakbeen naar een ziekteproces waarbij het volledige gewricht alsook subtiele inflammatie een rol spelen.

Het was dan ook de doelstelling van deze thesis om bij te dragen aan een beter begrip van factoren betrokken in het ontstaan en verloop van OA. Er werd hierbij gekozen om te focussen op de stromale gewrichtscomponenten. Enerzijds werd de rol van kraakbeen-ondersteunende gewrichtsstructuren bestudeerd, met name het acetabulair labrum. Anderzijds werden eerste stappen gezet in het begrijpen van de rol van twee specifieke

proteïnenfamilies in het OA ziekteproces, short leucine rich repeat proteins (SLRP) en small heat shock proteins (sHsp).

In Hoofdstuk I komen we terug op het actabulair labrum en de SLRP. Onze studie toont een OA-specifiek expressiepatroon aan voor labrum fibrochondrocyten. Hoewel dit expressiepatroon zeker sterke gelijkenissen vertoont met het OA fenotype van kraakbeen chondrocyten, zoals blijkt uit de verhoogde productie van pro-inflammatoire en katabolische cytokinen en de verlaagde expressie van matrixcomponenten en TGF β pathway-moleculen, zijn er ook belangrijke verschillen. Opvallend verschillend was de regulatie van SLRP in OA labrum fibrochondrocyten in vergelijking met kraakbeen chondrocytes. Daar waar in kraakbeen de expressie van osteomoduline (OMD), osteoglycine (OGN) en asporine (ASPN) verhoogd zijn, stelden we vast dat in labrum een sterke reductie van deze drie SLRP optrad. Mogelijks wordt deze reductie gemedieerd door pro-inflammatoire cytokines, wat gesuggereerd wordt door een IL-1 β geïnduceerde reductie van expressie *in vitro*. Gebaseerd op de dosisafhankelijke inductie van aggrecan expressie in fibrochondrocyten door osteomoduline, suggereren we verder dat deze SLRP betrokken kunnen zijn in het behouden van matrix homeostase, als anti-katabolische regulatoren.

Hoofdstuk II verschaft ons een vernieuwend inzicht in de expressie en functionele rol van sHsp in OA stromale gewrichtsstructuren. Deze resultaten duiden een consistent panel van 4 specifieke sHsp aan (HspB1, HspB2, HspB5 en HspB6) dat een sterke gereduceerde aanwezigheid kent in OA chondrocyten en fibrochondrocyten. De data suggereren dat

deze reductie gemedieerd wordt door IL-1 β . Hoewel verder onderzoek noodzakelijk is om de upstream regulator(en) van deze sHsp binnen een OA context te bepalen, wijzen initiële *in vitro* en *in silico* analyses C/EBP β aan als mogelijke transcriptiefactor voor dit specifieke sHsp panel. Onze *in vivo* experimenten duiden verder aan dat deze reductie een protectief effect op kraakbeen homeostase heeft. Muizen deficiënt voor zowel HspB2 als HspB5 zijn immers sterk beschermd tegen kraakbeendestructie en botnieuwvorming in zowel inflammatoire als trauma geïnduceerde artritis modellen.

Samenvattend kunnen we stellen dat labrum fibrochondrocyten functioneel betrokken zijn in OA ziekteverloop en dat hun fenotype geen kopie is van het OA chondrocyte fenotype, hoewel er ook sterke overeenkomsten zijn. Twee families kleine proteïnen werden dieper onderzocht en geven ons een dieper inzicht in cellulaire events binnen de stromale gewrichtscomponent tijdens OA. Beide groepen zijn betrokken in het OA ziekteproces en zouden mogelijks als protectief kunnen beschouwd worden. Zeker voor sHsp beschrijven we hiervoor sterke *in vivo* evidentie. Door de verscheidenheid aan cellulaire effecten en de alom vertegenwoordiging van deze eiwitten is het echter onwaarschijnlijk dat deze proteïnen als direct target te gebruiken voor behandelingen. Verder onderzoek naar upstream mediators zal uitwijzen of indirecte targetingstrategieën mogelijk zijn.

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Magna cum laude, Howest-departement Kortrijk

2007-2010: Bachelor in Science in industrial engineering
biochemistry

Magna cum laude, Howest-departement Kortrijk

2006-2007: 1^{ste} year bachelor in Law: Succeeded; Catholic
University Leuven

Poster and presentations

5 posters at national and international meetings

3 oral presentations at national and international meetings

Best abstract award, Belgian Rheumatology Conference, 2014,
Brussels

Travel grant, FOCIS, 2014, Phoenix, USA

Publications

Stijn Lambrecht, Nele Juchtmans, Dirk Elewaut. *Heat-shock proteins in stromal joint tissues, innocent bystanders or disease initiating proteins?* Rheumatology. 2014; 53(2): 223-32.

Nele Juchtmans, Aad A.M. Dhollander, Julie Coudenys, Emmanuel A. Audenaert, Christophe Pattyn, Stijn Lambrecht, Dirk Elewaut. *Distinct dysregulation of the small leucine-rich repeat protein (SLRP) family in osteoarthritic labrum compared to articular cartilage.* Arthritis & Rheumatology. 2015.

