The importance of mechanical loading for TGF-β signaling

in articular cartilage

Wojciech Maciej Madej
The importance of mechanical loading for TGF-β signaling in articular cartilage

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General introduction
and thesis outline
1. **ARTICULAR CARTILAGE**

Articular cartilage is an avascular and a-neural connective tissue with a highly specified mechanical function. Cartilage, by covering the ends of opposing bones, not only provides the bearing surfaces with highly reduced friction but also facilitates in load distribution in a synovial joint. Importantly, the mechanical properties of articular cartilage that allows it to perform its mechanical function are determined by its building components. Cartilage tissue is composed of cells (chondrocytes) surrounded by a multi-component extracellular matrix (ECM) [1]. ECM of articular cartilage consists of three principal phases:

I. A solid phase which is mainly composed (on a weight base) of a collagen fibrillar network and proteoglycan aggregates. The collagen network, with type II collagen as the predominant type of collagen, is responsible for the tissue tensile strength [2]. Moreover, other collagen types, mostly with regulatory functions, are also present in the tissue [2]. Proteoglycans are highly glycosylated proteins containing a high number of negatively charged sulphate chains and attract/retain a high amount of water within the cartilage matrix [3, 4].

II. A water based fluid phase which constitute 70 to 85 % of the weight of the tissue [5].

III. An ion phase with number of ionic species (Na\(^+\), Ca\(^{2+}\), K\(^+\)) that counteracts the negative charge of proteoglycans [6].

In humans, articular cartilage is composed of three zones: the superficial, middle and deep zone. In the superficial zone, the collagen fibers are oriented along the surface and cartilage cells are disc-shaped (Figure.1). The middle zone which is characterized by a high proteoglycan content, contains spherical shape cells and collagen fibers oriented more perpendicularly to the
surface (Figure 1). Finally, the deep zone with fibers oriented perpendicularly to the surface and round cells grouped in columns [1].

Chondrocytes are the only cells in the tissue and they actively control the composition of the cartilage ECM. By this, chondrocytes control cartilage ECM turnover, so the combination of matrix degradation and production. Importantly, both of these processes can be triggered in chondrocytes as a response to chemical, biological and mechanical signals [7, 8]. A sustained balance between catabolic and anabolic processes is necessarily to maintain intact and healthy cartilage. However, any persisting disturbance in this balance with higher degradation rate than synthesis will lead to tissue degradation and initiation of cartilage disease [9].

![Cartilage Structure](image)

**Figure 1. Structure of articular cartilage.** Adult human articular cartilage can be divided into three district zones: a superficial zone which contains collagen fibers aligned parallel to the tissue surface and disc-shaped chondrocytes, a middle zone with more perpendicularly oriented collagen fibers and more round cells and a deep zone with collagen fibers oriented perpendicularly to the surface and round chondrocytes grouped in columns. Calcified cartilage is separated from the deep zone cartilage by an interface called the tidemark, which is the boundary between cartilage and the underlying subchondral bone.

2. **BIOMECHANICAL PRINCIPLES OF ARTICULAR CARTILAGE**

The mechanical resistance of cartilage to load is mainly determined by tissue ECM and it is based on the interaction of swelled proteoglycan aggregates molecules with the entangling collagen framework [10]. When cartilage is compressed, the negatively charged sites of proteoglycans are pressed closer together and their mutual repulsive forces significantly contribute to the compressive stiffness of the tissue [11]. However, because proteoglycan aggregates accumulate such high amount of water in the tissue (more than 80% by weight), the mechanical response of the cartilage is also strongly determined by the interstitial fluid flow through the tissue. When cartilage deforms, the fluid entrapped by the ECM flows through the matrix and may be extruded from the tissue [12].

In a view of this combination of events, cartilage response to load is a combined response of interstitial fluid and solid (the porous matrix) components to the applied load. For the same reason, cartilage in biomechanical modeling is often described as a biphasic material [13]. As a result of these unique material properties, cartilage is able to withstand high compressive stresses without being damaged. The mechanism that provides the efficient method of shielding the solid matrix and chondrocytes from high stresses and strains generated by joint loading during physical activity is based on a very low permeability of the cartilage matrix [14]. Articular cartilage under the impact of load behaves as a single-phase, incompressible and elastic solid material. This means that under impact load, there is no time for the fluid to flow as a response to the rapid applied load and the impact is being mainly received by the fluid accumulated within the ECM [14].

Importantly, the deformation-dependent impermeability of cartilage is an essential determining factor of tissue condition and it might be related to cartilage diseases [15]. If creep
of the articular cartilage is more rapid than normal, the solid matrix will have to bear more stress and it will be more prone to fail. Moreover, the increased permeability of the cartilage which results in a more rapid deformation of the tissue and by that might influence the response of chondrocytes to mechanical signals and affect their mechanically-mediated metabolic activity [16]. However, there are more changes in the physical properties of the articular cartilage that accompany disease-mediated changes in ECM composition and structure. This include cartilage compressive modulus which has been shown to decrease in human osteoarthritic cartilage [17, 18] and shows that cartilage ECM properties are highly affected by OA development.

3. TGF-β SIGNALING AND ITS ROLE IN ARTICULAR CARTILAGE PHYSIOLOGY

Number of growth factors play a regulatory role in cartilage homeostasis. Although many growth factors have been shown to control this process, TGF-β might be particularly involved in the relationship between mechanical signals and cartilage maintenance. The Transforming growth factor beta (TGF-β) super-family consist of over four different subfamilies and together it contains over 35 members [19]. It includes, but is not limited to, a number of isoforms of TGF-β, bone morphogenetic proteins (BMPs) and activins [19]. Members of TGF-β superfamily, by controlling key cell processes like proliferation, differentiation, migration and apoptosis, play a fundamental role in the development and maintenance of wide range of tissues, including articular cartilage [20].

In mammalian cells, all three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3) are produced in an inactive form. TGF-β is secreted in inactive complex in which matured factor ligand is constrained by Latency Associated Peptide (LAP), called small latent complex. In most cells, this small latent complex is secreted in association with Latent TGF-β binding protein (LTBP) forming large latency complex (LLC) [21]. After secretion, LLC can bind to a number of ECM proteins and by this it can be stored until presentation of the complex to definite activating signal, either chemical, biological or mechanical [22]. Importantly, only activated TGF-β, so dissociated from the latent complex, is able bind to its specific receptors and induce subsequent signaling.

Activated TGF-β ligand binds to the TGF-β type II receptor which recruits the TGF-β type I receptor (also known as Activin receptor-like kinases- ALks) and activates it by phosphorylation of c-terminal serine/threonine residues. Subsequent, intracellular TGF-β signaling is mainly executed by transcription factors called Smads. After phosphorylation by the receptor, receptor-Smads (RSmads) form complex with common-Smad4 and translocate to the cell nucleus to modulate gene expression [23]. The canonical TGF-β receptor ALK5 induces intracellular signaling via activated Smad2 and Smad3 (Figure 2) [24]. Nevertheless, it has been shown that under certain conditions TGF-β can signal via the alternative receptor ALK1 inducing intracellular signaling via activated Smad1 and Smad5 (Figure 2) [25]. Importantly, Smad2/3 and Smad1/5 signaling pathways regulate different set of genes and generate contrasting physiological effects in chondrocytes [26]. Moreover, although signaling via the Smad pathway is the main signaling pathway induced by TGF-β, other pathways have been showed to be TGF-β dependent. This includes MAP kinase, Rho-like GTP-ase and phosphatidylinositide 3-kinases (PI3K) signaling [27].

TGF-β is a critical factor for articular cartilage physiology and maintenance [28]. It plays a pivotal role in cartilage development and maintenance. In chondrogenesis TGF-β is a key inducer of mesenchymal condensation and chondrocyte proliferation, matrix production and differentiation [28]. However, after an early stimulatory role of TGF-β in developing articular cartilage, TGF-β signaling via Smad2/3P pathway stabilizes the phenotype of articular chondrocytes and inhibits deleterious terminal differentiation of chondrocytes (Figure 2) [29].
that TGF-β signaling in cartilage can have opposing effects when being modified by external factors like inflammation, aging or mechanical loading.

4. **REGULATION OF ARTICULAR CARTILAGE BIOLOGY BY MECHANICAL SIGNALS**

Articular cartilage highly depends on the mechanical loading induced by joint movement. Studies show that mechanical forces are absolutely essential to maintain healthy articular cartilage. It has been demonstrated that in case of lack or reduced loading on the cartilage, tissue exhibit progressive loss [31, 32].

During joint movement cartilage experiences loading that results in compression of the tissue. Subsequently, compression-induced changes within the cartilage include deformation of matrix together with chondrocyte deformation, interstitial fluid flow, changes in osmotic pressure and in local ion concentrations [33]. Consequently, different physical activities, e.g., different mechanical loads, will lead to different compressive forces acting on the cartilage and will induce different load–induced responses in the tissue. In the human knee joint the magnitudes of contact pressures ranges between 1 - 6 MPa (depending on joint activity) [34]. However, these values can increase by two to three-fold during pathological conditions, for instance after meniscectomy [35]. Importantly, physiological levels of compression are related with protective and anabolic stimulation of cartilage but on the other hand high levels of pressure can lead to degenerative tissue overloading and induction of catabolic processes in cartilage [36]. Additionally, prolonged static compression is related with reduced transport of bioactive solutes in cartilage together with reduced proteoglycan synthesis and general induction of catabolic activity in chondrocytes [37-39].
Such distinctive reactions of cartilage tissue to mechanical load can be explained by the
fact that mechanical signals are precisely sensed by cartilage cells. This relation is important in
view of the fact that chondrocytes are the only cells in the tissue and they control cartilage ECM
remodeling processes. Chondrocytes are able to sense and respond to a wide range of
mechanical signals including mechanical tension [40], hydrostatic and mechanical compression
[41, 42] and fluid flow-induced shear stress [43]. Chondrocytes possess a series of receptors which
function is to detect and transduce events induced by loading applied on articular cartilage. A
number of key mechano-transduction mechanisms have been investigated and described [43].
These mechanisms by which chondrocytes sense the mechanical signals and transduce them to
intercellular signaling to promote adequate physiological response are described in Chapter 2-
“In search for the optimal model to investigate the role of mechanical signals in articular cartilage
biology”.

5. THE SIGNIFICANCE OF MECHANICAL SIGNALS IN ARTICULAR CARTILAGE AND SYNOVIAL
JOINT DISEASE DEVELOPMENT AND PROGRESSION

Abnormal or increased loading of articular cartilage has been described for decades as one
of the most significant and substantial risk factor for development of degenerative joint disease
like osteoarthritis (OA) [44]. Osteoarthritis is often called a disease of mechanics and there are
number of examples showing that increased physical forces cause degeneration of not only
cartilage but also other tissues of synovial joints [45].

Already more than 60 years ago it has been shown that injury-motivated, surgical removal
of the meniscus (meniscectomy) is causing substantial anatomical changes in the knee joint and,
already then, these changes were associated with further degenerative alterations in the joint
[46]. Later, detailed epidemiological research showed that meniscal removal is a highly significant
risk factor for OA development. These data demonstrated that around 48% of patients that
underwent meniscal resection did develop radiographic OA after 21 years’ follow-up [47]. This is
due to the fact that the menisci have important biomechanical functions in the knee joint, being
an essential element in distributing the load acting between femur and tibia (Fig.3) and stabilizing
the knee joint [48]. Because of this key function, removal of the meniscus leads to a decrease in
the contact areas in the knee joint of approximately 75% and an increase in peak contact
pressures acting on the cartilage of approximately 235% (Fig.3) [49]. Shortly, it has been noticed
that meniscectomy-induced increase in the peak contact pressures and subsequent cartilage
overload result in cartilage fibrillation, increased cartilage cell proliferation and clustering.
Additionally, an induction of biochemical changes like proteoglycan loss, proteoglycan
disaggregation, and increase in proteoglycan synthesis has been noticed [50].

Moreover, abnormal cartilage loading related with anterior crucial ligament (ACL) injury
has also been shown to be a significant factor in mechanically-driven OA development. Reports
show that that ACL deficiency is associated with abnormal knee kinematics with loads shifted to
act on infrequently loaded areas of the cartilage [51]. Further, higher prevalence of OA has been
noticed in the patient with combined injuries that significantly affect joint biomechanics [52]. This
clear connection of overload-induced cartilage fibrillation has been used in establishing of
mechanically-driven animal OA disease models [53].

Recently however, it is becoming clearer that OA development in patients after
meniscectomy or ACL injury is not only and/or purely mechanically-driven. Current reports show
that additional factors like proinflammatory cytokines that are also related with joint injury, might
play a significant role in post-traumatic OA development [55].
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Figure 3. Consequences of the meniscal removal from the knee joint- schematic explanation. Menisci function as a load-transmitting and energy-absorbing element of the joint. Because menisci facilitate in load distribution, in the presence of meniscus (A) the applied force is distributed across bigger contact areas between femur and tibia. As a result, the generated peak contact pressures are within physiological range (A). However, removal of the menisci from the knee joint, result in a dramatic decrease of the contact areas which subsequently leads to an increase of peak contract pressures acting on the cartilage (B). Adapted from McDermott et al. [54].

Proinflammatory cytokines are released in the acute post-traumatic period [56] and they are claimed to facilitate in deleterious changes in the cartilage related to tissue overload [55]. Moreover, advanced age, besides being a significant risk factor for OA development by itself, also facilitates in mechanically-driven OA initiation and progression [57]. In addition, age-related changes have been shown to play a role in modulating of TGF-β signaling in articular cartilage [26] which might further impair the mechanically-driven process of tissue maintenance [58].

6. AIM AND OUTLINE OF THIS THESIS

The aim to this thesis was to investigate the role of mechanical loading on the activation of TGF-β signaling in intact articular cartilage. The first part (Chapter 2), as a literature review, discusses the importance and rationale of using the best possible experimental model for investigation the role of mechanical signals in articular cartilage physiology. The second part (Chapter 3, 4, 5 and 6), as research articles, investigate the role of mechanical signals in the form of dynamic mechanical compression in process of activation of TGF-β signaling in articular cartilage. These investigations were carried out under different conditions which could reflect clinically oriented pathological or deleterious events that might interfere with mechanically-mediated activation of TGF-β signaling. The following outline explains in more details the specific aspects of each chapters of this thesis.

In search for the optimal model to investigate the role of mechanical signals in articular cartilage biology

Mechanobiology is the process of mechanical signal detection, recognition and subsequent transduction to appropriate biological effects and is the key regulatory mechanism in tissues that highly depend on the constant presence of load, like articular cartilage. However, a precise connection between load transducer and the load effectors is necessary for the physiologically correct biological response to mechanical signals. Nevertheless, in most experimental models used to investigate the response of articular chondrocytes to mechanical signals, the key connections between cartilage ECM and chondrocytes are disintegrated or are highly compromised. Although the fact that this is mostly directed by practical reasons (intact
cartilage is difficult material for deep biomolecular research) this approach might lead to
generation of not fully physiologically relevant conclusions about cartilage mechanobiology
events. Motivated by this, **CHAPTER 2** aimed to discuss the most common experimental models
which are used for the investigation of the role of mechanical signals in chondrocyte
mechanobiology. Subsequently, **CHAPTER 2** aimed to analyze the relevance of these models for
identification of physiologically accurate mechanisms and events of cartilage mechanobiology.
The conclusions from the **CHAPTER 2** provided the rationale for the use of intact articular
cartilage explants as a model for the research included in the experimental part of this thesis.

**Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P
signaling**

The aim of **CHAPTER 3** was to verify if mechanical signals in the form of dynamic
mechanical compression are able to activate TGF-β signaling in intact articular cartilage.
Secondly, because excessive mechanical compression is strongly associated with cartilage
degradation, the role of different levels (physiological or excessive) of dynamic mechanical
compression in activation of alternative TGF-β signaling via deleterious Smad1/5/8P route was
investigated. For this purpose, intact bovine articular cartilage explants were loaded with
physiological or excessive dynamic mechanical compression. Later, at different time points, the
activation of distinctive TGF-β signaling routes was studied by analysis of expression levels of
downstream genes, specific for the Smad2/3P or Smad1/5P signaling pathways. Additionally, in
**CHAPTER 3** the effect of physiological or excessive compression on the expression of alternative
TGF-β receptors was analyzed. Finally, the receptor blocker- SB505124 was used to confirm if
observed effects of the mechanical compression were mediated by ALKS receptor.

**Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in
articular cartilage**

Epidemiological data show that advanced age is one of the most significant risk factor for
the development of cartilage degenerative disease like OA. Furthermore, separate studies
showed that age-related changes in the cartilage affect TGF-β signaling. In a view of these facts,
the aim of **CHAPTER 4** was to investigate if aged articular cartilage has impaired
mechanically-mediated activation of TGF-β/Smad2/3P signaling- a signaling known to protect
chondrocytes from deleterious process of terminal differentiation. To investigate that, articular
cartilage from two different age groups were subjected to dynamic mechanical compression with
physiological or excessive load. Subsequently, the ability for mechanically-mediated activation of
TGF-β/Smad2/3P signaling was compared in cartilage from different ages. Additionally, in
**CHAPTER 4** the ability of cartilage for mechanically-mediated regulation of mechanosensitive
genes known to be essential for tissue physiology was compared in different cartilage age groups.

**Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling
in articular cartilage**

Reports show that after joint trauma articular cartilage is being affected by deleterious
excessive loading conditions combined with catabolic stress of proinflammatory cytokines
produced by inflamed synovium. Regarding the fact that mechanical loading is known to induce
TGF-β/Smad2/3P signaling whereas proinflammatory cytokines are known to regulate or
interfere with this signaling in articular cartilage, the aim of **CHAPTER 5** was to evaluate if
proinflammatory cytokines are able to impair the mechanically-mediated activation of
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TGF-β/Smad2/3 signaling. For this purpose, intact bovine articular cartilage explants were loaded with physiological or excessive dynamic mechanical compression in presence of IL-1β or osteoarthritic synovium-conditioned medium (OAS-CM). At chosen time points the activation of Smad2/3P signaling was studied by analysis of the expression levels of specific downstream genes for this pathway. Subsequently, in CHAPTER 5 the effect of the mechanical compression applied in presence of proinflammatory conditions on the expression levels of the receptors required for intact TGF-β/Smad2/3P signaling were examined.

Unloading results in rapid loss of TGF-β signaling in articular cartilage: Role of loading-induced TGF beta signaling in maintenance of articular chondrocyte phenotype?

For decades, it was known that articular cartilage has to be regularly loaded to maintain its integrity. In many different models, it has been shown that non-loaded articular cartilage shows signs of atrophy and in longer perspective it deteriorates. Furthermore, we showed that dynamic mechanical compression is very potently activating TGF-β/Smad2/3P signaling, known as an essential factor to maintain cartilage integrity. Considering these facts, CHAPTER 6 aimed to analyze if regularly loaded cartilage maintains stable high TGF-β/Smad2/3P signaling and if removal of loading from cartilage results in decline of this signaling. Subsequently, CHAPTER 6 aimed to determine if mechanic compression is able to repeatedly restore TGF-β/Smad2/3P in the cartilage after previous unloading. Finally, the physiological significance of stable high mechanically-induced TGF-β/Smad2/3P was investigated. Based on the results from CHAPTER 6, a hypothesis model for compression-mediated protection of articular cartilage integrity was proposed.

General discussion and future perspectives and Summary

In CHAPTER 7 the main findings of this thesis were discussed in order to create a broad picture of the work included in this dissertation. Additionally, a proposition for future concepts that should endorse a further continuation of a research line started with this project was proposed. Finally, in CHAPTER 8, English, Dutch and Polish summaries of this thesis were included.
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REFERENCES


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In search for the optimal model to investigate the role of mechanical signals in articular cartilage biology

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ABSTRACT

Articular cartilage is a tissue which physiology greatly depends on the continuous mechanical loading. Therefore, much attention has been directed into detailed investigations of the role of mechanical signals in cartilage development, maintenance and degradation. Nevertheless, in a view of difficulties with the use of advanced biomolecular tools in native intact articular cartilage, many alternative experimental models, using isolated chondrocytes or cartilage fragments have been developed and widely used in mechanobiology studies of chondrocytes. As artificial, these experimental models are not truly replicating the key connections between mechanical signal transducer- the extracellular matrix (ECM) and the effectors- chondrocytes, which are present in native cartilage. Considering the fact that physiologically correct and accurate connections between cartilage ECM and chondrocytes are the major determinants of the way how cartilage cells respond to mechanical stimuli, two questions arise:

1. How well does our present knowledge about cartilage mechanobiology, which is mainly based on artificial experimental models, reflects the actual situation in situ?
2. How relevant are the current in vitro models in helping to unravel the chondrocyte mechanobiology events?

To answer these questions in this review we first discuss the most widely used experimental models for chondrocytes mechanobiology studies. Secondly, we analyzed the most important events associated with cartilage mechanobiology in the context of experimental models used to identify them.
In search for the optimal model to investigate the role of mechanical signals in articular cartilage biology

1. INTRODUCTION

Articular cartilage is a highly specialized connective tissue that covers the endings of long bones. Articular cartilage is an avascular and aneural tissue and consists of cells (chondrocytes) sparsely distributed in the extracellular matrix (ECM). ECM consists of a solid phase which is composed of predominantly collagens and proteoglycan aggrecan [1]. A number of additional and essential minor components are also present, but in much smaller amounts on a weight base. Aggrecan, due to its negative charged side chains, potently attracts and retains water within the tissue. Because of this, the fluid phase (water and ion phase) of hyaline cartilage is more than 80% of its wet weight [2]. The unique composition of cartilage tissue allows it to perform its biomechanical function and provides almost frictionless surfaces allowing efficient joint movement.

To sustain cartilage homeostasis, cartilage ECM is under continuous turnover, a combination of matrix degradation and production. Importantly these processes are controlled by the chondrocytes, the only cells in the tissue. Chondrocytes control matrix composition in response to external chemical, biological and mechanical signals. Articular cartilage is highly dependent on the constant presence of mechanical signals. Loading has been shown to play a crucial role in various processes in articular cartilage, from development through tissue maintenance, but also in its degradation [3, 4]. In all of the mentioned processes, the mechanically-driven control of tissue physiology involves an uninterrupted transmission of mechanical signals coming from the environment, being transduced via tissue ECM to the cartilage cells (scheme 1). Only highly specific and intact connections between signal transducer, so ECM, and signal effectors, the chondrocytes, allows the undisturbed and physiologically correct response of the cells to mechanical stimuli [5]. The feedback of the matrix to the chondrocytes can be achieved by direct interaction through specific receptors or by release of soluble factors e.g. growth factors, or ions that induce the required cellular response (scheme 1). However, large numbers of studies that tried to unravel the mechanically sensitive signaling pathways in articular cartilage are based on experimental models lacking a complete and physiologically correct ECM. This clearly raises the questions: How well does our present knowledge reflects the actual situation in situ and are current models relevant in unravelling the chondrocyte mechanobiological regulation? In this review, we attempted to answer these questions and therefore we analyzed the most important aspects of cartilage mechanobiology in context of experimental models used to identify them. First, we discuss the most commonly used experimental models, hereafter we review our current understanding of mechanotransduction and discuss how good current models reflect the actual situation in situ.

![Scheme 1. Signal transduction via cartilage ECM is a crucial determinant of the tissue response to mechanical stimuli. Cartilage ECM is the central transducer of mechanical signals in the articular cartilage thus, deformation of the tissue ECM generated by mechanical signals can be sensed via direct connection between](image-url)
In search for the optimal model to investigate the role of mechanical signals in articular cartilage biology

the ECM and the cell receptors. However, mechanical signals can also generate the release/activation of
soluble factors from the cartilage ECM. Both routes of sensing the mechanical signals may lead to the
activation of the specific intracellular signaling that subsequently regulates defined gene expressions. By these
mechanisms, chondrocytes can generate an adequate response for mechanical stimuli and induce an
autocrine or paracrine response or ECM remodeling.

2. RATIONALE OF DIFFERENT EXPERIMENTAL MODELS USE FOR CHONDROCYTE AND
ARTICULAR CARTILAGE MECHANOBIOLOGY STUDIES

Intact articular cartilage is a relatively difficult experimental material for biomolecular
studies. Articular cartilage is not vascularized and the its dense matrix forms a size barrier for
large chemicals and proteins [6]. Consequently, chondrocytes encapsulated in cartilage ECM are
hardly available for any kind of gene modification by direct use of most recombinant vectors or
siRNA [7, 8]. For the same reason, also direct/live microscopic examination is extremely difficult.
Because of these limitations, many laboratories have decided to use alternative models instead
of intact articular cartilage for their research. As a result, many alternative experimental models
to study chondrocyte mechanobiology have been developed, making use isolated cells. However,
in most of these models the tight interaction between chondrocytes and ECM is highly
compromised.

2.1. CHONDROCYTE MONOLAYER

Most alternative models to intact cartilage use an enzymatic digestion of cartilage ECM
to obtain chondrocytes. These cells can then be cultured in non-confluent mono-layers or in high
density cultures. In this design, chondrocytes can be subjected to direct fluid flow-induced shear
stress, resembling compression-induced interstitial fluid flow. It has been shown that monolayer
chondrocytes respond to these stimuli by regulation of a wide spectrum of genes relevant for
cell physiology [9] and cell proliferation [10]. Furthermore, monolayer chondrocytes can be
cultured on flexible-bottomed culture plates or membranes where they can be subjected to
controlled mechanical stretch. Monolayer chondrocytes respond to mechanical stretch by
elevated expression and production of both catabolic and anabolic mediator [11], matrix
production [12] and cell proliferation [13]. Importantly, monolayer cultured chondrocytes are well
available for direct gene modification with recombinant vectors [14] or siRNA [15].

However, when using chondrocyte monolayer for mechanobiological investigations, several
disadvantages of these experimental models have to be taken into consideration. First, in
these settings, chondrocytes lose their intimate contact with the intact cartilage ECM.
Furthermore, enzymatic digestion of articular cartilage generates a large amount of ECM
break-down products (e.g. collagens, fibronectin). It has been shown that chondrocytes react to
these fragments by an increased production of catabolic enzymes [16], catabolic cytokines [17]
and by activation of catabolic intracellular signaling pathways like NF-kappa B [18]. Furthermore,
has also been shown that enzymatic digestion of articular cartilage yields only a fraction (<25%)
of the total chondrocytes from the available cell pool, which might lead to uncontrolled and
unwanted selection of certain chondrocyte subpopulations [19]. It is known that different
chondrocyte sub-populations respond differently to mechanical stimuli and this can thus skew
results [20]. Moreover, it is important to realize that after the isolation and from the moment
onwards when chondrocytes attach to the culture plate, they profoundly change their gene
expression profile and progressively lose their chondrogenic phenotype [21]. Importantly, this
change significantly impacts chondrocyte mechanotransduction processes [22]. When
chondrocytes are cultured on tissue plates in monolayer, the cells produce a new matrix of a
different constitution compared with native ECM [23]. For example, this new matrix is rich in
In search for the optimal model to investigate the role of mechanical signals in articular cartilage biology

collagen types I, III and V, small molecular weight PG or versican which are molecules common for fibroblast or pre-chondrocytes but not in mature chondrocytes [23]. Moreover, the vast majority (over 70%) of newly synthesized proteoglycans is being released into the surrounding culture medium [24] showing that the matrix lacks GAG binding capacity which is so characteristic of native cartilage. Based on these observations, it is unlikely that the sustainable chondrocyte-ECM connections, which are so important for biomechanical functions, in a monolayer culture system resemble those in native cartilage.

2.2. CHONDOCYTES EMBEDDED IN AN ARTIFICIAL THREE-DIMENSIONAL MATRIX

Based on the three-dimensional distribution of chondrocytes in cartilage ECM, efforts have been directed to create artificial matrices for chondrocytes. An optimal matrix should allow an easy embedding of cells in a three-dimensional environment and should at the same time be permeable for nutrients and oxygen and available for tools used in cell signaling investigations. Frequently used culture systems are chondrocytes encapsulated and cultured in alginate [25] or agarose [26] gel. Use of these culture systems has many benefits and is more physiologically relevant than monolayer chondrocyte culture. Chondrocytes cultured in alginate beads maintain their typical spherical shape and keep their chondrocyte-like phenotype even in long term culture, in contrast to chondrocytes in monolayer [27]. The same effect can be achieved with the use of agarose culture systems [26]. Importantly, chondrocytes cultured in alginate/agarose beads synthesize new ECM [28], including pericellular matrix (PCM) which is important for mechanotransduction [29]. Additionally, because preparation of the alginate/agarose chondrocyte constructs involves cartilage ECM enzymatic digestion, chondrocytes are accessible for bio-molecular tools like gene expression modification with vectors or siRNA during construct preparation. Based on these advantages, chondrocytes cultured in alginate/agarose beads have been widely used in mechanotransduction studies. Chondrocyte alginate/agarose beads constructs can be subjected to a range of mechanical stimuli like hydrostatic pressure [30], dynamic and static mechanical compression [31] as well as to fluid flow [32]. Moreover, this model has been also widely used to investigate the role of mechanical signals in chondrocyte intracellular signaling [33-39]. Nevertheless, it has to be remembered that chondrocyte alginate/agarose constructs are artificial experimental models and despite of all its advantages they do not replicate all the complicated connections and interactions of chondrocytes and ECM. It has been shown that the mechanical properties of these cell-seeded constructs are relatively poor, even after long time culturing [40]. When compared to native cartilage, the compressive modulus of chondrocyte-seeded alginate constructs is about 25 times lower than that of native cartilage, while the dynamic shear modulus is about 30 times lower [40]. Moreover, the GAG content of alginate constructs is 3 times lower than in native cartilage and the collagen content is approximately 10 times lower [40]. This is important especially in investigations in which the complex interplay between cartilage ECM, cartilage interstitial fluid and ions and ECM mechanical properties have an impact on chondrocyte response to mechanical stimuli [5]. Furthermore, preparation of chondrocyte agarose constructs still involves enzymatic digestion of cartilage, with all of its disadvantages, discussed above.

Stimulated by the growing interest in tissue engineering, many alternative artificial cartilage matrices have been developed [41]. The design of the “perfect” tissue engineering scaffold for articular cartilage regeneration involves a significant attention on recreation of the connections between chondrocytes and their native matrix [42]. Together with the fact that before seeding on the scaffold, chondrocytes are accessible for most bio-molecular tools, this makes chondrocytes seeded on highly specified three-dimensional scaffolds a very attractive
model for specific biomechanical studies. However, because of the diversity of available scaffolds, variety of particular approaches and limited studies with detailed investigation of biomechanical sensitive pathways, tissue engineering models will not be discussed here.

2.3. INTACT ARTICULAR CARTILAGE SPECIMENS

As already mentioned, intact articular cartilage specimen is a challenging experimental material because of its impermeability for most of the biomolecular tools. Nevertheless, because this experimental model preserves all of the crucial connections and interactions between chondrocytes and cartilage ECM, it is being endorsed by many research groups as most suited experimental model for mechanobiology studies of articular cartilage. Except maintaining the unaffected connections between force acceptor (ECM) and force effector (chondrocytes), intact articular cartilage specimens as an experimental model possess more substantial aspects which make them appropriate for mechanobiology studies. Most importantly, it has been shown that intact cartilage explants cultured in controlled conditions with strictly defined medium composition maintain the mechanical properties of native cartilage [43]. Furthermore, reports indicate that cartilage explants can be cultured for substantial amount of time in serum-free conditions without their physiology being negatively affected [44, 45]. This aspect gives a possibility to study chondrocytes mechanobiology in fully controlled conditions and eliminate effect of serum from experimental variation. However, it has to be remembered that non-serum culture for prolonged amount of time can negatively affect cartilage explants physiology, inducing GAG loss and reduction of responsiveness of cartilage cells to mechanical stimuli [45]. Other aspect, confirming the fact that articular cartilage explants are most suitable experimental model to study chondrocytes mechanobiology lays in their preserved morphology after isolation.

Since ageing has been shown to significantly affect articular cartilage physiology and hence cartilage response to mechanical stimuli [46], age-related changes in cartilage mechanobiology need to be studied in experimental that maximally preserves these alterations while transferred to experimental conditions. Importantly, recent reports show that cartilage explants can substantially serve as well-defined experimental model to study ageing in this tissue [47].

Nevertheless, intact cartilage explants as any experimental model are not devoid of weak points. First of all, isolation of explants involves cutting what induces cell death at the cutting sites and possibly might have a direct, negative impact on model morphology [48]. However, it has been shown that cutting-induced cell death cannot progress within time and is not exceeding inside to inner regions of the explant, but is strictly associated with cutting site. Still, isolation induces further undesirable artefact as it creates artificial surfaces, normally not present in the cartilage. Reports show that these artificial surfaces have high permeability and can have a significant impact on specimen mechanical properties [49]. Nevertheless, in order to overcome this issue, a special system for applying mechanical force on the cartilage have been introduced, e.g. confined compression [5].
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### Table 1: Comparison of the experimental models for mechanobiology studies of cartilage/chondrocyte.

<table>
<thead>
<tr>
<th>Experimental model</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold for articular cartilage</td>
<td>1. Design of the ideal scaffold considers the recreation of the connection between ECM and chondrocytes as most authentic as possible [42]</td>
<td>1. Involves enzymatic digestion what produces ECM breakdown products that can induce catabolic responses in the cells [16-18]</td>
</tr>
</tbody>
</table>

### 3. Consequence of use different cartilage experimental models in mechanobiology studies

#### 3.1. The consequence regarding use of cartilage models in integrin-mediated mechanotransduction

As mentioned, both monolayer and 3D chondrocyte experimental models being used to study chondrocyte mechanobiology, have considerable limitations, but how far do these limitations reach? The major and obvious limitation is that both of these models lack native ECM. This is crucial, regarding the fact that the link between cartilage ECM and chondrocytes by a series of cell receptors provide important interactions that mediate a number of key cell processes like proliferation, differentiation and homeostasis. These connections are executed by receptors called integrins which are cell surface receptors consisting of two subunits, α and β and operating as heterodimers [50]. Integrins have a large extracellular domain that binds to matrix ligands and short intracellular domain which interacts with cellular proteins and initiates kinase-mediated intracellular signaling [51]. Integrins can recognize and bind specific matrix proteins including, but not limited to, collagen type II [52], type VI [53], fibronectin [54], vitronectin, osteopontin [55] and laminin [56], representing a wide range of cartilage ECM components. As major connectors of chondrocytes and ECM, integrins play a key role in cartilage mechanotransduction [57]. When mechanical loading is applied on the tissue, the cartilage ECM together with associated interstitial fluid is acting as a signal transducer that receives the mechanical input and generates an output to the cells. Integrins, as a major connector of ECM molecules and chondrocytes, are able to sense these mechanical signals and convert them into specific intracellular signals. Already from the late 90’s researchers started to identify the integrin-dependent mechanically-driven...
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signaling pathways, including protein kinase C (PKC) [51] FAK kinase [59] a wide range of MAP kinases, like ERK [60] JNK and p38 [61] and G-protein signaling [62].

The involvement of integrins in mechanically-mediated activation of mentioned signaling pathways was first discovered in monolayer models. However, as already mentioned, it has been shown that chondrocyte dedifferentiate when cultured in monolayer and change their gene expression profile. This is associated with altered expression of genes encoding ECM proteins [63] but importantly also integrins [64] and integrin binding proteins [63]. Remarkably, changes in the type of the integrins expression during cartilage development [65] and cartilage degenerative disease progression, like osteoarthritis (OA) [66], have been demonstrated. These changes might underline the change of integrin expression as an adaptation to changes in the ECM component composition (known to occur during these processes) and provide evidence that the ECM molecules surrounding chondrocytes can determine the expression of the cell adhesion molecules [64, 67]. It has been shown that isolated chondrocytes when cultured in monolayer show changes in the integrin expression with increased α5β1 integrin expression [64]. This confirms the importance and complexity of the integrin-mediated connection between chondrocytes and ECM and supports the fact that care should be taken when choosing the experimental model for analysis of processes where these connections play a key role.

However, integrins are not the only mechanoreceptors. Recently, a glycocalyx layer located on the cell membrane has been proposed as a mechanosensor and transducer of fluid shear stress in the ECM embedded chondrocytes [68]. However, it has been shown also that glycocalyx-mediated interstitial flow mechanotransduction depends on integrin-mediated cell-matrix adhesions [69]. Monolayer culture of chondrocytes also affects glycocalyx composition [70], but the presence of a glycocalyx layer analogous to the one present in the chondrons of adult articular cartilage layer has been documented in chondrocyte agarose constructs [71]. Regrettably, the role of glycocalyx-mediated mechanotransduction in chondrocytes or in the intact cartilage is not commonly studied and the role of the mentioned sensor has not been fully identified yet.

3.2. THE CONSEQUENCE REGARDING USE OF CARTILAGE MODELS IN THE ION CHANNELS-MEDIATED MECHANOSENSTIVE TRANSDUCTION

In cartilage, mechanical signals are not only transduced via direct contact between the ECM and chondrocytes executed via cell adhesion molecules like integrins. Mechanical stimulation generates the deformation of the tissue ECM together with chondrocyte cell membrane what might play a role in signal transduction [72]. Moreover, the deformation of the tissue will also induce an interstitial fluid flow within cartilage ECM. Importantly, created fluid flow generates changes in the electrochemical potential within the tissue [73] and induces changes in membrane potential of articular chondrocytes [74]. This occurs because the cartilage ECM is negatively charged (it contains a high amount of the anionic proteoglycan aggrecan) and the interstitial fluid contains an abundance of positively charges ionic species (e.g. Na⁺, Ca²⁺, K⁺), to counteract the charge.

Physical stretching of cell membrane as well as changes in the membrane potential can be sensed by a number of ion channels located on the chondrocyte cell membrane [75-78]. Ion channels under certain conditions allow the influx of specific ions into the cell that will lead to specific intracellular signaling activation. Recently attention has been concentrated on the role of mechanosensitive ion channels in chondrocyte mechanotransduction. TRPV4 is Ca²⁺ permeable, cell membrane ion channel [79]. Recent studies indicated that TRPV4 is a chondrocyte mechano-sensitive ion channel and is responsible for a mechanically-mediated intracellular Ca²⁺
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gradient [33]. Moreover, the same studies proved that mechanically-dependent TRPV4 activation and subsequent Ca\(^{2+}\) signaling has several functional consequences on chondrocytes, like activation of anabolic and inhibition of catabolic cell activity. Furthermore, latest studies of Lee et al. described other mechanosensitive Ca\(^{2+}\) permeable channels, directly mechanically activated (MA) ion channels (Piezo1 and Piezo2) and identified their role in high-strain mechanosensitivity [80].

For technical reasons, research about the role of ion channels in chondrocyte mechanobiology has been performed in non-intact cartilage models. Measurement of the transmembrane electrical currents of ion channels with patch clamp technique is simply not possible in intact cartilage. However, it has to be remembered that the highly specific ion composition of cartilage interstitial fluid and highly defined cartilage ECM molecules (especially keratan sulphate and chondroitin sulphate) act as important regulators of chondrocyte physicochemical environment [5, 81] and thus lack thereof might affect the mechanically-mediated calcium signaling. Han et al. suggested that calcium signaling in intact cartilage is different from signaling in cell-gel constructs [82]. They showed that calcium signaling in situ occurs immediate after cartilage loading whereas in cell-gel constructs a time-delayed calcium signaling is observed [82]. They also demonstrated that the duration of mechanically-mediated calcium signaling observed in in situ chondrocytes was shorter than calcium signaling observed in chondrocytes lacking intact ECM, like monolayer cells or cell-gel constructs, however were similar in duration to calcium signaling induced by osmotic load in the intact cartilage [82]. Moreover, it is being shown that chondrocyte stretch-activated ion channels are interconnected with the cytoskeleton via \(\beta 1\) integrin [83]. Models like chondrocyte alginate constructs and especially monolayer culture are lacking these essential regulators or have altered their expression. Certainly, these studies suggest that the ion channels related signaling behavior of chondrocytes in the intact tissue might differs substantially from isolated cells or cell-gel constructs.

3.3. THE CONSEQUENCE REGARDING USE OF CARTILAGE MODELS IN GROWTH FACTORS MEDIATED MECHANOSENSATION

Mechanical stimulation of cartilage is not only directly sensed by chondrocytes via physical interactions, either via integrins or cell membrane deformation, but mechanical forces also play an important, indirect role in the signaling of many growth factors. Mechanical stimulation of cartilage can lead to release/activation of soluble growth factors from cartilage ECM. Subsequently, these active mediators are recognized by specific receptors on the cell membrane and are able to induce specific intracellular signaling pathways. Considering the fact that articular cartilage ECM binds and stores a number of different growth factors and cytokines which are crucial for cartilage physiology, like TGF-\(\beta\) and bFGF [29, 84-87], loading-mediated release/activation of these factors can indirectly control key processes in this tissue.

3.3.1. TRANSFORMING GROWTH FACTOR BETA (TGF-\(\beta\))

Articular cartilage ECM contains an abundant amount of TGF-\(\beta\) growth factor (up to 300 ng/g of all three isoforms), however it is bound to the ECM in the latent form [84]. In the latent TGF-\(\beta\) complex, the growth factor is constrained by a Latency Associated Protein (LAP) and it has to be activated, by release from the complex, before it can bind the TGF-\(\beta\) receptor and generate intracellular actions [88]. Active TGF-\(\beta\) signaling in the chondrocytes has been well studied and it has been shown that TGF-\(\beta\)/Smad2/3P plays a critical role in inhibition of
chondrocyte hypertrophy and articular cartilage maintenance [89]. Recently our group has shown that mechanical compression of intact articular cartilage plays an essential role in activation of canonical TGF-β/Smad2/3P signaling [90]. Moreover, other authors showed that mechanically driven activation of TGF-β/Smad2/3P signaling is present in other experimental models [14, 39].

However, the interaction of ECM components and integrins with latent TGF-β complex appears to be a fundamental factor of mechanically-mediated activation of latent TGF-β complex [91]. Moreover, recent reports show that ECM rigidity can have critical impact on the process of mechanically-mediated latent TGF-β activation [92]. Taking this into consideration, it is important that investigations of mechanically driven activation of TGF-β/Smad2/3P signaling use the experimental model that reflects the complexity of this process and the key role of ECM in it. Importantly, as it has been shown by our group that TGF-β signaling in articular cartilage is highly affected by age [93]. Changes in this signaling are also reflected in a loss of the mechanically-mediated activation of TGF-β signaling in aged cartilage [46]. This investigation would not be possible in any other model than intact articular cartilage because chondrocytes lose age related changes in TGF-β signaling (especially changed receptor balance) when isolated from the ECM (data unpublished) and their integrin expression is affected.

3.3.2. BASIC FIBROBLAST GROWTH FACTOR (bFGF)

TGF-β is not the only growth factor being stored in cartilage ECM which can be activated/released by mechanical loading. It has been shown that mechanical loading of articular cartilage also potently releases bFGF from ECM and further induce extracellularly regulated MAP kinases [94]. Later Vincent et al. proved that bFGF is bound to perlecan in the cartilage pericellular matrix and it can be involved in transduction of mechanical forces into biological signals [29]. The initial experiments for this work were carried out by loading of articular cartilage in situ that was attached to the subchondral bone, to approximate physiologic conditions as closely as possible. However, later as more technically advanced analyses of chondrocytes signaling were needed, the experimental model was changed to chondrocyte alginate beads. This approach shows a common sequence of events in mechanobiological research of articular cartilage. Start of the investigation in the most physiological model possible, but switch to more artificial model when more detailed biomechanical experiments are needed. Notably, when Vincent et al. investigated the role of FGF in chondrocyte mechanotransduction in alginate, they first showed the presence of crucial interactions of bFGF with pericellular matrix in the experimental model used. Later, it has been anticipated that bFGF plays a role in cartilage protection as it has been shown that Fgf2 deficient mice exhibited accelerated spontaneous and surgically induced OA [95].

However, the investigation of the role of FGF activation by mechanical signals is an example how the use of different experimental models of chondrocytes can influence the conclusions and lead to contradictory results [96]. FGF2, when signaling through FGFR1, mediates catabolic response of chondrocytes like induction of induction of ECM degradation proteases [97]. However, when FGF2 signals via FGFR3 it leads to anabolic response [98]. That is why, the response of chondrocytes to FGF2 can highly depend on FGF receptor balance. It has been shown that FGFR3 can be negatively regulated by external factors like FGF2 or positively by BMP7. Because of that, the presence of cartilage ECM and ECM bounded growth factors, like FGF2 or BMP7, in the experimental model for investigation of FGF2 role in cartilage physiology is crucial [96].
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4. CONCLUDING REMARKS AND PERSPECTIVES

The role of mechanical signals in articular cartilage physiology has been already studied for many years. Although, early studies provided only limited and cursory knowledge on how mechanical signals affect cartilage, they demonstrated that mechanobiology of cartilage is a highly complex process and many players are involved in its regulation. That is why, later, use of more profound biomolecular tools and methods were needed on order to better understand the processes of cartilage mechanobiology. Initial pilot studies showed that articular chondrocytes are not accessible for many available biomolecular tools because they are encapsulated in cartilage dense ECM. To overcome this problem, many alternative experimental models lacking the intact cartilage ECM, were introduced to let deep biomolecular analysis of chondrocytes response to mechanical stimulation.

In this review, we discussed that this is not always the most suitable approach and because of that in this review article, we pointed to the importance of the proper cartilage ECM and chondrocytes connection in mechanobiological studies of this tissue. We think that we were able to show that the use of a model without key factors regulating chondrocyte response to load might lead to contradiction or not fully accurate results. That is why we propose that more emphasis should be directed into use of intact cartilage experimental models in mechanobiology studies of this tissue. To achieve this, more effort should be directed into developing and use of techniques that allow advanced biomolecular examination of the chondrocytes in intact cartilage.

Some of these techniques already exist. Recently, Venkatesan et al. proved that effective gene transfer of chondrocytes in intact cartilage is possible [99]. This was achieved with the use of direct application of a recombinant adeno-associated virus (rAAV) vector [99]. Using this method an effect of stable and efficient TGF-β overexpression was observed later proved by enhanced chondrocyte proliferation, survival and anabolic activity in intact cartilage [99]. Latest research of Pi et al. showed also that successful delivery of siRNA into articular chondrocytes in intact cartilage is possible [101]. Using chondrocyte-homing peptides Pi et al. were able to deliver siRNA to the chondrocytes to silence Hif-2α in the cartilage and prevent cartilage degeneration in arthritic mice joint [100]. Moreover, current development of engineered antibody fragments provides technology of highly improved penetration abilities of dense tissues and provides new possibilities in the antibody targeted biomolecular techniques [102]. The antibody formats used today are in a size range between 80 to 15 kDa so they might be good candidates to target chondrocytes in intact cartilage. Moreover, together with the development of confocal microscopy research, also in articular cartilage mechanobiology [72], they serve as potential new tools for visualization of chondrocytes response for mechanical loading in the intact cartilage.

Notably, adjustable and non-invasive murine joint loading model shows a great potential for articular cartilage mechanobiological studies [103]. This experimental model allows studying changes within the cartilage after externally controlled murine joint loading [103]. This model not only keeps all of key advantages of in-vivo models, but also takes also into account the role of other joint compartments like synovial fluid in articular cartilage mechanobiology.

As we show in this review, there are a number of limitation of todays commonly used experimental models for articular cartilage mechanobiology since they lack the main transducer of mechanical signals to the chondrocytes, the cartilage ECM. We think that it is important to be aware of these limitations and put more effort into use of experimental models that can truly replicate all the crucial connection of chondrocytes and ECM. This will allow a better understanding of the process of cartilage mechanobiology and avoid contradictory or conflicting results.
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Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling

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ABSTRACT

**Objective**: Transforming growth factor beta (TGF-β) in articular cartilage can signal via two routes, the ALKS/Smad2/3P and the ALK1/Smad1/5/8P route, the first being protective and the latter favoring chondrocyte terminal differentiation. Since biomechanical factors are known to play an essential role in osteoarthritis (OA) initiation and progression, we investigated if excessive mechanical compression can alter TGF-β signaling in cartilage shifting it from ALKS/Smad2/3P to ALK1/Smad1/5/8P pathway, favoring terminal differentiation of chondrocytes.

**Design**: Articular cartilage explants were harvested from bovine metacarpophalangeal joints. After equilibration, explants were subjected to unconfined dynamic mechanical compression (1 Hz) with 3 MPa (physiological) or 12 MPa (excessive) stress. After different time intervals samples were frozen and mRNA levels of selected genes were examined using real-time polymerase chain reaction.

**Results**: In articular cartilage compressed with 3 MPa and also 12 MPa stress the expression of Smad2/3P responsive genes bSerpine1, bSmad7 and bAlk5 was up-regulated, whereas the expression of Smad1/5/8P responsive gene bId1 was down-regulated. Furthermore, the expression of bTgfβ1 was significantly up-regulated in both compression groups. When ALKS/Smad2/3P pathway was blocked with a selective ALK4/5/7 inhibitor, the effect of excessive mechanical compression on bSmad7 and bAlk5 expression was prevented.

**Conclusions**: Here we show that excessive mechanical compression alone is not able to shift TGF-β signaling toward the ALK1/Smad1/5/8P pathway. In contrast, we show that mechanical compression not only with physiological but also with excessive stress can activate Smad2/3P signaling, which is known to be protective for articular cartilage and to block chondrocyte terminal differentiation.
1. INTRODUCTION

The major characteristic of osteoarthritis (OA) is articular cartilage destruction [1]. Notwithstanding the increasing interest in OA research, its etiology is still poorly understood. However, it is known that conditions that result in increased load transmission, like joint instability or local articular cartilage mechanical overloading e.g. due to meniscus resection, lead to a dramatically increased risk for OA development [2, 3].

Articular chondrocytes are highly mechanosensitive cells. In various experimental models, from monolayer, 3D cultures to tissue explants, it has been shown that physiological mechanical stimulation leads to activation of anabolic processes in chondrocytes, resulting in increased matrix production and deposition [4-6]. In contrast, excessive or injurious mechanical loading has catabolic effects and is related to reduced extracellular matrix (ECM) proteins biosynthesis, elevated production and activity of proteolytic enzymes and subsequent increased matrix destruction [7-9]. Interestingly, mechanical stimulation can stimulate chondrocytes directly but can also induce the release and activation of growth factors being stored in cartilage ECM [10].

One of the most abundant and potent growth factors in articular cartilage is Transforming Growth Factor beta (TGF-β). TGF-β is not only a potent inducer of cartilage ECM production [11] but also is a highly potent counteracting agent of catabolic factors, like IL-1β [12]. These studies suggest that TGF-β has chondroprotective capabilities. However, it has been shown that TGF-β can also induce OA like changes in healthy articular cartilage under specific conditions [13, 14].

TGF-β initiates cellular responses by signaling through a complex of transmembrane receptors. Recently, we and others have shown that in articular chondrocytes TGF-β not only binds to the TGF-β type II receptor and thereafter forms a complex with the TGF-β type I receptor, ALKS [15] but TGF-β can alternatively form a complex with another type I receptor, ALK1 [16, 17]. Importantly, activated ALKS signals by phosphorylation of intracellular transcription factors Smad2 and Smad3, while ALK1 signals by phosphorylation of Smad1, Smad5 and Smad8 [17, 18]. Whereas the Smad2/3 pathway, restrains chondrocytes terminal differentiation [19], Smad1/5/8 signaling promotes this process [20]. Terminally differentiating chondrocytes undergo phenotypic changes resembling alterations taking place in chondrocytes during OA development [21]. This fact not only explains enigmatic findings of TGF-β effect on articular cartilage, but also demonstrates a mechanism how TGF-β might contribute to OA development.

Recently two publications have shown that mechanical stimulation can activate TGF-β signaling in chondrocyte-like cells [22, 23]. However, the chondrocytes in these studies were embedded in an artificial matrix or cultured in monolayer. This means that the experimental models did not resemble physiological conditions, since these models were lacking a well-integrated and intact ECM, a crucial transducer of mechanical signals [24] and storage of growth factors [10]. We think that it is essential to study the effects of dynamic compression on TGF-β signaling in articular chondrocytes within their natural ECM.

We investigated if TGF-β signaling could be activated by dynamic mechanical compression in intact articular cartilage. Furthermore, since excessive loading is strongly associated with OA development, we examined if an excessive level of mechanical compression results in altered TGF-β signaling. We hypothesized that excessive mechanical compression (12 MPa) could be a trigger for altered TGF-β signaling favoring ALK1/Smad1/5/8P over ALKS/Smad2/3P in articular cartilage and in this way, promote the terminal differentiation of chondrocytes.
2. MATERIAL AND METHODS

2.1. ARTICULAR CARTILAGE EXPLANTS CULTURE

Full thickness articular cartilage explants were harvested from metacarpophalangeal (MCP) joints of skeletally mature cows (age range 3-6-year-old) obtained from the local abattoir within 3 hours post-mortem. Explants were isolated with a 4 mm biopsy punch (Kai-medical, Japan), and were 0.7 ± 0.12 mm thick. Afterwards, explants were placed in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco®, UK) with addition of Antibiotic-Antimycotic (contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (Gibco®, USA). No serum was added to the cultures. Explants were equilibrated for 48 hours in standard culture conditions (37°C, 5% CO₂ and 95% humidity).

2.2. DYNAMIC MECHANICAL COMPRESSION OF ARTICULAR CARTILAGE EXPLANTS

Following 48 hours equilibration explants were randomly assigned to two stimulation groups with a corresponding unloaded control specimen. Stimulation groups were: 3 MPa compression (physiological stress) and 12 MPa compression (excessive stress).

Chosen pressure levels were based on literature evaluation. The magnitude of contact pressure in natural knee joint during light to moderate activity ranges between 1 and 6 MPa [25]. However, immediately after meniscectomy contact stresses in articulating areas increase with two-to three-folds [26, 27].

The cartilage sample to be loaded was placed into the loading bioreactor chamber filled with DMEM/F-12 medium, between two plates. First, a preset compression force of 3 N was applied to guarantee a contact between plates and specimen (this value included into the desired pressure calculation). Then, explants were subjected to force controlled, unconfined, dynamic mechanical compression using a BOSE® ElectroForce® BioDynamic™ bioreactor (5160 BioDynamic System) equipped with a 50 lbf load-cell (BOSE Bose Corp. ElectroForce Systems Group, MN, USA). Compression was performed as a sine wave with frequency of 1Hz and desired pressure. Duration of compression was 30 min (1800 cycles). The whole compression procedure was performed in a cell culture incubator under standard culture conditions (37°C, 5% CO₂ and 95% humidity).

With each stimulation, a paired unloaded control explant was placed in the same medium as the loaded sample and positioned in the bioreactor’s incubator to guarantee the same environmental conditions.

After the compression procedure, the loaded articular cartilage and the unloaded control sample were placed in a culture incubator. At desired time intervals samples were frozen in liquid nitrogen and stored at -80°C.

To validate our compression model, we prepared paraffin sections, stained with Safranin O and Fast Green. Analysis of cartilage condition confirmed that 3 MPa compressed cartilage had an intact surface, normal matrix architecture, no enlargement/distortion of chondrons and no proliferative changes of chondrocytes. In contrast, 12 MPa compressed cartilage had surface discontinuity and vertical fissures extending into the mid zone (Supplementary Fig. 1).

2.3. INHIBITION OF ALKS DURING DYNAMIC MECHANICAL COMPRESSION

To block the ALKS/Smad2/3P pathway, the selective kinase activity inhibitor of ALK4/5/7 (SB505124) was used. SB505124 (Sigma-Aldrich, St. Louis, MO, USA) was applied in a
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concentration of 5 µM. Inhibitor was dissolved in Dimethyl sulfoxide (DMSO), which was used as a vehicle control. First, specimens were pre-incubated with SB505124 for 1 hour prior stimulation.

Then, articular cartilage explants were subjected to unconfined dynamic mechanical compression with 12 MPa (protocol described above) in a presence of SB505124 (5 µM). At 2 hours after compression samples were frozen and stored at -80°C. 5 ng/ml TGF-β1 (BIOlegend, CA, USA) stimulation for 6 hours was used as a positive control of SB505124 capability to block Smad2/3P signaling.

2.4. TOTAL mRNA ISOLATION AND QUANTITATIVE POLYMERASE CHAIN REACTION RT-PCR (Q-PCR)

Deep frozen articular cartilage samples were homogenized using a micro-dismembrator (B. Braun Biotech International, Melsungen, Germany) at 1500 RPM for 1 min. RNA was isolated from homogenate using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocol. Isolated RNA was reverse transcribed and produced cDNA was used to perform Q-PCR reaction with the StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s protocol. Primers used are included in Table 1. All Ct values for gene of interest were corrected for Ct values of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to obtain dCt values. All Q-PCR reactions were performed in duplicate.

Table 1. Primers used in Q-PCR. Only primers with standard curve slope deviated no more than 0.3 from standard curve slope of bovine GAPDH were used. Another criterion for primers selection was only one product showed by melting curve. Slopes of all selected primers were determined by standard curve from a series of five cDNA water dilutions, in duplicate

<table>
<thead>
<tr>
<th>Gene</th>
<th>Slope</th>
<th>Forward primer (5‘ – 3‘)</th>
<th>Reverse primer (5‘ – 3‘)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Gaddh</td>
<td>3.2</td>
<td>CACCGACGGCAAGTTCACAC</td>
<td>TCTCGCTCCTGGAAGATGGT</td>
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<tr>
<td>Bovine All1</td>
<td>3.3</td>
<td>ACAACACAGTGCGTCAGACAGCA</td>
<td>TGTCGTGAGTGTCGAGAT</td>
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<tr>
<td>Bovine All5</td>
<td>3.5</td>
<td>CAGGACCACCTGCAAATAAATAGAATT</td>
<td>TGCCAGTTCAACAGGACCA</td>
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<tr>
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<td>3.1</td>
<td>GCTCGGTGACGCACCTTCCA</td>
<td>GATCGTCCGCTGGAACACA</td>
</tr>
<tr>
<td>Bovine Serpine1</td>
<td>3.3</td>
<td>CGAGCCAGCGGGACTTC</td>
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</tr>
<tr>
<td>Bovine Smad7</td>
<td>3.2</td>
<td>GGGTTTTCAGATTCACACATT</td>
<td>CTCCCATGATGCGCACCCAG</td>
</tr>
<tr>
<td>Bovine Tgfb1</td>
<td>3.2</td>
<td>AGTGGACATTAAACGGGTTTCAGT</td>
<td>GAATCCACTTCCAGCCCGAG</td>
</tr>
</tbody>
</table>

2.5. STATISTICAL ANALYSIS

All quantitative data were expressed as a grouped column scatter of multiple repeats with displayed mean (all experiments were repeated five times using cartilage isolated from different animals, N = 5). For each analysis data were checked for normality using the Shapiro-Wilk test. Linear mixed models with Bonferroni multiple comparison post tests were used to estimate the effect of time and compression (and interaction terms) on gene expression. Linear mixed models take into account the correlated nature of repeated measures on cartilage isolated from the same subject. One-Way ANOVA with Tukey’s multiple comparison post-test was used to determine the effect of the compression on gene expression in a presence of SB505124. The statistical
Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling

3. RESULTS

3.1. PHYSIOLOGICAL AND EXCESSIVE MECHANICAL COMPRESSION UP-REGULATES DOWNSTREAM GENES OF SMAD2/3P SIGNALING PATHWAY

We examined the expression of a specific downstream gene Serpine1 (PaI), being activated by Smad2/3P route [17, 28]. Serpine1 gene promoter has been shown to contain specific Smad3/Smad4 binding elements [29], which substantiates the role of Serpine1 gene as a highly specific marker of the activated Smad2/3P signaling route.

At the 2 hours time point, 3 MPa mechanical compression up-regulated bSerpine1 with 14.9- fold ($2^{5.9}$) (p = 0.001) while 12 MPa compression up-regulated it with 32- fold ($2^{5.09}$) (P < 0.0001) (Fig. 1A). At 6 hours after compression the expression of bSerpine1 in unloaded control increased when compared to 2 hours time point. At the 6 hours time point, in both stimulation groups the bSerpine1 expression was no longer up-regulated when compared to unloaded control (Fig. 1A). At 24 hours after compression, changes of bSerpine1 expression were noticed in unloaded control. Comparison of bSerpine1 expression among related stimulation groups within 2 and 24 hours time point showed a very significant down-regulation of bSerpine1 with 11.3- fold ($2^{5.15}$ in 3 MPa, P = 0.001) and with 10.5- fold ($2^{4.5}$) in 12 MPa compressed articular cartilage (P = 0.002) (Fig. 1A).

![Fig. 1. Relative mRNA expression of Smad2/3P signaling pathway downstream genes (bSerpine1 (A) and Smad7 (B)), at 2, 6 and 24 hours after dynamic mechanical compression.](image-url)
bSerpine1 expression was unstable in unloaded controls with time and this complication could obscure the effects of loading on TGF-β signaling in dynamically compressed articular cartilage. Therefore, we decided to analyze the expression of a second gene that has been shown to be responsive to Smad2/3P signaling, Smad7 [30, 31].

At the 2 hours time point, 3 MPa compression up-regulated bSmad7 expression by 8-fold (2.13, P < 0.0001) and 12 MPa compression up-regulated it by 12.1-fold (21.12, P < 0.0001) (Fig. 1B). Most importantly, the expression of bSmad7 in unloaded control was not affected by culture time and remained stable. This allowed us to draw more clear conclusions than analyzing bSerpine1 expression. At 6 hours after compression bSmad7 remained up-regulated only in 12 MPa compressed articular cartilage. This up-regulation was significant when compared to the unloaded control (P = 0.032) as well as when compared to 3 MPa compressed cartilage (P = 0.005). However, in both stimulation groups bSmad7 expression was significantly lower (P < 0.0001) when compared to its expression in analogous stimulation groups at 2 hours after compression (Fig. 1B). At the 24 hours time point, bSmad7 expression levels in both compression groups continued to decrease. This reduction was significant in both compression groups (P < 0.0001 for 12 MPa) (P = 0.048 for 3 MPa) when compared to 6 hours time point.

3.2. PHYSIOLOGICAL AND EXCESSIVE MECHANICAL COMPRESSION INHIBITS EXPRESSION OF DOWNSTREAM GENE OF SMAD1/5/8P SIGNALING PATHWAY

Id1 expression was influenced by compression not earlier than at the 6 hours time point.

Id1 expression was influenced by compression not earlier than at the 6 hours time point. Then Id1 was down-regulated in both compression groups (P = 0.017 for 3 MPa) (P = 0.004 for 12 MPa) when compared to unloaded control (Fig. 2). At the 24 hours time point, the Id1 expression continued to decrease. In 3 MPa compressed cartilage it was almost 13-fold lower (21.7, P < 0.0001) and in 12 MPa compressed cartilage was nearly 78.8-fold lower (214.3, P < 0.0001) than in unloaded controls.

![Fig. 2. Relative mRNA expression of Smad1/5/8P signaling pathway downstream gene (Id1) at 2, 6 and 24 hours after dynamic mechanical compression](image-url)

Id1 is a highly specific downstream gene for the Smad1/5/8P signaling route [17, 28]. Id1 promoter has been shown to contain Smad1/Smad4 binding elements, which establish Id1 gene as a highly specific marker for activation of Smad1/5/8 route [32]. That is why we analyzed...
3.3. PHYSIOLOGICAL AND EXCESSIVE MECHANICAL COMPRESSION UP-REGULATES EXPRESSION OF bTGF-β1

To evaluate if dynamic mechanical compression can influence the expression of bTGF-β1 we analyzed its expression in 3 MPa and 12 MPa compressed articular cartilage at 2, 6 and 24 hours after compression and compared it to unloaded controls.

At 2 hours, Tgfβ1 was significantly up-regulated with 3-fold (2\(^{16}\) \(^{15}\)) in 3 MPa compressed articular cartilage (\(P < 0.0001\)) as well as in 12 MPa compressed cartilage (2.8-fold \(2^{15}\), \(P < 0.0001\)) when compared to the unloaded controls (Fig. 3). Even after 6 hours, a significant \(bTgfβ1\) up-regulation could be observed, as in 3 MPa compressed cartilage \(bTgfβ1\) was up-regulated with 3.2-fold (\(2^{17}\), \(P < 0.0001\)) and in 12 MPa compressed cartilage \(bTgfβ1\) was up-regulated with 4.5-fold (\(2^{12}\), \(P < 0.0001\)) (Fig. 3).

At 24 hours after mechanical compression \(bTgfβ1\) expression in both stimulation groups was significantly down-regulated (\(P = 0.005\) for 3 MPa and \(P = 0.028\) for 12 MPa compressed cartilage) (Fig. 3). Remarkably, within all-time intervals after stimulation we did not observe any significant differences between \(bTgfβ1\) expression in 3 MPa and 12 MPa compressed articular cartilage.

3.4. PHYSIOLOGICAL AND EXCESSIVE MECHANICAL COMPRESSION INFLUENCES THE EXPRESSION OF TGF-β TYPE I RECEPTORS ALK5 AND ALK1

Since the chondrocytes response characteristics for TGF-β stimulation can be determined by the balance between receptors ALK5 and ALK1, we decided to investigate whether 3 MPa and 12 MPa mechanical compression of articular cartilage can affect the expression patterns of these receptors.

At the 2 hours time point, in 3 MPa and 12 MPa compressed articular cartilage the expression of \(bAlk5\) was increased when compared to unloaded control. 12 MPa mechanical compression up-regulated \(bAlk5\) expression significantly with 4.9-fold (\(2^{2.5}\), \(P = 0.003\)), 3 MPa mechanical compression also up-regulated \(bAlk5\) expression with 2.6-fold (\(2^{14}\)) but in this case the increase was not significant (Fig. 4A). At 6 hours after compression, \(bAlk5\) expression had restored to control levels (Fig. 4A). At the 24 hours time point, \(bAlk5\) expression decreased significantly in both stimulation groups not only when compared to unloaded control from this time point (\(P = 0.001\) for 3 MPa and 12 MPa compressed cartilage) but also when compared to \(bAlk5\) expression in analogous experimental groups at 6 and 2 hours after mechanical compression (Fig. 4A).
Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling

Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling.

Fig. 4. Relative mRNA expression of TGF-β type I receptors - ALK5 (A) and ALK1 (B) at 2, 6 and 24 hours after dynamic mechanical compression. In both compression groups bALK5 expression was influenced by dynamic mechanical compression. bALK5 was significantly up-regulated only in 12 MPa compressed group when compared to unloaded control at 2 hours after compression. Already at the 6 hours after compression bALK5 expression went back to control levels. bALK1 expression was strongly down-regulated in both compression groups at the 24 hours time point. Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal N = 5). All significant outliers were detected by Grubbs' test and removed from dataset (for bALK1 dataset, N = 4). * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

The effect of compression on bALK1 expression was observed not earlier than at 6 hours time point (Fig. 4B) when bALK1 expression decreased in both stimulation groups in comparison to unloaded control. However, this decrease was significant only in 12 MPa compressed cartilage

(P = 0.006) (Fig. 4B). Analysis of bALK1 expression at the 24 hours time point showed significant decrease in expression of this gene, observed in 3 MPa compressed cartilage (P = 0.010) as well as in 12 MPa compressed cartilage (P < 0.0001) when compared to the unloaded control (Fig. 4B).

3.5. INHIBITION OF ALK4/5/7 BLOCKS COMPRESSION-INDUCED UP-REGULATION OF SMAD2/3P-RELATED GENES

We wanted to confirm whether the observed up-regulation of Smad2/3P response genes caused by 12 MPa mechanical compression was driven by active signaling via ALK4/5/7 and subsequent Smad2/3 signaling pathway. To achieve this, we analyzed whether the elevated expression of bSmad7 and bALK5 could be blocked with the ALK4/5/7 kinase activity inhibitor-S8505124.

We decided to analyze the expression of bSmad7 instead of bSerpine1 since this gene showed more stable basal expression than bSerpine1, observed in previous experiments. Moreover, bSmad7 expression showed to be more susceptible for different compression stresses.

Analysis of bSmad7 expression showed as expected, that addition of exogenous TGF-B1 to static culture, significantly up-regulated bSmad7 expression (P = 0.036) when compared to vehicle control group. This up-regulation could be completely blocked by S8505124 (P < 0.0001) (Fig. 5A). 12 MPa mechanical compression significantly up-regulated expression of bSmad7 in vehicle control group (P < 0.0001).
Physiological and excessive mechanical compression of articular cartilage activates Smad2/3 signaling

However, in a presence of SB505124, 12MPa mechanical compression was not able to up-regulate bSmad7 expression (P < 0.0001) (Fig. 5A). It is important to mention that results of this experiment clearly confirmed the validity of bSmad7 as a relevant and specific indicator of active Smad2/3 signaling pathway in our experiments.

As expected, the addition of exogenous TGF-β1 to the culture up-regulated bAlk5 expression significantly when compared to vehicle control (P < 0.0001). In a presence of SB505124 this up-regulation was no longer present (P < 0.0001) (Fig. 5B). In vehicle control, 12 MPa compression up-regulated bAlk5 expression significantly (P = 0.010) However, in the presence of SB505124 up-regulation of bAlk5 caused by 12 MPa dynamic mechanical compression was no longer observed (P < 0.0001) (Fig. 5B).

4. DISCUSSION

There is strong evidence that biomechanical factors play a crucial role in the events leading to OA initiation and progression [2, 3]. We have investigated if excessive mechanical compression can be a trigger for altered TGF-β signaling in the intact articular cartilage and influences pathways that have previously been shown to be involved in chondrocyte function and differentiation. We report that mechanical compression of articular cartilage with a physiological as well as an excessive stress activates Smad2/3 signaling pathways equally.

It has been previously reported that excessive TGF-β action can induce OA-like changes in articular cartilage [13, 14]. Furthermore, it has previously been reported that TGF-β, when signaling via the alternative ALK1 receptor and subsequent Smad1/5/8 activation, can be involved in chondrocyte terminal differentiation [14, 17]. Since increased load transfer is associated with OA initiation and progression, we postulated that excessive mechanical compression might be a
factor that modulates TGF-β signaling. However, we found that physiological as well as excessive mechanical compression up-regulated downstream factors, associated with Smad2/3 activation. This indicates that a signaling pathway which inhibits terminal, hypertrophic differentiation of chondrocyte and is essential for maintaining of healthy articular cartilage [33] is activated in both stimulation protocols.

We considered several reasons why no functional differences were observed between the two loading forces applied. First of all, we show the effect of a relatively short time compression. In the majority of the experiments showing the effect of compression on articular cartilage or chondrocytes, the time of loading procedure was longer, differing from hours to days of duration [5, 7, 37, 38]. However, we have chosen a short time of the compression to be able to observe the direct effects of stimulation on TGF-β signaling in chondrocytes with minimal effect of secondary pathways, like MAPK kinase, which have been shown to be activated already at 4 hours within/after dynamic mechanical compression [39]. Secondly, we have chosen to apply levels of the mechanical compression measured in human knee joint although we have used bovine tissue. It is highly possible that loading patterns and peak contact pressures in bovine MCP joint differ from the ones in human’s knee. However, bovine articular cartilage is a well-accepted model for biomechanical studies of cartilage [40-43] and it has been shown to respond to mechanical compression in a range from 6 to 10 MPa with increased apoptosis [44]. Third of all, our compression model was applying unconfined mechanical dynamic compression. We decided to use the unconfined compression since the confining chamber prevents nutrients and additional factors like growth factors or specific inhibitors (which we wanted to use in our experiments) from accessing the specimen during stimulation procedure [45]. We cannot exclude that the effect of confined compression in our experiments would show differences between two applied compression stresses. However, in the unconfined compression test, while the stress in the axial direction is always compressive, the stress in the radial direction is always tensile [45]. This means that chondrocyte at any location within the explant are squashed in the axial direction and stretched in the radial direction. That is why, we believe that unconfined compression is more representative to in situ contact conditions than confined compression since the latter does not produce any tensile stress [46].

Considering the onset of activation of downstream factors for Smad2/3P signaling we showed that this activation is most likely mediated by the ALK4/5/7 receptors, since this effect could be fully blocked with a specific ALK4/5/7 inhibitor. Despite using SB505124 we expect to observe the same results with use of another specific ALK4/5/7 inhibitor, e.g. SB431542. Not ruling out a role of ALK4 and ALK7, we speculate that the observed effects are a consequence of active TGF-β bound to the receptor ALK5 and activation of subsequent Smad2/3 signaling. We think that fast activation of Smad2/3P specific downstream genes caused by both compression stresses might indicate the activation of TGF-β in extracellular space from a pre-existing pool rather than by production and release of TGF-β de novo by the stimulated chondrocytes. It has been reported that TGF-β can be stored in large amounts (up to ~300 ng/g of all isoforms of TGF-β) in EMC of articular cartilage, however in the latent form [47]. Recent research showed that mechanical loading can operate as a significant activator of latent TGF-β [48] as Albro et al. demonstrated that shearing of synovial fluid can activate substantial amounts of latent TGF-β. However, they observed no activation of latent TGF-β in dynamically compressed devitalized articular cartilage [49]. This shows that TGF-β activation in mechanically compressed devitalized articular cartilage is controlled by living chondrocytes. Indeed, it has been shown that cells, can strictly control the activation of latent TGF-β by the action of proteolytic enzymes, especially MMP3 [50]. However, we cannot exclude totally that the observed effects are the consequence of signaling induced by other
members of the TGF-β superfamily, for instance activin A, which also signals via the Smad2/3P pathway.

We report also that dynamic mechanical compression induces the expression of bTgfb1 in intact articular cartilage (explants). Since TGF-β1 is considered a central regulator of articular cartilage repair and homeostasis, this indicates that mechanical compression is a crucial indirect mediator of these processes via induction of TGF-β production. Moreover, these results show that whereas TGF-β is being activated in articular cartilage by dynamic mechanical compression, the same signals might stimulate the production of TGF-β and renewal of its pool in ECM.

Our results are in line to Bougault et al. who showed that dynamic compression (0.5 Hz, 20-40 kPa, 5-30 min) of chondrocyte-agarose constructs promotes the phosphorylation of Smad2/3 but not Smad1/5/8 [22]. They have speculated that the observed effect is a consequence of mechanically driven release of soluble TGF-β which then binds to its receptor and triggers Smad2/3P signaling. We cannot exclude this process happening also in our model, but regarding the fact that TGF-β is always released in an inactive form and we observe fast up-regulation of TGF-β signaling downstream genes we think that our observations are the consequences of activation of latent TGF-β stored in the ECM.

The major limitation of our study is fact that we based our conclusions on gene expression data only. However, our experiments were performed on articular cartilage explants, where cellular proteins represent only 0.01% to 0.1% of the entire tissue volume. This makes the detection of specific proteins extremely difficult and reproducibility of the result at the protein level is poor. We have shown that physiological and excessive mechanical compression activates ALK5/Smad2/3P signaling pathway being known to protect articular cartilage and to block chondrocytes terminal differentiation. Furthermore, we show that Smad1/5/8P pathway is suppressed not only in physiologically and also in excessively compressed cartilage. Since, we have observed down-regulation of this Smad1/5/8P signaling pathway downstream gene caused by exogenous recombinant TGF-β1 in articular cartilage explants cultures (unpublished data) we postulate that observed suppression is a consequence of mechanically initiated activation of latent TGF-β and subsequent ALK5/Smad2/3P signaling at an earlier time point.

Results we report in this article point out, that excessive mechanical compression alone in the regime we applied, is not stimulating altered TGF-β signaling toward the potential deleterious Smad1/5/8P pathway. Furthermore, our findings demonstrate that short term, excessive mechanical compression induces the activation of Smad2/3P signaling pathway. However, we propose that additional factors, like inflammatory conditions and/or another type of loading (shear stress and/or combination of multiple types of loadings) which are also present after meniscectomy could alter TGF-β signaling in articular cartilage to promote TGF-β mediated terminal differentiation and OA like changes in overloaded articular cartilage.

5. ACKNOWLEDGMENTS

This study was supported by a grant from the Dutch Arthritis Association.

6. CONFLICT OF INTEREST

The authors have no conflict of interest.
Physiological and excessive mechanical compression of articular cartilage activates Smad2/3 signaling.

7. REFERENCES


Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling

8. SUPPLEMENTARY MATERIALS

Supplementary Fig 1. Effect of unconfined dynamic mechanical compression on articular cartilage explants. To validate our compression model, histology sections of compressed cartilage explants were prepared. Paraffin sections were stained with Safranin O and Fast Green. (A) 3 MPa Dynamically compressed articular cartilage with an intact surface, normal matrix architecture, no enlargement/distortion of chondrons and no proliferative changes of chondrocytes. (B) 12 MPa Dynamically compressed articular cartilage with surface discontinuity and vertical fissures extending into mid zone. (C) Unloaded control articular cartilage. Scale bar = 1000 μm.
Ageing is associated with reduction of mechanically induced activation of Smad2/3P signaling in articular cartilage
ABSTRACT

Objective: Mechanical signals control key cellular processes in articular cartilage. Previously we have shown that mechanical compression is an important ALK5/Smad2/3P activator in cartilage explants. However, age-related changes in the cartilage are known to affect tissue mechanosensitivity and also ALK5/Smad2/3P signaling. We have investigated whether ageing of cartilage is associated with an altered response to mechanical compression.

Design: Articular cartilage explants of two different age groups (young- 6 - 36 months old, aged- 6 – 13 years old) were subjected to dynamic mechanical compression with 3 MPa (physiological) or 12 MPa (excessive) load. Subsequently, essential cartilage extracellular matrix (ECM) components and tissue growth factors gene expression was measured in young and aged cartilage by QPCR. Furthermore, the ability of young and aged cartilage, to activate the Smad2/3P signaling in response to compression was analyzed and compared. This was done by immunohistochemical Smad2P detection and Smad3-responsive gene expression analysis.

Results: Aged cartilage showed a highly reduced capacity for mechanically-mediated activation of Smad2/3P signaling when compared to young cartilage. Compression of aged cartilage, induced collagen type II (Col2α1) and fibronectin (Fnt1) expression to a far lesser extent than in young cartilage. Additionally, in aged cartilage no mechanically-mediated up-regulation of bone morphogenetic protein 2 (Bmp2) and connective tissue growth factor (Ctgf) was observed.

Conclusions: We identified age-related changes in cellular responses to mechanical stimulation of articular cartilage. We propose that these changes might be associated with age-related alterations in cartilage functioning and can underlie mechanisms for development of age-related cartilage diseases like osteoarthritis.
1. INTRODUCTION

Mechanical signals have been shown to play a crucial role in cartilage formation as well as in tissue maintenance [1]. One of the major consequences of mechanical stimulation on cartilage is regulation of cartilage tissue matrix proteins expression, including aggrecan [2], collagen type II [3], fibronectin [4] and perlecan [5]. Furthermore, in chondrocytes, the expression of many growth factors crucial for cartilage maintenance like transforming growth factor beta 1-Tgfβ1 [6] connective tissue growth factor- Ctgf [7] and bone morphogenetic protein 2- Bmp2 [8] is modulated by mechanical signals. Additionally, various of intracellular signaling cascades are also mechanosensitive, including Smad2/3P [6], FAK [9] and ERK [10].

Age-related changes in cartilage affect the extracellular matrix as well as the chondrocytes. In articular cartilage the size, structure and sulfation characteristics of aggrecan in the extracellular matrix (ECM) change during ageing [11, 12]. Because aggrecan is the main determinant of the water content in cartilage ECM, changes in aggrecan result in reduction of tissue resiliency, hydration and finally volume. Furthermore, age-related glycation of collagens has been shown, causing increase in stiffness of the cartilage ECM [13, 14]. In addition, chondrocytes are susceptible to senescence during ageing [15]. Importantly, many studies have shown an age-related declined responsiveness and/or disrupted signaling of key cartilage growth factors, including IGF1 [16], BMP7 [17] and TGF-β [18, 19].

Particularly TGF-β is an essential anabolic growth factor in articular cartilage as it prevents deleterious chondrocyte terminal differentiation [20]. Importantly, TGF-β can signal via two different type I receptors- ALK5 and ALK1 in chondrocytes, being able to induce opposing effects in cartilage. There is evidence that TGF-β action in restriction of cartilage terminal differentiation is limited to TGF-β signaling via the type I receptor-TGFBRI (ALK5), followed by Smad2 and Smad3 phosphorylation [18, 20]. Recently we showed that mechanical compression potently activates Smad2/3P signaling in young mature articular cartilage which was apparently TGFBRI (ALK5) controlled [6]. Independently, in vivo studies in mice demonstrated a strong reduction in ALK5 expression in ageing articular cartilage [18, 21].

Considering the fact that current understanding of mechanotransduction events is based on the studies of tissues from young experimental models, there is a need to investigate how age-related changes, in the cartilage ECM and cells, influence cellular response in situ to mechanical stimulation. Evidence for altered mechanosensitivity of articular cartilage in many aspects could provide more insight into understanding age-related articular cartilage disease like osteoarthritis (OA).

The purpose of this study was to investigate if aged articular cartilage, responds differently to dynamic mechanical compression than young cartilage. To investigate this, we analyzed the effect of physiological (3 MPa) and excessive (12 MPa) mechanical compression on the gross structural changes, expression of ECM components together with essential cartilage growth factors and activation of Smad2/3P signaling in young and aged articular cartilage.

2. MATERIALS AND METHODS

2.1. ARTICULAR CARTILAGE EXPLANTS CULTURE

Bovine articular cartilage explants were harvested from metacarpophalangeal joints (MCP) of two different age groups (exact ages, established on original abattoir documentation are provided in figure legends of each experiment and in Supplementary Table 1 and 2). Joints were obtained from the local abattoir within 3 hours post mortem. Full cartilage thickness
2.4. ARTICULAR CARTILAGE STRAIN MONITORING DURING DYNAMIC MECHANICAL COMPRESSION

The displacement of cartilage explants was monitored during the complete duration of dynamic, force controlled mechanical compression with 3 MPa or 12 MPa pressure. Compressions were performed as described before [6]. Data were recorded with WinTest® software (BOSE ElectroForce, USA). Displacement values were corrected for the thickness of the specimen (measured on histological section of unloaded controls) to calculate strain values.

2.5. TOTAL mRNA ISOLATION AND QUANTITATIVE RT-PCR (Q-PCR)

mRNA isolation and quantitative RT-PCR were performed exactly like described before using primers included in Table 1 [6]. Full detailed description is included in supplementary materials.

2.6. IMMUNOHISTOCHEMICAL (IH) ANALYSIS

At 2 hours after compression, samples were fixed over night at 4°C in 10 % phosphate buffered formalin. Specimens were dehydrated and embedded in paraffin. 7 μm thick sections were cut and mounted on Superfrost™ Plus Microscope Slides (Thermo Scientific, USA). Then, the immunohistochemistry for c-terminally phosphorylated SMAD2P (rabbit mAb anti Phospho-Smad2 (Ser465/467) (1:100) (Cell Signalling Technology, Danvers, Massachusetts, USA) was performed as previously described [21].
Table 1. Primers list. Only primers with efficiency between 93% and 105% were used

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Full gene name</th>
<th>Ref seq</th>
<th>Product Length</th>
<th>Efficiency</th>
<th>Forward 5’→ 3’</th>
<th>Reverse 5’→ 3’</th>
</tr>
</thead>
</table>
| bSmad7      | transforming growth factor, SMAD family member 7 | NM_1740 | 90             | 100.92%    | CACCCACGGGGATTTCA | TGTGGCTTCGTAAGAT
| bHspg2      | heparan sulfate proteoglycan 2 | NM_1741 | 80             | 93.07%     | GGAAGTGGCGGATTTCA | CAGGAAGATCGGTTG
| bFn1        | collagen, type I, alpha 1 | NM_1742 | 72             | 100.92%    | CGGGTGCTTTGAGATTT | TCAAAGGACTGTTG
| bFn1        | fibronectin 1 | NM_1743 | 55             | 105.35%    | CCAGCTACCCGACTATT | TCTTCTACCACGACGACT
| bMappl      | bone morphogenetic protein 2 | NM_1744 | 73             | 105.35%    | TGATCGAGTACCGGTCA | TCAAGGACTGTTG
| bMappl      | bone morphogenetic protein 2 | NM_1745 | 105.35%        |            |                |                |
| bMappl      | bone morphogenetic protein 2 | NM_1746 | 139            | 105.35%    |                |                |
| bMappl      | bone morphogenetic protein 2 | NM_1747 | 60             | 105.35%    |                |                |
| bMappl      | bone morphogenetic protein 2 | NM_1748 | 86             | 105.35%    |                |                |
| bMappl      | bone morphogenetic protein 2 | NM_1749 | 30.2           | 105.35%    |                |                |

2.7. COMPUTATIONAL SCORING OF SMAD2P IHE

To score the load induced Smad2P staining, first the detection threshold was set to detect only intense Smad2P staining. Obtained values of positive Smad2P staining were first corrected for ROI (region of interest). Then values of each experimental group were corrected for values of staining in unloaded controls (to show the load-induced Smad2P in each age). Finally, the load-induced Smad2P staining was corrected to the average cell number in a certain age group.

To score the nuclear localization of Smad2P staining, first the threshold was set to detect only Smad2P nuclear staining. In each scored section, it was verified that staining detected after thresholding was always located in the cell nucleus but not in the cytoplasm. Obtained values were corrected for ROI (region of interest), and then corrected for average cell number in the certain age group.

All scoring values were expressed as a % of young. Scoring was performed with LAS (Leica Application Suite, Leica Microsystems, Germany).

2.8. STATISTICAL ANALYSIS

Quantitative data of gene expression analysis were expressed as a grouped column scatter of multiple repeats with displayed mean. All experiments were repeated 5 times on material isolated from different animals, N = 5 (experimental setups are included in Supplementary Table 1 and 2). First all datasets were checked for normality using the Shapiro-Wilk test. Linear mixed models were used to estimate the effects of compression level and age on gene expression. Linear mixed models take into account the correlated nature of repeated measures on cartilage isolated from the same subject. All the analyses were performed with the statistical software packages: SPSS 20.0 (SPSS, Chicago, USA).

Additionally, linear mixed models were used to fit the individual strain profiles of dynamically compressed cartilage explants of different ages (Supplementary Table 2). The dependent variable was strain. The independent variables were the loading condition and age. Interaction terms between loading condition and age were included in the model. The intercept and the regression coefficients of time were treated as random effects. The estimated regression parameters with standard errors were used to calculate the mean strain profiles with 95% confidence intervals for each loading/age condition. These statistical analyses were performed using R version 3.1.2 with package ‘nlme’ (R Development Core Team).
3. RESULTS

3.1. AGE-RELATED CHANGES IN ARTICULAR CARTILAGE STRUCTURE

Histological comparison of articular cartilage of 2 years and 13 year old revealed a very prominent age-related reduction in cartilage thickness (Fig.1). In young cartilage an irregular transition of cartilage to bone, active remodeling and abundant vascularity was observed. In contrast, in aged cartilage the transition to the subchondral bone was straight and a clear tide mark was present (Fig.1). Furthermore, a significantly reduced number of chondrocytes was present in aged when compared to young cartilage (P = 0.0002) (Fig.1 and Supplementary Fig. 2).

![Fig. 1. Age-related changes in articular cartilage structure.](image)

Representative full thickness cross section of bovine articular cartilage with subchondral bone of a 2 years old individual (A) and of 13 years old individual (B).

3.2. ARTICULAR CARTILAGE OF DIFFERENT AGES Responds DIFFERENTLY to DYNAMIC MECHANICAL COMPRESSION

Histological evaluation showed that 3 MPa mechanical compression did not cause any severe structural changes in 1 year or 10 years old cartilage (Fig.2B and 2E, respectively) when compared to corresponding unloaded controls (Fig.2A and 2D, respectively). In both cases, intact surfaces, normal matrix architecture, no enlargement/distortion of chondrons were observed. However, the disappearance of the unstained superficial cartilage zone caused by 3 MPa compression was noticed. 12 MPa compression did not result in severe structural changes of 1 year old cartilage either (Fig.2C). However, in 10 years old cartilage, 12 MPa mechanical compression induced surface discontinuities in a form of vertical fissures (Fig.2F). Examination of the sections under polarized light confirmed that no vertical fissures were present in the unloaded control (Fig.2G) and 3 MPa (Fig.2H) compressed cartilage but in 12 MPa compressed 10 years old cartilage vertical fissures penetrated the mid-zone (Fig.2I).

Cartilage deformation as a function of time can deliver information about mechanical properties and water content of the tissue [22]. We analyzed load-induced cartilage deformation to compare young and aged cartilage mechanical properties. Individual strain (deformation) profiles showed typical patterns over time, starting with a steep increase in strain followed by a gradual increase and flattening of the strain profile (Fig.2I). Analysis of strain profiles showed that young cartilage was able to deform significantly more, starting from 1550 seconds (p = 0.04) compared to aged cartilage when subjected to 3 MPa mechanical compression (Fig.2I). Comparison of cartilage deformation during 12 MPa mechanical compression revealed no significant differences in the amount of the deformation between young and aged cartilage (Fig.2I).
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

3.3. AGEING AFFECTS THE INFLUENCE OF DYNAMIC MECHANICAL COMPRESSION ON THE EXPRESSION OF ARTICULAR CARTILAGE EXTRACELLULAR MATRIX COMPONENTS

Expression of cartilage ECM components is highly sensitive to mechanical signals [3]. We measured the impact of compression on expression of ECM components in young and aged cartilage. Dynamic mechanical compression had no effect on the expression of bAcan (Aggrecan) (Fig.3A). However, at 2 hours after compression a significantly diverse (3.9-fold, \(2^{\pm 0.5} \), \(P = 0.048\)) regulation of bCol2a1 expression with up-regulation in young and down-regulation in aged cartilage by 12 MPa compression was found (Fig.3B). Analysis of the bFn1 expression at 6 hours time point showed a significantly different regulation of bFn1 expression by 12 MPa compression in cartilage of different age (Fig.3C) (2.1-fold, \(2^{\pm 0.5} \), \(P = 0.001\)), with up-regulation in young cartilage and down-regulation in aged cartilage. Remarkably, at the 6 hours time point, in both stimulation groups, a down-regulation of perlecan (bHspg2) was observed in aged cartilage whereas no changes in bHspg2 expression were observed in young cartilage (Fig.3D). This resulted in significantly different changes in expression levels of bHspg2 in cartilage of different age (3.4-fold, \(2^{\pm 0.5} \), \(P = 0.002\) for 3 MPa and 2.3-fold, \(2^{\pm 0.5} \), \(P = 0.025\) for 12 MPa compressed cartilage) (Fig.3D).

3.4. AGEING AFFECTS MECHANICALLY-MEDIATED EXPRESSION OF ESSENTIAL CARTILAGE GROWTH FACTORS

Expression of many key cartilage growth factors, same like ECM components, is also mechanosensitive [6-8]. We studied if aged articular cartilage has an altered mechanically-mediated regulation of key tissue growth factors.
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

At 2 hours time point, a potent and significant (P = 0.001 for all cases) up-regulation of bTgfb expression was measured in both age and stimulation groups (Fig.4A). At 6 hours time point, in 12 MPa compressed cartilage up-regulation was significant for both age groups (P < 0.0001 for young cartilage and P = 0.001 for aged cartilage) (Fig.4A). At 2 hours after compression, a down-regulation of bCtgf in aged cartilage compressed with 12 MPa (P = 0.027) was observed. This down-regulation was significant when compared to young cartilage compressed with 12 MPa (6.3-fold, 2.15 ± 1 P = 0.003) (Fig.4B). At 6 hours time point, in both stimulation groups, an up-regulation of bCtgf was observed in young cartilage with down-regulation in aged cartilage. At this time point, a significantly opposed effect of mechanical compression on bCtgf expression regulation (10-fold, 2.15 ± 1 P < 0.0001 for 3 MPa and 48.1-fold, 2.15 ± 1 P < 0.0001 for 12 MPa compressed group) was identified in cartilage of different age. At 2 hours time point, a pronounced up-regulation of bBmp2 induced by both compression levels (P < 0.0001 for both stimulation groups) was seen, however only in young cartilage (Fig.4C). Remarkably, in aged cartilage bBmp2 was not affected by any level of mechanical compression (Fig.4C). This revealed significantly different regulation of bBmp2 expression by compression between young and aged cartilage compressed with 3 MPa (3.0-fold, 2.15 ± 1 P = 0.045) and 12 MPa (4.2-fold, 2.15 ± 1 P = 0.011) (Fig.4C). The up-regulation of bBmp2 expression in young cartilage was still present at 6 hours after the compression in both stimulation groups (P = 0.002 for 3 MPa and P < 0.0001 for 12 MPa) whereas in aged cartilage still no regulation of bBmp2 was observed.

Thus, significantly different regulation of bBmp2 in cartilage of different age was present in 3 MPa (2.2-fold, 2.15 ± 1 P = 0.048) and in 12 MPa group (5.2-fold, 2.15 ± 1 P < 0.0001).

Fig. 3. Influence of dynamic mechanical compression on the expression of cartilage ECM components in cartilage of different age. Relative expression of bAcan (A), bCol2a1 (B), bFn1 (C) and bHsp2 (D) in young and aged cartilage. 12 MPa dynamic mechanical compression induced significantly different regulation of bCol2a1 (B), and bFn1 (C) expression in cartilage of different age. Both levels of mechanical compression down-regulated bHsp2 only in aged cartilage (D). Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal). Age of cartilage was as follows: Young-three 7 months, and two 11 months old, aged-12, 10, 8 and two 9 years old. ~ P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

3.5. AGEING REDUCES MECHANICALLY-MEDIATED PHOSPHORYLATION OF SMAD2 IN ARTICULAR CARTILAGE

Previously we showed that mechanical compression can act as a significant inducer of Smad2/3P signaling in mature articular cartilage [6]. Here, we analyzed if ageing affects mechanically-mediated phosphorylation of Smad2 in articular cartilage.

A clear induction of Smad2P staining was observed in young cartilage compressed with 3 MPa (Fig.5A, e, h) when compared to unloaded control (Fig.5A, d, g). Evident induction of Smad2P staining was also observed in 12 MPa compressed young cartilage (Fig.5Ac, f, i) when compared to unloaded control (Fig.5Aa, d, g). A reduced level of Smad2P staining in aged compressed cartilage was detected when compared to young cartilage. This was visible in 3 MPa (Fig.5Ak, n) and 12 MPa (Fig.5Al, o) compressed aged cartilage as well as in unloaded control (Fig.5Aj, m). Computational scoring of Smad2P IH confirmed these observations (Fig.5B). Aged cartilage showed highly reduced load-induced Smad2P staining when compared to young cartilage. This was the case for 3 MPa (P < 0.0001) as well as for 12 MPa compressed cartilage (P < 0.0001) (Fig.5B).

Furthermore, prominent differences between dynamically compressed young and aged cartilage were observed in the localization of Smad2P staining. In young cartilage compressed with 3 MPa as well as with 12 MPa, Smad2P staining was mainly localized in the cell nuclei (Fig.5Ca, b) whereas in aged cartilage Smad2P staining was more predominantly present in the chondrocyte cytoplasm (Fig.5Cc, d). Computational scoring of Smad2P nuclear staining showed that in aged cartilage there is a significant reduction of nuclear Smad2P localization when compared to young cartilage (P < 0.0001). This was the case for both loading conditions (Fig.5D).
3.6. AGING REDUCES MECHANICALLY-INDUCED ACTIVATION OF SMAD3P SIGNALING REPORTER GENES

To investigate if the reduction of mechanically-induced TGF-β signaling in aged cartilage, indicated by the reduced Smad2P, is reflected in gene expression, the expression of downstream reporter genes for Smad3P in compressed cartilage isolated from individuals of different age was examined. These included - Serpine1 (Pai1), JunB and Smad7. All of these genes contain a Smad Binding Element in their promoter (23-25).

Because in cartilage, TGFBR1 (ALK5) is the main receptor activating Smad2/3 signaling pathway, the influence of age on basal expression of bTgfbr1 (bALK5) in bovine cartilage was analyzed. Comparison of bTgfbr1 expression levels between young and aged cartilage demonstrated significantly lower (2.4-fold, 21.3CI P = 0.002) bTgfbr1 expression levels in aged cartilage than in young cartilage (Fig.6A).

At 2 hours after compression a profound up-regulation of bSerpine1 expression was observed, especially in young cartilage (Fig.6B); 3 MPa mechanical compression of young cartilage up-regulated bSerpine1 expression (32-fold, 21.3CI P < 0.0001) whereas the same compression level in aged cartilage did not up-regulate bSerpine1 expression significantly (Fig.6B). Therefore, bSerpine1 expression was significantly different up-regulated by 3 MPa compression in cartilage of different ages (6.1-fold, 21.3CI P = 0.002) (Fig.6B). In 12 MPa compression group, a significantly higher bSerpine1 up-regulation was observed in young compared to aged cartilage (3.7-fold, 21.3CI P = 0.015) (Fig.6B). At 6 hours after compression significant bSerpine1 up-regulation was observed in young cartilage (P = 0.003 in 3 MPa and P < 0.0001 for 12 MPa compressed group) and in 12 MPa compressed aged cartilage (P = 0.006) (Fig.6B).
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

Both age groups, bSmad7 up-regulation was observed (D). Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal). Age of cartilage was as follows: Young-three 7 months, and two 11 months old, aged-12, 10, 8 and two 9 years old. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

However, only in 12 MPa compressed cartilage, a significantly different level of bSerpine1 up-regulation levels between young and aged cartilage were observed (5.9-fold, 2^1.053 p = 0.005) (Fig.6B) with higher expression levels in young cartilage.

Expression analysis of an alternative Smad3P responsive gene- bJunB confirmed the results of the bSerpine1 measurements. Only in young cartilage, bJunB was greatly induced at 2 hours after both levels of mechanical compression (22-fold 2^1.533 P < 0.0001 for 3 MPa and 25-fold 2^1.753 P < 0.0001 for 12 MPa) (Fig.6C). At the same time in aged cartilage, bJunB expression showed no response to any level of compression (Fig.6C). Therefore, bJunB expression responded significantly different to mechanical stimulus in young versus aged cartilage (17-fold, 2^1.03 P < 0.0001 for 3 MPa and 10-fold, 2^1.853 P < 0.0001 for 12 MPa stimulation group). At 6 hours time point the age-related differences were no longer detectable.

Expression levels of another Smad3P responsive gene- bSmad7 were analyzed but no age-related differences in regulation of bSmad7 expression by mechanical compression were detected (Fig.6D).

4. DISCUSSION

Articular cartilage performs a very important biomechanical function being at the same time a highly mechanosensitive tissue. Cartilage experiences various forms of loads and these loads have been shown to play an important role in tissue formation, physiology and
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage

maintenance [1]. However, cartilage accumulates a number of age-related changes in the ECM as well as in its cells [26]. Notwithstanding growing interest in the role of mechanical signals in cartilage biology, the knowledge on how age-related changes influence tissue mechanosensitivity is scarce. That is why the response of young and aged cartilage to physiological and excessive mechanical compression was investigated and compared. We report a reduced or loss of ability for regulation of various ECM components and essential tissue growth factors in aged cartilage by mechanical signals. However, most importantly, we identified a diminished ability for activation of Smad2/3 signaling as a response to mechanical load in aged cartilage. This indicates that age-related changes in articular cartilage have significant impact on the characteristic of tissue response to mechanical signals.

Previously our group has demonstrated that mature cartilage, when compressed, is activating Smad2/3 signaling which we hypothesized to be a consequence of latent TGF-β1 activation and subsequent signaling via TGFBR1 (ALK5) receptor [6]. In the present study, we show a highly reduced ability for mechanically-mediated Smad2/3P signaling activation in aged cartilage. Moreover, we observed that Smad2P in young compressed cartilage is localized in cell nuclei whereas in aged cartilage is more predominantly present in the cytoplasm. This indicates that particularly in young dynamically compressed cartilage Smad2/3P was translocated to the nucleus to control transcription of genes. Not ruling out the role of other receptors known to be able to activate Smad2/3P signaling (ALK-4 and ALK-7) we think that this might be a consequence of age-related loss in bTGFBR1 (ALK5) expression in articular cartilage which was shown in this study and also previously in murine cartilage [18]. The loss of ALK5 receptor would disable the function of the mechanically activated TGF-β1 growth factor and subsequent Smad2/3P signaling activation. Moreover, a reduced synthesis of TGF-β ligands [21, 27], could decrease the content of this ligands in aged cartilage ECM. This could negatively affect the extent of growth factor activation mediated by mechanical signals.

As previously reported, cartilage shows an age-related decrease in tensile fracture stress what indicates an alteration in biomechanical properties of this tissue when aged [28]. We show that higher pressure had to be applied on aged cartilage to achieve the same amount of deformation as in young cartilage. This indicates an increase in stiffness together with loss of water content in aged cartilage ECM. It is known that cells from many tissues are able to sense and respond to changes in ECM elasticity [29]. Because cartilage ECM is the major transducer of mechanical signals to the chondrocytes, increase in ECM stiffness could result in lower mechanosensitivity of aged cartilage and negatively affect mechanical signal transduction.

However, an alternative explanation for altered mechanically-mediated regulation of essential tissue growth factors and cartilage ECM components could be found in age-related changes in the expression of integrins [30] which are the main cell surface receptors transducing mechanical signals from the ECM and translating it to cell signaling pathways [31]. Because it has been shown that numerous of chondrocyte mechanosensitive signaling pathways are integrin-dependent [31], it can be speculated that alterations in the expression of integrins could have a significant impact on the mechanical signal transduction and further downstream gene expression regulated by load.

Numerous factors can control chondrocyte response to loads. Importantly, this also includes the cell’s epigenetic status. Additionally, many studies reveal an epigenetic drift with aging also in cartilage [32]. This could indicate that in our experiments genes which are not responding to mechanical compression in aged cartilage compared to young cartilage, like bBmp2, are epigenetically repressed or silenced. Indeed, an age-related epigenetically silencing in a promoter of another growth factor from the same family (BMP7) was found in cartilage [33].
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Moreover, according to the Encyclopedia of DNA Elements data (ENCODEx, http://genome.ucsc.edu/, release date of the genome assembly- 20.01.2015), two CpG islands are present in the Bmp2 promoter. It is known that DNA methylation, resulting in gene silencing, occurs mostly in CpG islands. Furthermore, ENCODE shows that several chromatin modifiers bind to the Bmp2 promoter, including Histone-lysine N-methyltransferase (EZH2), which also in cartilage is involved in silencing of gene expression [34]. Independently, it has been reported that direct modulation of histone deacetylase (HDAC) activity, so the modulation of cell epigenetic status, can interfere with mechanically-mediated gene expression [35]. Remarkably not all key growth factors genes were less responsive for mechanical signals in aged cartilage. The expression of bTGFb1 gene was as potently induced in aged cartilage as in young. This shows that aged cartilage is not less responsive in all studied aspects and there are pathways induced by mechanical signals which are not altered by ageing.

There are some limitations of our study. First of all, as we showed, aged cartilage has highly reduced thickness when compared to young cartilage. By this, during unconfined compression of cartilage explant the force could possibly be differently distributed in samples with different thickness. However, our experiments were performed with force controlled set up, so there was always the same amount of force applied on the explant, not depending on specimen thickness. Moreover, age-related decrease of cartilage thickness appear also in vivo [26], but aged cartilage will in general experience a similar magnitude of loading like young. Secondly, our conclusions are based on a bovine animal model and might not be directly applicable to humans. However, as we demonstrated, bovine cartilage shows age-related changes in tissue gross appearance similar to changes observed in humans [26].

There are numerous studies showing the importance of mechanical signals in articular cartilage homeostasis. There are also many studies demonstrating age-related changes in the articular cartilage cells and ECM. Nevertheless, to our knowledge this is the first report, connecting these two aspects and showing that aged cartilage responds differently to mechanical load compared to young cartilage. Here we report a disruption in Smad2/3 phosphorylation in aged articular cartilage, a pathway which is known to be protective for articular cartilage [20]. Our results demonstrate that the age of the articular cartilage greatly affects the response of this tissue to mechanical signals. Age-related alteration in cartilage mechanotransduction can point to mechanisms of age-related articular cartilage diseases like OA.

5. ACKNOWLEDGMENTS

This study was supported by a grant from the Dutch Arthritis Association (grant LLP-15).

6. CONFLICT OF INTEREST

The authors have no conflict of interest.
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage

7. REFERENCES


33. Loeser RF, Im HJ, Richardson B, Lu Q, Chubinskaya S. Methylation of the OP-1 promoter: potential role in the age-related decline in OP-1 expression in cartilage. Osteoarthritis Cartilage 2009; 17: 511-517.


8. SUPPLEMENTARY MATERIALS

Supplementary Table 1 Experimental setup for the gene expression analysis.

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<td>Old</td>
</tr>
<tr>
<td>Time point after compression</td>
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<td>Compression level</td>
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<td>12 MPa</td>
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</table>

Supplementary Table 2 Experimental setup for the articular cartilage strain monitoring during dynamic mechanical compression.

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<tr>
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<tr>
<td>Compression level</td>
<td>3 MPa</td>
<td>12 MPa</td>
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Supplementary Fig. 1 Schematic representation of Base Electroforce 5160 BioDynamic System set-up used for dynamic mechanical compression of cartilage explants. The load-cell on the top (above the specimen) and motor at the bottom.

Supplementary Fig. 2 Cellularity of articular cartilage in different age. Aged articular cartilage showed high reduction in cellularity when compared to young cartilage. Cartilage cellularity was scored on hematoxylin stained, full thickness (with sub-chondral bone) cartilage sections. This was done by computational system LAS (Leica Application Suite, Leica Microsystems, Germany). Age of cartilage was as follows: young-1, 2 and 3 years old, aged-two 10 and one 13 years old. *** P ≤ 0.001. Each dataset of certain age group consists of N = 18 measured values (6 different areas of the section measured in 3 different individuals).
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

**Supplementary Fig. 3.** Articular cartilage stained with Rabbit isotope negative control immunoglobulin. No unspecific staining was noticed in Rabbit IgG (DakoCytomation, Denmark) control antibody stained articular cartilage.
Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage

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Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage

ABSTRACT

Objective: Joint trauma which is frequently related with mechanical overloading of articular cartilage and is a well-established risk for osteoarthritis (OA) development. Additionally, reports show that trauma leads to synovial joint inflammation. In consequence, after joint trauma, cartilage is influenced by deleterious excessive loading combined with the catabolic activity of proinflammatory mediators. Since, the activation of TGF-β signaling by loading is considered to be a key regulatory pathway for maintaining cartilage homeostasis, we tested the effect of proinflammatory conditions on mechanically-mediated activation of TGF-β/Smad2/3P signaling in cartilage.

Design: Cartilage explants were subjected to dynamic mechanical compression in the presence of interleukin-1 beta (IL-1β) or osteoarthritic synovium-conditioned medium (OAS-CM). Subsequently, the activation of Smad2/3P pathway was monitored with QPCR analysis of reporter genes and additionally the expression of receptors activating the Smad2/3P pathway was analyzed. Finally, the ability for mechanically-mediated activation of Smad2/3P was tested in human OA cartilage.

Results: IL-1β presence during compression did not impair the up-regulation of Smad2/3P reporter genes, however the results were affected by IL-1β-mediated up-regulations in unloaded controls. OAS-CM significantly impaired the compression-mediated up-regulation of bSmad7 and Tgfb1. IL-1β suppressed the compression-mediated bAki5 up-regulation where 12 MPa compression applied in presence of OAS-CM down-regulated the Tgfb1. Mechanically-driven up-regulation of Smad2/3P reporter genes was present in OA cartilage.

Conclusions: Proinflammatory conditions partly impair the mechanically-mediated activation of the protective TGF-β/Smad2/3P pathway. Additionally, the excessive mechanical compression,
applied in presence of proinflammatory conditions diminishes the expression of the type II TGF-β receptor, a receptor critical for maintenance of articular cartilage.

1. INTRODUCTION

Mechanical signals have been shown to play a pivotal role in articular cartilage development, tissue maintenance but also tissue degradation. The mechanical microenvironment acts as a regulator of stem cell fate and leads the chondrogenesis in early embryonic development [1]. Later on, during normal daily activity, cartilage experiences loading within the physiological range of forces [2]. This has been shown to play an essential role in cartilage preservation and in the anabolic production of cartilage matrix molecules [3, 4]. On the other hand, abnormal or excessive cartilage loading, often related with joint trauma and local cartilage mechanical overloading e.g. as a result of meniscus resection [5], not only leads to direct cartilage damage but can drive the activation of catabolic processes in chondrocytes and cell apoptosis which leads to further tissue damage [6, 7].

Furthermore, trauma-related damage of articular cartilage and other joint tissues, like meniscus or ligaments, cause the release of tissue debris into joint space contributing to synovial inflammation [8]. Subsequently, inflamed synovium will secrete a number of soluble inflammatory mediators into the joint space, including cytokines and chemokines [9]. Mostly studied are interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), which have both been shown to suppress matrix synthesis and to promote catabolic processes in cartilage [10]. However, also other proinflammatory mediators, like interleukin-6 (IL-6) and interleukin-8 (IL-8), have been identified in synovial fluid of injured joints [9]. In consequence, joint trauma leads to a condition in which articular cartilage is influenced by deleterious excessive loading combined with a catabolic action of proinflammatory mediators.

Transforming growth factor beta (TGF-β)-induced SMAD family member protein Smad2/3 signaling has been shown as a crucial signaling in cartilage, since it prevents the deleterious terminal differentiation of cartilage cells [11]. Recently, we have shown that dynamic mechanical compression of healthy articular cartilage is a potent inducer of Smad2/3P signaling in chondrocytes [12]. Nevertheless, data indicate that the effects of cartilage excessive compression with a combination of catabolic action of proinflammatory mediators play a role in development of degenerative joint disease like osteoarthritis (OA) [13, 14]. That is because, joint injury is a well-established risk factor for OA [15] and inflammation has been shown to play a key role in OA development in posttraumatic joints [14].

Here, we hypothesized that compression in combination with proinflammatory conditions will be less effective in the induction of Smad2/3P signaling in articular cartilage. Subsequently, a lack of mechanically-mediated Smad2/3P would disrupt cartilage maintenance and lead to tissue loss. To test this hypothesis, we investigated the influence of proinflammatory mediators, IL-1β or osteoarthritis-conditioned medium, (OAS-CM) on 3 MPa (physiological) and 12 MPa (excessive) compression-mediated activation of Smad2/3P signaling in healthy bovine articular cartilage. Additionally, we tested if this combination of mechanical and biological factors affects the expression levels of the key TGF-β receptors. Finally, we tested if human osteoarthritic cartilage shows mechanically-mediated activation of TGF-β/Smad2/3P.
2. MATERIAL AND METHODS

2.1. ARTICULAR CARTILAGE EXPLANTS CULTURE

Metacarpophalangeal joints (MCP) of skeletally mature cows (age range 3-6-years-old), obtained from the local abattoir, were processed within 3 hours post-mortem to isolate full-thickness articular cartilage explants. Explants were isolated with use of a 4 mm biopsy punch (Kai-medical, Tokyo, Japan). After isolation, all explants were cultured in Dulbecco’s modified Eagle’s medium: nutrient mixture F-12 (DMEM/F-12) (Gibco®, Paisley, UK) containing Antibiotic-Antimycotic (containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (Gibco®, Carlsbad, CA, USA). Cartilage explant culture was carried out without serum. Afterward, explants were left for equilibration for 24 hours in standard culture conditions (37°C, 5% CO₂ and 95% humidity). The same protocol was used for human OA cartilage explants isolation and culture. Human OA cartilage was obtained from anonymized patients that underwent total knee joint-replacement surgery. Explants were isolated from adjacent areas with intact tissue structure (macroscopically inspected).

The study protocol for experiments with animal material obtained from abattoir material did not need animal ethics committee approval.

No consent from patients whose human material was used in this study was needed, because material was obtained from anonymized patients and no personal data was available and needed for our study. Therefore, the study protocol for the experiments with anonymized human material did not need the ethics committee approval.

2.2. DYNAMIC MECHANICAL COMPRESSION OF ARTICULAR CARTILAGE EXPLANTS IN THE PRESENCE OF PROINFLAMMATORY CONDITIONS

After 24 hours after isolation, explants were stimulated with hrIL-1β (1 ng/ml) (R&D Systems, Minneapolis, MN, USA) or with osteoarthritic synovium-conditioned medium OAS-CM 10% (v/v) (16)). Following 24 hours of culture in proinflammatory conditions (48 hours after explant isolation) explants were randomly assigned to compression groups with corresponding unloaded controls. Compression groups were: 3 MPa compression (physiological stress) and 12 MPa compression (excessive stress). Chosen pressure levels were based on previous evaluations, calculations and published data (12). Explants from stimulation groups were subjected to force-controlled, sinusoidal, unconfined, dynamic mechanical compression with 3 or 12 MPa pressure and frequency of 1 Hz for 30 minutes (1800 cycles), following the previously published protocol (12). HrIL-1β (1 ng/ml) or OAS-CM 10% (v/v) were present in the medium during the compression procedure. After the compression, the loaded articular cartilage and the unloaded control samples were placed back into medium with refreshed hrIL-1β (1 ng/ml) or OAS-CM 10% (v/v) and back into the culture incubator. At 2 hours after compression, samples were frozen in liquid nitrogen and stored at -80°C. Additionally, in parallel to the compression experiment, cartilage explants from another group were stimulated with TGF-β1 (10 ng/ml) (Biolegend, San Diego, CA, USA) in combination with hrIL-1β (1 ng/ml) or OAS-CM 10% (v/v) for 6 hours (these samples were also pre-incubated with proinflammatory mediators starting at 24 hours before compression).
2.3. TOTAL mRNA ISOLATION AND QUANTITATIVE RT-PCR (Q-PCR)

A micro-disembrator (B. Braun Biotech International, Melsungen, Germany) was used to homogenize deep-frozen articular cartilage samples with 1500 RPM for 1 minute. Afterward, using the RNasey Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s protocol, total RNA was isolated from homogenate. Isolated RNA was used in reverse transcription reaction to produce complementary DNA (cDNA). Obtained cDNA was used in the QPCR reaction which was carried out with the StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s protocol. Primers used are listed in Table 1. Ct values for genes of interest were corrected for the average Ct values of bovine glyceroldehyde-3-phosphate dehydrogenase (bGAPDH) and bovine ribosomal protein S14 (bRps14) to obtain Ct values (in case of bovine material) or they were corrected for the average Ct values of human GAPDH and human ribosomal protein S27 (human RPS27) (in the case of human material).

Table 1. Primers used for QPCR.

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</table>
3. RESULTS

3.1. THE EFFECT OF IL-1β ON MECHANICALLY-MEDIATED ACTIVATION OF TGF β/SMAD3P RESPONSIVE GENES

Previously, we have shown that dynamic mechanical compression can potently induce Smad2/3P signaling activation and TGF-beta1 gene upregulation in articular cartilage [12]. Here, we examined if IL-1β can affect mechanically-mediated upregulation of Smad3P-responsive genes (e.g., Serpine1, Smad7, and JunB) and bTgfb1.

A significant induction of bSerpine1 was observed in both compression groups when compared to unloaded controls (13.7-fold (23.8Ct), P < 0.0001 for 3 MPa and 38.2 25.3Ct, P < 0.0001 for 12 MPa) (Fig. 2A). In the presence of IL-1β (1 ng/ml), a significant bSerpine1 upregulation was still noticed in both compressed groups. Compression with 3 MPa induced upregulation of bSerpine1 by 5.3-fold (22.4Ct) (P = 0.03) and compression with 12 MPa induced 13-fold upregulation (23.7Ct) (P < 0.0001) when compared to unloaded controls treated with IL-1β (Fig 2a). Comparable level of upregulation was noticed in cartilage explants stimulated with 10 ng/ml of exogenous rTGF-β1 in a presence of IL-1β (7.7-fold, 22.9Ct) (P = 0.005) (Fig 2A).

Analysis of bSmad7 expression showed a potent and significant upregulation of Smad7 in both compression groups (17.5-fold, 24.1Ct, P < 0.0001 for 3 MPa and 35.1-fold, 25.1Ct, P < 0.0001 for 12 MPa) (Fig 2B). Remarkably, IL-1β treatment stimulated significant upregulation of bSmad7 in unloaded controls (P = 0.044), regardless of loading treatment. Nevertheless, in the presence of IL-1β, the compression-mediated upregulation of Smad7 was still present when compared to unloaded controls treated with IL-1β (Fig 2B). 3 MPa compression upregulated bSmad7 by 6.8-fold (22.8Ct, P < 0.0001) whereas 12 MPa compression upregulated it by 17.6-fold (24.1Ct, P < 0.0001). The upregulation of Smad7 caused by compression was similar to the one present in explants stimulated with 10 ng/ml of rTGF-β1 in the presence of IL-1β (10.4-fold, 23.4Ct) (P < 0.0001) (Fig. 2B). Further, analysis confirmed that bJunB was also potently and significantly upregulated by cartilage compression (Fig. 2C). 3 MPa compression induced a 14.6-fold upregulation of bJunB (23.9Ct) (P < 0.0001) and the 12 MPa compression caused a 25.8-fold upregulation of this gene (24.7Ct) (P < 0.0001). However, a very significant upregulation of bJunB (P < 0.0001) was also noticed in unloaded controls treated with IL-1β, regardless of loading treatment (Fig. 2C). Because of that, in the presence of IL-1β, a significant bJunB upregulation was observed only in 12 MPa compressed cartilage, 4.8-fold, 22.2Ct (P < 0.0001) (Fig. 2C). The level of this upregulation was comparable to the one detected in explants stimulated with 10 ng/ml of rTGF-β1 in the presence of IL-1β (Fig 2C).

Analysis of the expression of bTgfb1 demonstrated a significant upregulation of this gene by both levels of compression when compared to the unloaded controls (Fig. 2D). 3 MPa compression induced the expression of bTgfb1 by 4.2-fold (22.1Ct, P < 0.0001) and 12 MPa induced it by 6.9-fold (22.8Ct, P < 0.0001) (Fig 2D). In the presence of IL-1β, a significant induction of bTgfb1 expression by the compression was also observed (Fig 2D). 3 MPa compression induced a 2-fold upregulation of bTgfb1 (21.0Ct) (P = 0.033) and 12 MPa induced a 4.2-fold upregulation of this gene (22.1Ct) (P < 0.0001) when compared to unloaded controls treated with IL-1β (Fig 2D). This effect was comparable to the effect of exogenous 10 ng/ml rTGF-β1 stimulation in the presence of IL-1β.

Overall, fold induction by compression in the presence of IL-1β was lower, although not significant, than an absence of IL-1β, but this appears mainly to be caused by elevated basal expression of responsive genes in the presence of IL-1β.
Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage.

3.2. THE EFFECT OF OAS-CM ON MECHANICALLY-MEDIATED ACTIVATION OF TGF-β/SMAD3P RESPONSIVE GENES

Subsequently, the effect of 10 % OAS-CM on the loading-mediated regulation of the Smad3P-responsive genes and *bTgfβ1* was analyzed.

As before, mechanical compression with 3 MPa, as well as with 12 MPa, induced significant and potent upregulation of *bSerpine1* (P < 0.0001 in both cases; Fig. 3a). Remarkably, addition of 10 % OAS-CM induced a potent upregulation of *bSerpine1* in unloaded controls, regardless of compression treatment (12.8-fold, 23.7Ct, P < 0.0001) (Fig. 3A). Because of these, high levels of *bSerpine1* in unloaded controls, no significant induction of *bSerpine1* was observed in 3 MPa compressed cartilage in the presence of 10 % OAS-CM, however it was observed in 12 MPa compressed cartilage (12.6-fold, 23.7Ct, P < 0.0001) (Fig. 3A). Comparable level of *bSerpine1* upregulation was noticed in cartilage stimulated with exogenous rTGF-β1 in the presence of 10 % OAS-CM (Fig. 3A). Analysis of *bSmad7* expression showed potent and significant upregulation in 3 MPa and 12 MPa compressed cartilage (P < 0.0001 in both conditions) (Fig. 3B). In the presence of 10 % OAS-CM, a significant upregulation of *bSmad7* was noticed in unloaded controls (P = 0.014, 2.0-fold, 21.0Ct (Fig. 3B). Nevertheless, in the presence of 10 % OAS-CM, the upregulation of *bSmad7* induced by 3 MPa compression was still noticeable. Remarkably, the level of this upregulation was significantly lower (5.5-fold, 22.5Ct, P = 0.009) compared to the one in 3 MPa compressed cartilage without OAS-CM (Fig. 3B). In cartilage compressed with 12 MPa in the presence of 10 % OAS-CM the upregulation of *bSmad7* (P < 0.0001, 14.1-fold, 23.8Ct) was as potent as the one induced by the 12 MPa compression in the condition without 10 % OAS-CM (Fig. 3B). Comparable levels of *bSmad7* upregulation were noticed in cartilage stimulated with exogenous rTGF-β1 in the presence of 10 % OAS-CM. Analysis of *bJunB* showed analogous results.
Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage.

As in the case of bSerpine1, mechanical compression with 3 MPa, as well as with 12 MPa, induced significant and potent upregulation of bSerpine1 (P < 0.0001 in both cases) (Fig. 3C). Also, a significant induction of bJunB was observed in unloaded controls stimulated with 10 % OAS-CM (5.3-fold, 22.4Ct, P < 0.0001) (Fig. 3C). Regardless of this effect, in the presence of 10 % OAS-CM in both compression groups a significant upregulation of bJunB was observed (3.6-fold, 21.8Ct, for 3 MPa and 6.3-fold, 22.6Ct, for 12 MPa, P < 0.0001 in both cases), and this upregulation was similar to the one present in cartilage stimulated with exogenous 10 ng/ml TGF-β1 (Fig. 3C).

In conditions without 10 % OAS-CM, in both compression groups, a significant upregulation of bTgfb1 was found (P < 0.0001 in both cases) (Fig. 3D). In the presence of 10 % OAS-CM, 3 MPa also induced bTgfb1 upregulation (2-fold, 21.0Ct, P = 0.040), however this induction was significantly lower (2.5-fold, 21.3Ct, P = 0.049) than without OAS-CM (Fig. 3D). In 12 MPa compression groups this difference was not noticeable, as in presence of OAS-CM, the 12 MPa-induced bTgfb1 upregulation was as potent as the one observed in 12 MPa compressed cartilage without OAS-CM (Fig. 3D). The effect of compression in the presence of 10 % OAS-CM was comparable to the effect of exogenous 10 ng/ml TGF-β1 stimulation in the same conditions.

Overall, the effect of the OAS-CM on the loading-mediated upregulation of bSerpine1 and bJunB has been disrupted by the strong effect of the OAS-CM on the induction of these genes in unloaded controls. However, when this effect was not observed, a significantly lower loading-induced upregulation of bSmad7 and bTgfb1 was noticed in the presence of OAS-CM.

![Fig. 3](image-url) The effect of OAS-CM on mechanically-mediated activation of Smad3P responsive genes in bovine articular cartilage. The influence of 3 and 12 MPa dynamic mechanical compression, carried out with OAS-CM (red squares) or without (blue dots), on relative expression of bSerpine1 (A), bSmad7 (B), bJunB (C) and bTgfb1 (D). Dynamic mechanical compression with 3 as well as with 12 MPa, potently up-regulated bSerpine1, bSmad7, bJunB and bTgfb1 in condition without OAS-CM. In unloaded controls treated with OAS-CM, a significant bSerpine1, bSmad7 and bJunB up-regulation was observed. Regardless this issue, in presence of OAS-CM a mechanically-mediated up-regulation of bSerpine1, Smad7 JunB and Tgfb1 was noticed. Nevertheless, in presence of OAS-CM, the 3 MPa compression-mediated up-regulation of bSmad7 and bTgfb1 was significantly lower than the effect of 3 MPa in condition without OAS-CM. The effect of compression in the presence of OAS-CM was comparable to the effect of exogenous TGF-β1 stimulation in the presence of OAS-CM. Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal N=4). * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.
3.3. THE EFFECT OF INFLAMMATORY CONDITIONS AND MECHANICAL COMPRESSION ON THE EXPRESSION OF TGF-β RECEPTORS

Response of chondrocytes to TGF-β is determined by the balance of specific receptors for this growth factor (transforming growth factor, beta receptor I [TGFBR1 (ALK5)]) and transforming growth factor, beta receptor II (TGFBR2)). Here we analyzed whether inflammatory conditions are able to influence the loading-regulated expression of TGF-β receptors.

Analysis of bAlk5 expression showed that mechanical compression with 3 MPa as well as with 12 MPa could significantly induce expression of this receptor (P = 0.018 in both cases; Fig. 4A). Remarkably, IL-1β was able to completely inhibit the mechanically-mediated upregulation of bAlk5 expression induced by 3 MPa as well as by 12 MPa compression (Fig. 4A). This was in contrast with the effect of exogenous TGF-β (10 ng/ml), which even in the presence of IL-1β was able to significantly upregulate bAlk5 expression (4.7-fold, 24.7 Ct, P = 0.003). In the presence of 10 % OAS-CM, 3 MPa mechanical compression was still able to upregulate bAlk5 when compared to unloaded controls (2.58-fold, 21.37 Ct, P = 0.029). However, in the same conditions, 12 MPa was not able to upregulate bAlk5 (Fig. 4A). Nevertheless, the upregulation of bAlk5 induced by exogenous TGF-β was still observed in OAS-CM conditions also (3.4-fold, 21.78 Ct, P = 0.004).

When bTgfrb2 expression was analyzed, no effect of compression with 3 MPa as well as with 12 MPa was noticed (Fig. 4B) in the absence of proinflammatory conditions. Also in the presence of IL-1β, no effect of compression was observed. Further, in the presence of 10 % OAS-CM no effect of 3 MPa on bTgfrb2 was observed. Remarkably, in the presence of OAS-CM, 12 MPa mechanical compression even induced a downregulation of bTgfrb2 (2.25-fold, 21.16 Ct, P = 0.010), (Fig. 4B).

Fig.4. The effect of mechanical compression carried out in presence of inflammatory conditions on the expression of TGF-β receptors. The influence of 3 and 12 MPa dynamic mechanical compression, carried out with IL-1β (red squares) or with OAS-CM (green triangles) or without inflammatory conditions (blue dots), on the relative expression of bAlk5 (A) and bTgfrb2 (B). Dynamic mechanical compression with 3 MPa as well as with 12 MPa potently up-regulated bAlk5 in condition without inflammatory conditions. However, in the presence of IL-1β, 3 MPa as well as 12MPa mechanical compression had no influence on bAlk5 expression. In the presence of OAS-CM, only 3 MPa compression up-regulated bAlk5. Dynamic mechanical compression
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3.4. MECHANICALLY-MEDIATED ACTIVATION OF TGF-β/SMAD3P Responsive Genes in Human OA Cartilage

Analysis of bSerpine1 expression showed that both mechanical compression levels caused an upregulation of this gene in OA cartilage of all four patients. Statistical analysis showed that, overall the effect of 3 MPa compression was a significant bSerpine1 upregulation of 6.8-fold (mean upregulation) (22.76 Ct, P < 0.0001) in all patients and the overall effect of 12 MPa was a significant upregulation of 8.1-fold (23.03 Ct, P < 0.0001) (Fig. 1A). Subsequent examination of bSmad7 expression confirmed these results of bSerpine1 analysis. In OA cartilage of all four patients, an upregulation caused by both mechanical compression regimes was noticed (Fig. 1B). Statistical analysis showed that the overall effect of 3 MPa compression in all patients was a significant bSmad7 upregulation for 5.8-fold (mean upregulation) (22.52 Ct, P < 0.0001) and the effect of 12 MPa was a significant upregulation of 5.4-fold (22.44 Ct, P < 0.0001) (Fig. 1B). In all four patients, an upregulation of bTgfβ1 was also noticed. Statistical analysis showed that the overall effect of 3 MPa compression was a significant upregulation of bTgfβ1 for 2.2-fold (21.14 Ct, P < 0.0001) whereas in the case of 12 MPa compression, the overall effect was a significant upregulation of this gene of 2.5-fold (21.36 Ct, P < 0.0001) (Fig. 1D). Generally, both levels of the compression were able to induce the upregulation of all investigated Smad3P-responsive genes in human OA cartilage.

![Graphs showing the effect of mechanical compression on gene expression](image)

**Fig. 1.** The effect of dynamic mechanical compression of human OA cartilage on the expression of Smad3P responsive genes. Statistical analysis showed that overall effect of the compression with 3 MPa as well as with 12 MPa of human OA cartilage explants was the induction of the up-regulation of bSerpine1, bSmad7, bTgfβ1 and bAlk5 in all four different donors. Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat of different human OA cartilage explant). * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
4. **DISCUSSION**

Previously we have shown that excessive dynamic mechanical compression alone was not able to induce deleterious alterations of TGF-β signaling in articular cartilage [12]. Of note, we observed that even excessive compression induced a pathway that has been shown to be crucial for cartilage maintenance- Smad2/3P signaling [11]. These results were not fully in line with previous observations, which suggested that overloaded cartilage has increased chondrocytes catabolic activity and matrix degradation [6, 17, 18]. However, as we discussed in our previous manuscript, additional proinflammatory conditions present during mechanical compression could alter or impair the mechanically-mediated activation of TGF-β signaling in articular cartilage. The rationale for this statement might lay in the data which shows that overloading of the cartilage, that lead to tissue degeneration and OA development, in vivo takes place predominantly after joint trauma [15], where inflammation of the joint is a common fact [9, 19]. That is why, as a follow-up, here we tested if proinflammatory conditions in combination with compression can impair the mechanically-mediated activation of TGF-β/Smad2/3P signaling in articular cartilage.

Interplay between proinflammatory mediators and TGF-β signaling has been shown already. Van Beuningen et al. showed that TGF-β is able to suppress IL-1β-induced proteoglycan degradation in vivo [20, 21]. Furthermore, TGF-β has been shown to counteract IL-1β effect on several levels, not only by down-regulation of its receptor but also by up-regulation of the IL-1 antagonist IL-1Ra [22, 23]. Separately, compression of articular cartilage has been shown to counteract the IL-1β-induced catabolic activity in chondrocytes [24] which might be associated with mechanically-mediated TGF-β action [12, 25]. On the other hand, our analysis showed that IL-1β in the concentration of 1 ng/ml has no significant effect on mechanically-mediated activation of Smad2/3P signaling response genes like Serpine1, Smad 7 or JunB in articular cartilage. This showed that IL-1β alone and/or IL-1β-induced signaling in cartilage are not able to interfere with activation of Smad2/3 signaling by compressive load in intact articular cartilage.

We observed however, that IL-1β was able to block the mechanically-mediated up-regulation of Smad2/3P activation receptor (ALK5). Considering the fact that mechanically-mediated up-regulation of ALK5 is actually depending on the ALK5 receptor [12] and no reduction of ALK5 receptor caused by IL-1β treatment only was observed, it can be concluded that inhibition of mechanically-mediated bALK5 up-regulation by IL-1β is regulated by modulation of intracellular pathways.

The canonical intracellular TGF-β signaling pathway involves phosphorylation of Smad2/3 followed by subsequent formation of a complex with Smad4, the common-Smad. Subsequently, this complex translocates to the nucleus where it binds DNA and regulates gene transcription [26]. On the other hand, in chondrocytes IL-1β signals mainly by activation of nuclear factor kappa B (NF-kB) signaling, a well-validated major catabolic pathway in cartilage degradation [27, 28]. In fact, intracellular interaction of TGF-β-induced Smad signaling and IL-1β-induced signaling has already been shown [29]. Roman-Blas et al. showed that IL-1β-induced NF-kB signaling is able to reduce DNA-binding activity of Smad3/4- the main TGF-β signaling-induced gene regulating complex in adult chondrocytes [30]. However, the lack of significant influence of IL-1β on mechanically-mediated induction of Serpine1, Smad 7 or JunB, so the Smad3P reporter genes that should also be regulated by Smad3/4 complex [31-33], appears in contrast with results of Roman-Blas et al. Nevertheless, in results published by Roman-Blas et al., the most prominent effects of IL-1β on DNA-binding activity of Smad3/4 were observed in human OA chondrocytes and not in healthy bovine cartilage cells [29]. Moreover, results published by Roman-Blas et al. were observed in the isolated monolayer cells but not in the intact cartilage explants like we used.
As it has been shown, chondrocyte isolation for later monolayer culture has an effect on catabolic intracellular signaling pathways [34] including NF-κB signaling [35] which might explain the differences in observations.

Nevertheless, inflamed synovium produces more proinflammatory cytokines than only IL-1β and as shown by Heldens et al. the catabolic effects of mediators produced by inflamed synovium extend beyond the effect of IL-1β only [36]. Because of that, we tested if OA synovium-conditioned medium, containing multiple proinflammatory mediators [9] can have an impact on the mechanically-mediated activation of the Smad2/3 pathway in articular cartilage. Our data showed that physiological as well as excessive mechanical compression is able to induce the up-regulation of Smad3 responsive genes (bSerpine1, blunB) also when applied in the presence of OAS-CM. Nevertheless, we also noticed a very prominent effect of OAS-CM in unloaded cartilage which up-regulated the expression of Serpine1 and JunB regardless of the loading treatment which was most likely caused by soluble factors contained in OAS-CM [37].

This effect was not observed in case of Smad7 and Tgfb1 expression. Stable expression levels of Smad7 and Tgfb1 allowed us to notice that inflammatory mediators contained in OAS-CM significantly impaired the activation of these genes by physiological compression. One of the possible explanations for this particular response might be explained by the action of TGF-β-activated kinase 1 (TAK1). TAK1 has been identified as a TGF-β/BMP activated intracellular component of mitogen-activated protein kinases (MAPK) pathways [38]. However, TAK1 has been also shown to be a central intracellular kinase for a number of important inflammatory cytokines [39]. Hoffman et al. has shown that TAK1 plays an essential role in Smad2/3 signaling modulation. They demonstrated that overexpression of TAK1 or activation of TAK1 leads to accumulation of all activated Smad2/3 in the cell cytoplasm with their parallel depletion from the nucleus [40], which would impair gene regulation by Smad3 and stay in line with our results.

These results might explain why in the presence of inflammatory mediators present in OAS-CM, the loading-mediated up-regulation of TGF-β responsive genes are impaired.

Analysis of the expression levels of the TGF-β/Smad2/3P responsive genes did not fully confirm our hypothesis that combination of proinflammatory conditions with excessive loading could alter the TGF-β signaling in cartilage. All analyzed TGF-β responsive genes were up-regulated by excessive mechanical compression applied in presence of either IL-1β or OAS-CM, however some to a significantly lesser extent demonstrating the inhibitory effect of the proinflammatory conditions on the effect of the physiological compression. Moreover, human OA cartilage, which is known to be exposed to proinflammatory conditions for a prolonged period [41], still demonstrated induction of Smad2/3 genes. However, we cannot conclude anything about the induction level since healthy human cartilage was not available for comparison.

Nevertheless, most importantly, the analysis of the bTgfb2 expression revealed a prominent down-regulation of the bTgfb2 by excessive compression only when applied in the presence of OAS-CM. TGF-β type II receptor (TGFBR2) is the receptor that directly binds TGF-β ligand which induces the recruitment and subsequent phosphorylation of the type I receptor, thereafter followed by further Smad signaling. It has been shown that TGFBR2 is absolutely critical for maintenance of articular cartilage and loss of this receptor results in total loss of responsiveness to TGF-β which drives chondrocyte terminal differentiation and development of OA [42]. Baugé et al. showed that proinflammatory mediators like IL-1β can reduce Tgfb2 expression in OA monolayer chondrocytes [43]. We did not observe a similar effect of proinflammatory conditions alone, but this might be due to different sensitivity of OA monolayer chondrocytes (used by Baugé et al.) for the used cytokines than healthy cartilage explants [44]. Here we observed the down-regulation of Tgfb2 at 2 hours after the compression. This time
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point is too early to see the functional consequences Tgfbr2 gene down-regulation on TGF-β responsive gene expression. In the future, it is important to check if the down-regulation of the Tgfbr2 caused by excessive mechanical compression applied in the presence of inflammatory conditions will result in reduced protein expression, if this loss TGFBR2 is temporary or permanent and most importantly if and what are the physiological consequences of this loss.

The major limitation of our study is fact that our conclusions were based only on gene expression data. However, this was mainly driven by the fact that our experiments were performed on the model of intact articular cartilage explants, where cellular proteins are only 0.01 to 0.1 % of the entire tissue volume. High amounts of big ECM protein make the detection of specific membrane or phosphorylated proteins extremely difficult and reproducibility of the result at the protein level is poor.

Joint trauma is a well-established risk for the development of OA and this is often attributed to the fact that after joint injury the cartilage is overloaded in the presence of inflammatory mediators. However, in this manuscript we point out that excessive mechanical compression with a combination of proinflammatory conditions partly suppresses the mechanically-mediated TGF-β/Smad3/2P signaling. Our observations suggest that in the presence of inflammatory conditions, compression is less able to effectively induce the TGF-β/Smad3/2P signaling. Moreover, in our view the most important observation of this study is that excessive compression applied in the presence of inflammatory factors causes a downregulation of the crucial TGF-β receptor TGFBR2. We hypothesize that the loss of TGFBR2 might explain how overloading will induce cartilage damage that transcends its purely mechanical effects. Additionally, this may also indicate why the surgical restabilization of the joint does not reduce the risk of progressive joint degeneration after joint trauma [45].

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors have no conflict of interest.
Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage

7. REFERENCES


Inflammatory conditions partly impair the mechanically-mediated activation of Smad2/3 signaling in articular cartilage.


Unloading results in rapid loss of TGF-β signaling in articular cartilage: Role of loading-induced TGF-β signaling in maintenance of articular chondrocyte phenotype?

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ABSTRACT

Objective: Recently it was shown that loading of articular cartilage explants activates TGF-β signaling. Here we investigated if in vivo chondrocytes express permanently high TGF-β signaling, and the consequence of the loss of compressive loading-mediated TGF-β signaling on chondrocyte function and phenotype.

Design: Bovine articular cartilage explants were collected within 10 min post mortem and stained immediately and after 30, 60 and 360 min for phosphorylated-Smad2, indicating active TGF-β signaling. Explants were unloaded for 48 h and subsequently repeatedly loaded with a compressive load of 3 MPa. In addition, explants were cultured unloaded for 2 weeks and the effect of loading or exogenous TGF-β on proteoglycan level and chondrocyte phenotype (Col10a1 mRNA expression) was analyzed.

Results: Unloading of articular cartilage results in rapid loss of TGF-β signaling while subsequent compressive loading swiftly restored this. Loading and exogenous TGF-β enhanced expression of TGF-β1 and ALKS. Unloading of explants for 2 weeks resulted in proteoglycan loss and increased Col10a1 expression. Both loading and exogenous TGF-β inhibited elevated Col10a1 expression but not proteoglycan loss.

Conclusions: Our data might imply that in vivo regular physiological loading of articular cartilage leads to enduring TGF-β signaling and TGF-β-induced gene expression. We propose a hypothetical model in which loading activates a self-perpetuating system that prevents hypertrophic differentiation of chondrocytes and is crucial for cartilage homeostasis.
1. INTRODUCTION

Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

Loading of articular cartilage is absolutely essential for its maintenance. Reduced joint loading leads to cartilage atrophy and degeneration, both in humans and animal models [1-5]. Patients with spinal cord injuries show progressive loss of knee cartilage at a pace faster than osteoarthritis (OA) patients [6, 7]. In dogs, it has been shown that joint motion in the absence of normal loading is not sufficient to prevent this cartilage loss [8]. Moreover, articular chondrocytes are mechanosensitive cells which has been clearly described in a number of excellent recent reviews [9-11]. However, the exact mechanism how unloading negatively affects articular cartilage homeostasis has not been elucidated yet.

Transforming growth factor-β (TGF-β) is stored in high amounts (up to ~300 ng/g of all isoforms) in the articular cartilage matrix but in a latent form [12]. TGF-β signaling has been shown to be essential for the preservation of articular cartilage. Loss of the intracellular TGF-β signaling molecule Smad3 or functional loss of the TGF-β type II receptor in chondrocytes results in loss of articular cartilage in mice [13-15]. In humans, an inactivating mutation of Smad3 results in early onset of OA while a Smad3 polymorphism is associated with the total burden of radiographic OA [16-18].

It has been shown that mechanical stimulation can activate TGF-β signaling in chondrocyte-like cells [19, 20]. Recently, we described that compressive in vitro loading of bovine articular cartilage explants rapidly induces TGF-β signaling [21]. Based on these observations we hypothesized that under normal in vivo conditions articular cartilage is always subject to active TGF-β signaling and that loss of in vivo loading will result in loss of signaling. Remarkably, this has never been investigated yet. In this study, it was investigated if TGF-β signaling was lost when articular cartilage was taken out of its natural environment and if the changes in TGF-β signaling could be reversed by in vitro compressive loading. Moreover, we studied the effect of prolonged unloading in vitro on proteoglycan content and chondrocyte phenotype, as measured by expression of Col10α1, and if induced changes could be prevented by loading or exogenously added TGF-β. Our data indicate that unloading results in rapid loss of TGF-β signaling and this might lead to changes in chondrocyte phenotype. Based on our observations a self-regulatory loading-driven model is proposed that keeps articular cartilage healthy, connecting compressive loading to cartilage homeostasis via TGF-β.

2. MATERIAL AND METHODS

Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

For all performed experiments full cartilage thickness explants were harvested from bovine metacarpophalangeal joints (MCP) of skeletally mature cows (age 4–5 years old) obtained from the local abattoir. 0.7 ± 0.12 mm thick explants were isolated with a 4 mm Ø biopsy punch (Kai-medical, Japan). All explants (if cultured) were cultured in standard culture conditions (37°C, 5% CO₂ and 95% humidity) in DMEM/F-12 medium (Gibco®, USA) containing Antibiotic-Antimycotic (contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®) (Gibco®, USA) unless stated differently. No serum was added to the medium unless stated differently.

2.1. EFFECT OF UNLOADING ON TGF-β SIGNALING IN ARTICULAR CARTILAGE (FIG. 1 A)

Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

Bovine articular cartilage explants were harvested from the MCP joint of skeletally mature cows (age 3–5 years old). Joints were processed within 10 min post mortem (joint loading stopped).
Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

For immunohistochemical (IHC) analysis, explants (4 mm Ø) were fixed in 4% phosphate buffered formalin (pH 7.0) directly after isolation or first cultured for 30 min, 2 h, 6 h or 24 h at standard culture conditions in DMEM/F-12 medium (Gibco®, UK). No serum was added to the medium.

For gene expression analysis, explants were isolated within 3 h post mortem. One group of explants was flash frozen in liquid nitrogen immediately after opening. The remaining groups were placed in medium with or without the ALK4/5/7 kinase blocker SB-505124 (Sigma-Aldrich, St. Louis, MO, USA) [22] (5 μM) or vehicle control (0.5 μl/ml Dimethyl sulfoxide (DMSO)) for 24 or 48 h. This experiment was repeated in seven animals.

Fig. 1. Schematic representation of experiments conducted in this study. (A) The effect of unloading on TGF-β signaling in articular cartilage was investigated by measuring Smad2P and Smad2/3P responsive genes on indicated time points. (B) Effect of repeated physiological mechanical compression on TGF-β signaling in articular cartilage was investigated by measuring Smad2P and Smad2/3P responsive genes on indicated time points before and 2 h and 48 h after compression for multiple compressions. (C) Effect of loading on GAG content and Col10a1 expression was measured by culturing cartilage explants for 2 weeks ex vivo. Explants were compressed every 48 h the first week and hereafter either cultured for 1 week in serum free medium for Col10a1 or compressed again for GAG measurement.

2.2. EFFECT OF REPEATED PHYSIOLOGICAL MECHANICAL COMPRESSION ON TGF-β SIGNALING IN ARTICULAR CARTILAGE (FIG.1B)

Five groups were used in this experiment (see Fig.1B). Explants were harvested within 3 h post mortem. After 48 h of equilibration, the first group of explants was frozen, whereas the other groups of explants were subjected to 3 MPa dynamic mechanical compression for 30 min with a frequency of 1 Hz [21] (Fig. 1(B)). At 2 h after the first compression, a second group of explants was frozen. The remaining groups of explants were again cultured for 48 h after which the third group of explants was frozen. At the same day, the last two groups of explants were subjected to mechanical compression. Two hours after the second compression, the fourth group of explants was frozen and the fifth group was further cultured for 48 h and then frozen.

The same experimental set up was repeated in the presence of SB-505124 (5 μM) or DMSO. The specimens were pre-incubated with SB-505124 (or DMSO) for 1 h prior to the compression to ensure penetration of the agent [21]. SB-505124 or DMSO was also present in the medium during and after dynamic mechanical compression. These experiments were repeated four times.

To immunohistochemically investigate the induction of pSmad2 by mechanical compression after 48 h of equilibration, explants were stimulated with 3 MPa for 30 min with 1 Hz. Then explants were fixed in 4% phosphate buffered formalin (pH 7.0) at 1 h after the compression. For the staining details see section: IHC Analysis.
2.2. EFFECT OF REPEATED PHYSIOLOGICAL MECHANICAL COMPRESSION ON TGF-β SIGNALING IN ARTICULAR CARTILAGE (FIG.18)

The first group of explants was isolated and flash frozen immediately after joint opening. After an equilibrium period of 48 h the medium of four other groups was changed for DMEM/F-12 containing 10% Fetal Bovine Serum, 20 ng/ml of rhGF-1 (PeproTech, NJ, USA) or 10 ng/ml rhTGF-β1 (Biolegend, CA, USA) or combination of 20 ng/ml of rhGF-1 + 10 ng/ml rhTGF-β1 and refreshed every 72 h. An additional group of explants was subjected to mechanical compression every 48 h for 14 days. At day 14, explants from all groups were flash frozen and GAG content was measured using Dimethylmethylene Blue (DMB).

To analyze if a lack of mechanical load on articular cartilage explants results in induction of Col10a1, a first group of explants was isolated and immediately frozen. Four other groups were cultured in unloading condition for 14 days in DMEM/F-12 medium supplemented with 10% FBS or 1 ng/ml rhTGF-β1 or 10 ng/ml rhTGF-β1 or 50 ng/ml of Activin A (R&D Systems, MN, USA). To investigate if mechanical compression is able to inhibit non-loading induced induction of Col10a1 expression an extra group of explants was subjected to mechanical compression three times during the first week of the experiment (every 72 h). During the second week of the experiment, only medium was changed every 72 h. This experiment was conducted 6 times.

2.4. DYNAMIC MECHANICAL COMPRESSION OF ARTICULAR CARTILAGE EXPLANTS

To compress cartilage, a BOSE® ElectroForce® BioDynamic™ bioreactor (5160 BioDynamic System) equipped with a 50 lbf load-cell was used (BOSE Bose Corp. ElectroForce Systems Group, MN, USA). First, a preset compression force of 5 N (0.3 MPa) was applied to guarantee contact between plates and specimen. Subsequently, explants were subjected to 3 MPa, force controlled, unconfined, dynamic mechanical compression using a 1 Hz sine wave and desired pressure for 30 min (1800 cycles). Unloaded controls were also placed in the bioreactor incubator but in a separate well.

2.5. GENE EXPRESSION ANALYSIS

Samples were homogenized using a micro dismembrator (B. Braun Biotech International, Melsungen, Germany). Total RNA was isolated using RNaseasy Fibrous tissue kits (Qiagen Inc., Valenzia, CA, USA) according to manufacturer’s protocol. Isolated RNA was transcribed into cDNA using M-MLV reverse transcriptase and single step RT-PCR: 5 min at 25°C, 60 min at 39°C, and 5 min at 95°C. Gene expression was measured using 0.5 μM of validated primers (see Table 1) (Biolegio, the Netherlands) in a quantitative real-time polymerase chain reaction (qPCR) using SYBR green (Applied Biosystems, Darmstadt, Germany). A melting curve was made to verify gene specific amplification. Two reference genes were used: glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and ribosomal protein S14 (Rps14).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Ref seq</th>
<th>Product length</th>
<th>Efficiency (%)</th>
<th>Forward 5′ → 3′</th>
<th>Reverse 5′ → 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>bActin</td>
<td>NM_1739812</td>
<td>144</td>
<td>97.3</td>
<td>TGAACACGTCCTCCCTCACTGCA</td>
<td>TCAAGAGCGTGCTGCTGACTCTC</td>
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<tr>
<td>bARF7</td>
<td>NM_00100147</td>
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<tr>
<td>bARF5</td>
<td>NM_1746212</td>
<td>75</td>
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<tr>
<td>bCol10a1</td>
<td>NM_1746341</td>
<td>74</td>
<td>92.2</td>
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<td>TGCTCTCCCTCTGCTATGACCTCCCT</td>
</tr>
</tbody>
</table>

Table 1. Template, efficiency and sequence of the primers used in this study.
Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

2.7 SPECTROPHOTOMETRIC ANALYSIS

Cartilage explants were weighed and digested overnight at 60°C using papain (1 mg/ml papain, 0.1 M sodium acetate, 10 mM l-cysteine hydrochloride and 50 mM ethylenediaminetetraacetic acid sodium salt, pH 6.0). After digestion, samples were centrifuged for 15 min at 15,000 RPM and supernatant was diluted 20 times in ultra-pure water. 200 μl of the DMB solution was added to 40 μl of diluted digest, and absorbance at λ = 595 nm was measured immediately using a 96-well plate reader (Biorad, CA, USA).

2.8 STATISTICAL ANALYSIS

All quantitative data analysis was expressed as a Tukey box plot with mean shown as '*' and outliers showed as '*'. All datasets were checked for normality using the Shapiro–Wilk’s test and then for equality of variances by Levene’s test.

Linear mixed models with Bonferroni multiple comparison post tests were used to estimate the effect of time and treatment (+DMSO or + SB-505124) on gene expression levels. One-way ANOVA with Fisher’s LSD post-test was used to estimate the effect of compression or the effect of lack of the compression on gene expression (LSD does not correct for multiple comparisons, however we compared only the effect of the compression on induction of the gene expression or the effect of lack of the compression on gene drop, no multiple comparisons were required). The same approach was used to estimate the effect of compression or lack of the compression and treatment (+DMSO or +SB-505124) on Smad7 expression levels. One-way ANOVA with Tukey’s post-test was used to estimate the effect of treatments on GAG content. The same approach was used to estimate the effect of treatments on bCol10a1 gene expression.

| BGLapdh | NM_00138403.4.2 | 90 | 100.5 | CACCCACGCGACGTCAAC | TCCCGCTCTTGAAAGATGCT |
| bPap7 | NM_074187.2 | 55 | 99.1 | CGAGCCAGGCGGACTTC | TGCCACGGTACAAAGACTCTTTGA |
| bPjg4 | NM_0017783.2 | 125 | 104.9 | CATCAGCTGCGCTACATCA | TTCACATTGCGCGAATCTTCA |
| bSmad7 | NM_00110889.0.1 | 88 | 103.8 | CATCGAGCCCCAGAGCAATA | GCTGTCATCTGGTGACTCT |
| bSmad7 | NM_0017826.5.1 | 72 | 105.4 | GGGCTTCAGATGCCAAGCTT | CTCCCGATATGCGACCAAC |
| bPb1 | NM_0016606.8.1 | 80 | 106.8 | GGTGAAATACGGCAACAAATCT | GCTCGGAGCTGTGGAAGAAC |
| bIm2 | NM_00199941.1 | 73 | 104 | CCGAGCTCTCATACGAA | AGAAGAATTCCGGCGGTGTT |

2.6 IHC ANALYSIS

Samples were fixed overnight in phosphate buffered formalin, dehydrated and embedded in paraffin. Six μm thick sections were cut and mounted on Superfrost™ Plus Microscope Slides (Thermo Scientific, Waltham, USA). After deparaffinization, citrate buffer (0.1 M sodium citrate and 0.1 M citric acid) was used for 2 hours at RT for antigen unmasking. Hydrogen peroxide 1% v/v in methanol was used for 30 min to block endogenous peroxidase. Afterwards, sections were incubated overnight at 4°C with specific primary antibodies against c-terminally phosphorylated SMAD2 (rabbit pAb anti Phospho-Smad2 (Ser465/467) (1:100) (Cell Signalling Technology, Danvers, Massachusetts, USA). Biotin-labelled secondary antibodies were used (Dako, Glostrup, Denmark). Together with a biotin–streptavidin detection system used according to the manufacturers’ protocol (Vector Laboratories, Baiklin Game, California, USA). Staining was visualized using dimethylaminobenzene (DAB) reagent [23].
Unloading results in rapid loss of TGF-beta signaling in articular cartilage:
Role of loading-induced TGF beta signaling in maintenance of articular chondrocyte phenotype?

Unpaired one tailed t-test was used to estimate the effect of time on Pail expression levels. One-way ANOVA with Tukey’s post-test was used to estimate the effect of addition of TGF-β1 or Activin A on Smad7, Pail and Tgb1 expression levels. The same approach was used to estimate the effect of dynamic mechanical compression on the expression levels of Alk1.

All the analyses were performed with the statistical software packages: SPSS 20.0 (SPSS, Chicago, USA).

3. RESULTS

3.1. UNLOADING RESULTS IN LOSS OF TGF-β SIGNALING

To investigate if unloading results in loss of TGF-β signaling, articular cartilage was obtained within 10 min post mortem from the MCP joint of mature cows and fixed immediately or after in vitro incubation. At the earliest time points, the majority of chondrocytes clearly stained positive for active TGF-β signaling (phosphorylated Smad2, Smad2P) throughout all zones of the articular cartilage (Fig. 2A). However, already after 2 h of unloaded culture, cells in middle zone of the cartilage had lost staining which was even more pronounced after 6 h. At 6 and 24 h only very few cells stained positive for Smad2P.

To ensure that this loss in Smad2P staining was not a cutting artifact we left intact MCP joints unopened for 6 h or overnight and thereafter isolated the cartilage. After 6 h, cartilage had highly reduced Smad2P staining. Moreover, cartilage stored overnight showed significantly reduced Pail (a marker for active TGF-β signaling) expression when compared to fresh tissue (Supplementary Fig. 2).

Fig. 2. Loss of active Smad2/3 signaling in unloaded cartilage. (A) Phosphorylated Smad2 levels in adult cartilage after unloading, as detected by IHC. Representative individual shown. (B) Relative gene expression of the TGF-β-responsive genes: Alk5, Smad7 and Pail 24 h and 48 h after unloading compared to fresh samples. Tukey box plot, + = mean, N = 7, ** P ≤ 0.01, *** P ≤ 0.001. (C) The effect of the ALK4/5/7 blocker SB-505124 on relative gene expression of bAlk5, bSmad7 and bPail 24 h and 48 after unloading, compared to fresh samples. DMSO was used as vehicle control. Tukey box plot, + = mean, N = 7, N.S. = not significant, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. (D) Relative gene expression of AggreCan or Smad3 24 h and 48 h after unloading. Tukey box plot, + = mean, N = 7.

To determine whether this rapid drop in Smad2/3 signaling was reflected in TGF-β signaling-dependent gene expression, expression of the known ALK5/Smad3 responsive genes Smad7 [24], Pail [25] and Alk5 (also known as TGFBRI) [26] was assessed. Expression of all
three genes was significantly reduced after 24 h and further lowered after 48 h (Fig. 2B). Addition of the ALK4/5/7 blocker SB-505124 which blocks TGF-β signaling did not result in a more severe loss of gene expression, indicating that no residual TGF-β signaling was present in the unloaded, cultured cartilage and that absence of loading has similar effects as actively blocking Smad2/3 signaling (Fig. 2C). Notably, a decrease in gene expression is not a general phenomenon in unloaded cartilage, as the expression levels of for example Acan (Aggrecan) and Smad3 were maintained (Fig. 2D).

3.2. RELOADING REPEATEDLY INDUCES TGF-β SIGNALING

Next, we evaluated if compressive reloading could restore Smad2P signaling and TGF-β-dependent gene expression and if this was a repeatable process. Therefore, explants were loaded 48 h after isolation and again 48 h after the first in vitro loading. Compressive loading rapidly induced Smad2P staining in cartilage explants (Fig. 3A).

Two hours after the first 30 min of loading, gene expression of Aik5, Smad7 and Paf1 was significantly induced, indicating that loading restores TGF-β signaling [Fig. 3(B)]. Strikingly, 48 h after the first in vitro loading gene expression had dropped again to unloaded levels. Two hours after a second loading for 30 min, again gene expression of Smad7 and Paf1 was strongly elevated. We could confirm our earlier observations [21] that the loading-induced expression of Smad7 can be fully blocked by the ALK4/5/7 inhibitor SB-505124 (Fig. 3C), indicating that compression-induced Smad7 expression indeed runs via active Smad2/3P.

![Image](image.png)

**Fig. 3. Repeatable restoration of active Smad3 signaling by dynamic mechanical compression.** (A) Induction of Smad2 phosphorylation by dynamic mechanical compression (3 MPa) of adult articular cartilage, as detected by IHC. (B) Relative gene expression of the TGF-β-responsive genes: Aik5, Smad7 and Paf1 in response to repeated dynamic mechanical compression and unloading. Tukey box plot, + = mean, N = 4, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001, n.d. = not detected. (C) Relative gene expression of Smad7 in response to repeated dynamic mechanical compression and unloading in the presence of the ALK4/5/7 inhibitor, SB-505124. DMSO was used as vehicle control. Tukey box plot, N = 4, + = mean, N.S. = not significant, *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001. (D) Scheme reflecting the effects of dynamic compression on TGF-β-signaling in the absence or presence of SB-505124. (E) Relative gene expression of Aik1 in (repeatedly) dynamically compressed (3 MPa) cartilage compared to unloaded controls 48 h after compression. Tukey box plot, + = mean, N = 8, ***= P ≤ 0.001.

Notably, SB-505124 did not inhibit all compression-induced gene expression. For example, a loading-induced ~4-fold increase in Bmp2 expression (Supplementary Fig. 3) was unaffected. Because Bmp2 expression was also not responsive to exogenously added TGF-β, regulation of this gene is most likely TGF-β-independent. This observation thus shows that SB-
505124 does not affect compression-induced gene expression that is induced independently of TGF-β. In Fig. 2D a schematic representation of the effects of loading and unloading is depicted showing the repeated mechano-responsiveness of TGF-β signaling in cartilage. Finally, we were also able to confirm our earlier observations that loading reduces ALK1 expression (Fig. 2E) [21].

As a possible source for the observed induction of Smad2/3P we investigated two ligands capable of inducing these Smads: TGF-β and Activin-A. Incubation of bovine explants with exogenously added TGF-β resulted in comparable up-regulation of gene expression as induced by compressive loading, in contrast to Activin-A, which did not induce expression of either Alk5, Smad7 or PaI1 although it was bioactive (Supplementary Fig. 4).

3.3. LOADING-INDUCED TGF-β SIGNALING BLOCKS CHONDROCYTE HYPERTROPHY

Subsequently, the potential physiological relevance of the loading-induced TGF-β signaling was investigated. We postulated that TGF-β signaling either sustains the proteoglycan (aggrecan) content of cartilage or blocks hypertrophic differentiation of chondrocytes or both. Culturing bovine explants for 2 weeks resulted in a significant loss of glycosaminoglycans (GAGs) (nearly 60%) from the extracellular matrix (ECM). Compressive loading was totally ineffective to prevent this loss (Fig. 4A). In contrast, this GAG loss could be prevented by addition of 10% fetal calf serum or 20 ng/ml insulin-like Growth Factor-1 (IGF1) to the medium. However, addition of 10 ng/ml TGF-β was completely ineffective and even lowered IGF1 effects on GAG content (Fig. 4B). We conclude that it is unlikely that TGF-β plays a direct role in maintenance of GAG content in articular cartilage.

Fig. 4. Dynamic mechanical compression protects against chondrocyte hypertrophy. (A) The sulfated GAG content (w/w) of adult articular cartilage; cultured for 2 weeks ex vivo either with or without 10% FCS, 10 ng/ml TGF-β1 or subjected to dynamic mechanical compression (3 MPa). Tukey box plot, = mean, N = 6; *** P ≤ 0.001. (B) GAG content (w/w) of articular cartilage cultured for 2 weeks ex vivo in serum free medium, or in the presence of either: 10% FCS, 20 ng/ml IGF1, 10 ng/ml TGF-β1, or a combination of IGF1 and TGF-β1. Tukey box plot, N = 9; *= P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. (C) Expression of the hypertrophy marker Col10α1 after 2 weeks ex vivo culture in serum free medium or in the presence of 10% FCS, 1 or 10 ng/ml TGF-β1, or 50 ng/ml Activin A. Tukey box plot, = mean, N = 4 (for Activin A, N = 2); ***P ≤ 0.001. (D) The effect of repeated dynamic mechanical compression (3 MPa) on Col10α1 expression after 2 weeks ex vivo culture. Tukey box plot, = mean, N = 6; ** P ≤ 0.01, *** P ≤ 0.001.

Apart from GAG loss, culturing of bovine explants in the absence of loading resulted in strongly increased expression of Col10α1, an accepted marker for early hypertrophic differentiation of chondrocytes [27]. The increase in Col10α1 expression was not affected by addition of 10% fetal calf serum, underscoring that serum factors are not able to inhibit hypertrophic
differentiation of articular chondrocytes (Fig. 4C). In contrast, addition of 1 ng/ml TGF-β fully blocked induction of Col10a1 gene expression. Of note, addition of 10 ng/ml activin did not affect the increase in Col10a1 expression. When we investigated whether compressive loading inhibited the up-regulation of Col10a1 expression, loading for 30 min at time point 48, 96 and 144 h after isolation significantly prevented the up-regulation of Col10a1 in bovine explants measured after 2 weeks (Fig. 4D). Unfortunately, in this experimental setting, a 14-day culture period, we were unable to include the inhibitor SB-505124 because addition of this compound for such a long period resulted in significantly decreased cell viability in the cartilage explants (Supplementary Fig. 5), making us unable to show the importance of Smad2/3P in this process.

4. DISCUSSION

This study is the first to demonstrate that removal of articular cartilage from in its in vivo situation results in rapid loss of TGF-β signaling and that subsequent compressive loading can repeatedly restore this signaling. This suggests that the absence of loading will result in the loss of TGF-β signaling in articular cartilage. The consequence of this is reduced expression of TGF-β1 and the TGF-β type 1 receptor ALK5, together with increased expression of ALK1 [21]. Moreover, prolonged unloading leads to proteoglycan loss and change in chondrocyte phenotype, as determined by increased Col10a1 expression. However, compressive loading or addition of exogenous TGF-β prevent the increase in Col10a1 expression but not of proteoglycan loss.

Our data demonstrate the repeated mechano-responsiveness of TGF-β signaling in cartilage, which shows rapid activation upon compression and inactivation upon unloading, we propose the following hypothetical model for this loss and activation of TGF-β signaling by compressive mechanical loading: Articular cartilage contains high amounts of TGF-β (up to 300 ng/g [12]), but inactive and bound to the latency-associated peptide (LAP) and ECM. LAP forms a so-called straitjacket that keeps the mature form of TGF-β1 associated with LAP, but unfolding of LAP by mechanical force (40 pN) can release active TGF-β [28,29]. Mechanical force (compressive loading) is thus able to release active TGF-β, and this has been shown in multiple systems [30,31]. However, upon unloading, all active TGF-β is sequestered again to the abundant binding places in the ECM [32], inactivating it again. Although no tools are available yet to investigate this mechanism in situ in intact articular cartilage, we propose that such a mechanism explains the repeated mechanosensitivity of TGF-β in cartilage.

We hypothesize that this loading-released TGF-β will bind to its receptors, but will also rapidly bind the ECM becoming unavailable again [32]. In the absence of mechanical force TGF-β signaling will rapidly diminish. The loading-induced TGF-β signaling will induce synthesis of TGF-β1 [33], but in an inactive form that will be bound to the ECM. Moreover, expression of ALK5 will be up-regulated whereas expression of ALK1 will be down-regulated, favoring TGF-β-dependent Smad2/3 signaling and decreasing Smad1/5/8 signaling (Fig. 5). Chondrocyte terminal differentiation is stimulated by Smad1/5/8 activation and inhibited the Smad2/3 [34]. This is in line with our current observation that regular loading appears to prevent early hypertrophic differentiation of chondrocytes.

Other factors than TGF-β will undoubtedly play a role in the mechanical regulation of articular cartilage and several mechanosensitive actors have been identified in chondrocytes [9]. For instance, compressive loading can induce BMP2 expression in cartilage [23]. Our current study shows that loading and exogenous TGF-β can block hypertrophic differentiation of chondrocytes, as measured by Col10a1 mRNA expression, but is not able to block proteoglycan
loss in vitro. In vivo, proteoglycan synthesis will be maintained by systemic levels of IGF-I and BMP9 and by (load-induced) factors such as BMP2 [35-37].

Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

TGF-β and compressive loading inhibited the up-regulation of the early hypertrophy marker Col10a1, suggesting that loading-induced TGF-β signaling blocks hypertrophic differentiation of chondrocytes in articular cartilage. Unfortunately, we were not able to use SB-505124 in our long-term cultures but in our short-term cultures we could show that loading-induced TGF-β signaling can be blocked by this inhibitor, in line with our previous work [21]. Our results are in line with earlier observations that TGF-β, via the Smad2/3 signaling route, is a potent inhibitor of chondrocyte hypertrophy [13, 39, 40]. Importantly, our results seem to indicate that loading and cartilage homeostasis are interconnected via TGF-β signaling.

Our study has a number of limitations. We used Col10a1 as a marker for changes in chondrocyte phenotype in the direction of hypertrophy. Because we had to perform our cultures without fetal calf serum, to prevent continuous presence of TGF-β, we were not able to perform our in vitro cartilage cultures endlessly. This made it impossible to demonstrate the induction of late hypertrophic markers, such as MMP13. However, although we only demonstrated elevated hypertrophic markers in the time span studied, we still think that our results indicate a phenotypic shift towards hypertrophy since this is supported by other studies that show that loss of TGF-β signaling results in chondrocyte hypertrophy [13, 15, 17].

Another limitation is our loading regime. We use simple compressive loading as a simplified model for the mechanical forces acting in vivo on articular cartilage. The loading protocol we used results in a permanent deformation during the 30 min loading cycle of approximately 10% [23]. The force we used is in a physiological range (3 MPa) but the loading itself will be quite different from in vivo loading. In addition, there are regions in articular cartilage which are considered non-load bearing in vivo. In our concept, it should be expected that these areas deteriorate. However, it can be argued if truly non-load bearing articular cartilage exists in joints. Furthermore, these locations might experience high shear stress, which has also been

Fig. 5. Proposed hypothetical model for compression-mediated protection of articular cartilage integrity. Compression of articular cartilage leads to release of active TGF-β from the ECM. This active TGF-β signals via ALK5, resulting in phosphorylation of Smad2/3. Subsequently, phosphorylated Smad2/3 inhibits hypertrophic differentiation of articular chondrocytes, as characterized by Col10a1. Additionally, phosphorylated Smad2/3 activates a positive feedback loop by not only inducing expression of ALK5 and lowering expression of ALK1, but also by inducing the expression of inactive TGF-β1, which, after production will bind to the ECM, returning the system to its original state.

The observation that the biological consequence of the loading/TGF-β driven process is not the direct maintenance of proteoglycan content might appear to be in contrast with the study of Morales et al. However, in that study cartilage of 6 months old calves was used, where growth still takes place, while cartilage from skeletally mature cows was used in our study [38]. Both
shown to be able to activate TGF-β [31]. Moreover, our data indicate that short physiological compressive loading once every day will be sufficient to maintain TGF-β-induced gene expression. Infrequent compressive loading might be experienced by this so-called "non-loaded" cartilage but still be sufficient to maintain homeostasis.

Finally, a considerable limitation is the lack of absolute proof that the observed processes run via TGF-β. SB-505124 gives an indication that an ALK4/5/7 ligand is important, but we were limited in its use due to its toxic long-term effects. Unfortunately, no tools are currently available to investigate our proposed hypothesis more deeply in situ. Ideally, we would knock out the TGF-β type II receptor TGFBR2 [41] to show the importance of TGF-β in our proposed model, but we have been unable to target chondrocytes in situ with the currently available tools to manipulate gene expression.

Absence of loading results in cartilage loss. Chondrocyte hypertrophy is associated with cartilage degradation and proteoglycan loss [42]. Our finding of early hypertrophic differentiation of chondrocytes in the absence of loading-induced TGF-β signaling might provide an explanation for the loss of articular cartilage that is observed after long term cartilage unloading. Increased numbers of hypertrophic chondrocytes and expression of matrix degrading enzymes have been described in articular cartilage of rats after immobilization [4, 5], which is in line with our hypothetical model.

This model, in the light of our observation that mechanically-induced activation of TGF-β signaling is impaired in aged cartilage [23], could be involved in the age-dependency of OA. If the hypothetical model we propose is valid, age-related loss of this loading-induced mechanism will make articular cartilage more prone to hypertrophic changes of articular chondrocytes and OA development. Furthermore, this suggests that unraveling the exact molecular mechanism underlying this system could provide tools to interfere with OA development and or progression.

5. ACKNOWLEDGMENTS

Dr Gerjon Hannink is kindly acknowledged for his assistance with the statistical evaluation. ReumaFonds (LLP-7) and ZonMW (40-00812-98-090200) are greatly acknowledged for their financial support.

6. CONFLICT OF INTEREST

None of the authors have any support or other benefits from commercial sources or other conflicts of interest regarding the work reported in the manuscript, or any other competing financial interests.
Unloading results in rapid loss of TGF-beta signaling in articular cartilage: 

Role of loading-induced TGF beta signaling in maintenance of articular chondrocyte phenotype?

References:


42. Tchetina EV, Squires G, Poole AR. Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions. J Rheumatol 2005;32:876-86.

8. **SUPPLEMENTARY MATERIALS**

**Supplementary Figure 1:** Counterstaining of bovine cartilage explants. Hematoxylin staining of bovine cartilage explants as used in Fig. 1.

**Supplementary Figure 2:** Active TGF-β signaling is lost in articular cartilage of intact MCP joints. (A) Phosphorylated Smad2 (left) and Hematoxylin (right) staining of cartilage explants isolated 6 h after unloading of the MCP joint. The MCP joint was left intact for the whole duration of this experiment. Arrows depict cells without pSmad2 staining. (B) Gene expression of Pals1 relative to the average expression of the reference genes Gapdh and Rps14, in cartilage explants isolated rapidly after unloading or after an overnight (O/N) period. Tukey box plot, + = mean, N = 8. **P ≤ 0.01.

**Supplementary Figure 3:** Compression induces Bmp2 expression but this is not affected by SB-505124. Relative gene expression of Bmp2 in dynamically compressed cartilage compared to unloaded controls 2 h after compression in the presence of SB-505124 (gray) or vehicle/DMSO (white). Additionally, explants were stimulated with 5 ng/ml TGF-β for 2 h but this did not significantly induce Bmp2 expression. Tukey box plot, + = mean, N = 5. ***P ≤ 0.001, n.s. = not significant.

**Supplementary Figure 4:** rhActivin A does not induce Smad7, Pals1 or Tgfβ1 expression in bovine cartilage explants. (A) Relative gene expression of Smad7, Pals1 and Tgfβ1 in cartilage explants 24 h after stimulation with rhTGFβ1 or rhActivin A compared to unstimulated samples. Tukey box plot, + = mean, N = 4, ***P ≤ 0.001. (B) Relative gene expression of Smad7 and Pals1 5 h after stimulation of primary bovine chondrocytes with various doses of rhActivin A showing that the used rhActivin A is bioactive and compatible with bovine cells. Experimental duplo shown.
Unloading results in rapid loss of TGF-beta signaling in articular cartilage:

Role of loading-induced TGF beta signaling in maintenance of articular chondrocyte phenotype?

Supplementary Figure 5: SB-505124 negatively affects chondrocyte viability in long term explants culture. Relative viability of cartilage explants after 2 weeks ex vivo culture in the presence of DMSO or 5 μM SB-505124 as measured by XTT assay according to manufacturer’s protocol (Roche Diagnostics GmbH, Germany). Tukey box plot, + = mean, N = 5, *- P ≤ 0.05.
General discussion and future perspectives
Numerous reports showed that mechanical loading is critical for articular cartilage maintenance and health [1]. However, the understanding of how cartilage cells sense their microenvironment and convert mechanical signals into biochemical and biological signals remains poor. This knowledge is crucial, considering the fact that deviations from normal, physiological loading of articular cartilage is the cause of cartilage tissue deterioration and development of osteoarthritis (OA) [2]. Consequently, investigation on the role of the mechanical factors in OA development and progression can deliver solutions for potential disease prevention. Studies that try to unravel the role of mechanical signals in cartilage physiology and disease, range from in vitro and animal models to large-scale randomized clinical trials and longitudinal cohort studies. In this doctoral dissertation, the role of mechanical signals in the activation of TGF-β signaling in articular cartilage was investigated. Dynamic mechanical compression appeared to be a key activator of TGF-β/Smad2/3P signaling [3]. Furthermore, it was shown that well known OA risk factors, like aging or trauma-related proinflammatory conditions, can significantly impair this activation [4, 5]. Finally, and most importantly it was proven that mechanical signals in the form of dynamic mechanical compression are essential for maintaining constant, high TGF-β/Smad2/3P signaling, which is extremely important to restrain deleterious chondrocyte terminal differentiation [6].

However, as described in detail in Chapter 2, not only TGF-β signaling is known to be mechanosensitive in chondrocytes. Especially calcium signaling has been a focus of several mechanotransduction studies in the past years [7-10]. In 2014, O’Conor et al. demonstrated a critical role of Transient Receptor Potential Vanilloid 4 (TRPV4) in mechanically-mediated cartilage matrix metabolism [7]. They showed that inhibition of TRPV4 blocks proanabolic and anti-catabolic genes and mechanically-induced matrix accumulation and function, however exact mechanism been shown. Furthermore, artificial activation of TRPV4 resulted in transcriptional
activation of anabolic growth factors and suppression of proinflammatory mediators [7]. Motivated by this promising data, in numerous of follow-up publications TRPV4 together with other ion channels emerged as a core of the mechanotransduction system in cartilage and therapeutically targeting TRPV4-mediated mechanotransduction for the treatment of OA has been proposed [11]. However, OA is a multifunctional disease and its development and progression is promoted by several different risk factors. Those factors involve, but are not limited to advanced age [12], abnormal cartilage overload [13] or trauma-related catabolic action of proinflammatory cytokines [14]. Considering an integrated approach with respect to all of those risk factors seems to be crucial, since it has been shown that they can significantly affect calcium signaling in many different tissues [15-18]. This shows that more detailed investigation of the role of OA-related changes on TRPV4-mediated mechanotransduction is needed in order to recognize its full function in OA affected joint.

Those ambiguity stays in contrast with the broad picture of the role of TGF-β signaling activation in articular cartilage followed by TGF-β-mediated control of cellular functions also on OA cartilage. Already for many years, TGF-β function in OA for numerous different disease-related conditions have been investigated and described [19-23]. Consequently, in this dissertation, first it was demonstrated that mechanical signals are very potent activators of TGF-β signaling in articular cartilage [3]. Afterwards, to create broad understanding of this finding it was examined how OA-related conditions would affect mechanically-mediated activation of TGF-β signaling [3-6].

Experimental model implemented and used in PhD project which resulted in this thesis, involved isolation of intact articular cartilage explants from bovine MCP joints. After equilibration, explants were subjected to mechanical stimulation and at different time intervals TGF-β signaling was investigated. As a source of mechanical stimulation for investigations included in this thesis the unconfined dynamic mechanical compression was used. However, during physical joint activity, cartilage and thus chondrocytes experience and respond not only to compressive force but also other types of mechanical stress, involving fluid flow-induced shear stress [24], hydrostatic pressure [25] and tensile stress [26, 27]. This is important since it has been shown by Smith et al. that different types of mechanical stress can differently alter chondrocyte physiology [28]. For instance, applying shear stress decreases the expression of cartilage matrix proteins whereas intermittent hydrostatic pressure increases matrix protein expression [28]. Moreover, in 2003 Lee et al. showed that hydrostatic pressure is able inhibit shear stress-induced pro-catabolic actions in chondrocytes [29]. Together, these data indicate that a balance of effects induced by different forms of mechanical stress is needed, in order to maintain healthy cartilage tissue. In the research included in this thesis only one type of mechanical stress was used. Nevertheless, it might be possible that the application of different types of mechanical stress or a combination of mechanical compression with other types of stress would lead to alternative effects. Like showed by Gunn et al. that shear stress can induce pro-catabolic factors including IL-1β which have been shown in this thesis to negatively affect mechanically-mediated activation of TGF-β signaling [5]. On the other hand, also other types of mechanical load, including tensile strain [30], and shear stress [31] have been shown to be able to activate TGF-β signaling, however in the isolated chondrocytes. Nevertheless, it remains important to confirm the effect of other types of mechanical stress on the activation of TGF-β signaling in the intact cartilage and not isolated cartilage cells. This would create a more complete picture of the role of mechanical signals in different forms on activation of TGF-β signaling in articular cartilage. The significance of such follow up research is exemplified also by the fact that contact between cells and ECM can control and modulate mechanotransduction [32]. This aspect seems important especially in regard to
mechanically-mediated activation of a growth factor like TGF-β, which is stored in inactive form in cartilage ECM and is described to be activated by mechanical signals [6].

Articular cartilage condition and its response to external factors, including mechanical stress, can be modulated by other components of a synovial joint, like meniscus, synovial fluid, synovial membrane or sub-chondral bone [33]. Considering this fact, it seems clear that expected follow up of research described in this thesis should be in vivo studies. It needs to be confirmed that in vivo joint loading results in TGF-β signaling activation in the cartilage. This type of research seems essential considering the reports of Albro et al. in which the authors showed that the latent form of TGF-β can be activated in synovial fluid by shear stress [34] and later this growth factor is transported and accumulated in articular cartilage as well as possibly in other joint tissues [35]. This phenomenon shows that mechanically activated TGF-β in one part of the joint can possibly play a role in maintaining the joint homeostasis by acting on other tissues. Moreover, it proves that activation of this signaling in articular cartilage can depend on other joint components than cartilage. Additionally, in vivo study that investigated the importance of loading on cartilage, brought direct prove that cartilage has to be loaded to stay intact. Campbell et al. proved that in immobilized joints, on a non-loaded region of the tibia, articular cartilage is thinning, GAGs are being lost and finally cartilage is being replaced by bone [36]. Independently, a recent report showed that disruption in muscle activity and hence joint movement is associated with diminished TGF-β signaling activity and distortion in cartilage condition [37]. Those reports illustrate that in vivo investigation of the role of mechanical signals in TGF-β and hence cartilage and other synovial joint tissues physiology is already taking place and provides a clear and functional information for physicians: articular cartilage has to be loaded in order to activate crucial TGF-β signaling in this tissue and hence to develop, stays intact and maintain healthy.

According to the newest reports, half of the world’s population, aged 65 and older, suffers from cartilage destruction and joint problems related to OA [38]. However, despite well known risk factors for OA development, no fully effective treatment for its symptoms have been developed and introduced to the clinical treatment yet. Notably, at the end stage of OA, in order to retaining joint functionality, the only effective treatment is total joint replacement (TJR) with an artificial implant [39]. Although majority of TJR are successful, problems related with aseptic prosthetic loosening, infection and post-operative instability of implant may require a revision procedure which is related to additional and serious complications for the patient [40, 41]. Despite good efficacy and high successful rate of TJR in improving the patient quality of life, there is no doubt that the effort should be focused on preventing total joint replacement by suppressing OA-related joint destruction and stimulate tissue regeneration.

For those patients, activation of TGF-β signaling in articular cartilage for protecting tissue integrity seems to be an obvious way of treatment [42]. The easiest way of doing so is a simple injection of recombinant TGF-β into the joint cavity. That kind of treatment showed number of positive aspects not only in stimulation of matrix synthesis [43] but also in inhibiting the deleterious process of cartilage hypertrophy [44]. Nevertheless, the effects of injecting TGF-β into a joint are not limited to cartilage. TGF-β supplementation into the whole joint, results in responses of other tissues that are in contact with the synovial fluid and those responses are not always desirable. Reports showed that TGF-β injection, directly into the joint cavity, cause synovial membrane fibrosis and joint stiffening as well as severe osteophyte formation [43]. In contrast to TGF-β injection, mechanically-mediated activation of TGF-β signaling in cartilage is an efficient and highly specific way of initiation of this signaling exactly where its actions are needed and without affecting other joint tissues with undesirable TGF-β activity. Unquestionably, results presented in this dissertation bring an excellent reasoning for physicians in explaining and sharing
with patients the results provided by Fransen et al. who showed that patients with early OA who start participating in a physical exercise program can improve their physical function immediately after treatment [45]. This is meaningful, since evidences like the one postulated by Fransen et al., reassure healthcare professionals and OA patients that physical activity and hence cartilage loading that is performed regularly and is closely monitored can improve quality of life related to knee OA. That is why this thesis with its important conclusions is an example of research that could bridge scientific discoveries about cartilage physiology to clinical applications and practical solutions which can be highly useful for patients and physicians.

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SUMMARY

Articular cartilage is a tissue covering the ends of long bones that form the joint. Cartilage function is fundamental in the human body, since it allows the movements of the joints with practically no friction. In addition, articular cartilage is involved in the absorption and distribution of forces acting on the joint. This means that articular cartilage performs a strictly mechanical function in the human body.

Importantly, cartilage tissue is able to effectively perform its functions due to its unique composition and the exceptional features of its distinct components. Articular cartilage is composed of cells called chondrocytes, which are embedded in an extracellular matrix. Chondrocytes are the only cell type in articular cartilage and are the only components of the tissue that are metabolically active, being able to actively control the composition and structure of the extracellular matrix. Chondrocytes control the processes of new matrix formation and its degradation. Both of these processes are regulated by chemical, biological and mechanical signals. What is important, in order to keep cartilage intact, the balance between degradation and synthesis of new extracellular matrix has to be strictly maintained.

Mechanical forces are absolutely critical to maintain healthy cartilage tissue. However, overloading is considered deleterious for articular cartilage and a driver for degenerative disease (OA). Results described in this dissertation provide not only additional confirmation of the dependence of articular cartilage health on regular loading but additionally explain a mechanism underlying this dependence. Data included in CHAPTER 3 demonstrated that articular cartilage compressed not only with physiological but also with excessive force activated cartilage preserving TGF-β/Smad2/3P signaling. Moreover, both compressive forces diminished deleterious Smad1/5P signaling. This indicates that excessive mechanical compression alone is
not able to shift TGF-β signaling towards cartilage destructive terminal differentiation induced by TGF-β/Smad1/5 signaling. However, we postulated that additional factors, like advanced age, inflammatory conditions and/or another type of loading could alter TGF-β signaling in articular cartilage to promote TGF-β mediated terminal differentiation and OA-like changes in overloaded articular cartilage.

Motivated by this, in follow-up study described in CHAPTER 4, cartilage was isolated from two different aged groups and its response to mechanical compression was analyzed. Activation of TGF-β signaling was examined using immunohistochemical (IH) detection and TGF-β signaling responsive gene expression analysis. Results showed that cartilage isolated from aged individuals had a highly reduced ability for mechanically-mediated activation of Smad2/3P signaling when compared to young cartilage. Additionally, in aged cartilage no mechanically-mediated up-regulation of growth factors considered to be important for the maintenance of cartilage health was observed. This reduced mechanically-mediated response could be a result of a change in matrix stiffness of old compared to young cartilage which made it harder to mechanically activate TGF-β signaling, or it could be a result of an age-related reduction in expression of TGF-β receptor ALKS, reported before to occur in aged cartilage.

From many years, scientists have provided evidence that abnormally increased load on the articular cartilage is one of the most important risk factors for the development of degenerative joint diseases (OA). However, recent detailed studies show that the development of OA in patients who underwent meniscectomy, might not only be a result of changes in the biomechanics of the joint. Additional factors, such as proinflammatory cytokines, produced during contusion and tissue damage, are thought to play an important role in the development of cartilage destructive disease. These cytokines which are produced in the initial phase after injury, stimulate catabolic changes and participate in the development of the disease.

In CHAPTER 5 we investigated the effect of proinflammatory conditions on mechanically-mediated activation of TGF-β/Smad2/3P signaling in cartilage. In order to do so, cartilage explants were subjected to dynamic mechanical compression in the presence of interleukin-1 (IL-1β) or osteoarthritic synovium-conditioned medium (OAS-CM). Subsequently, the activation of the TGF-β/Smad2/3P pathway was monitored with reporter gene expression analysis. Additionally, the expression of key receptors, activating the TGF-β/Smad2/3P pathway was analyzed. Results showed that proinflammatory conditions partly impaired the mechanically-mediated activation of the protective TGF-β/Smad2/3P pathway. Furthermore, excessive mechanical compression, applied in the presence of proinflammatory conditions diminished the expression of the type II TGF-β receptor, a receptor critical for maintenance of healthy articular cartilage.

For decades, it is known that reduced joint loading leads to cartilage progressive loss and degeneration, both in humans and animal models. Patients with spinal cord injuries show progressive loss of knee cartilage at a pace faster than patients with diagnosed OA. In CHAPTER 6 we found that in adult articular cartilage unloading resulted in immediate loss of TGF-β signaling in this tissue. Remarkably, subsequently applied compressive loading, rapidly restored this loss. Furthermore, loading stimulated the expression of the protective receptor ALK5 and blocked expression of the deleterious receptor ALK1. Most importantly, both loading as well as exogenous TGF-β inhibited un-loading-induced elevated Col10a1 expression but not proteoglycan loss. Our observations provided evidences for hypothetical model in which loading activates a self-perpetuating system that prevents hypertrophic differentiation of chondrocytes and is crucial for cartilage homeostasis. Moreover, it shows that in vivo regular physiological loading of articular cartilage leads to enduring TGF-β signaling and TGF-β-induced gene expression.
There is no doubt that data described in this thesis and concussions made based on this
data, provide explanations how loading results in enhanced TGF-β signaling and maintenance of
cartilage homeostasis and how OA risk factors (ageing, inflammation) and loading interact,
impairing this elegant control system. Certainly, unraveling the exact molecular mechanism
underlying these interactions and relations could provide tools to interfere with OA development
and/or progression.

STRESZCZENIE

Chrząstka stawowa to tkanka, pokrywająca nasady kości tworzących staw. Chrząstka
pozwała na ruchy kości w stawach praktycznie bez tarcia. Ponadto, chrząstka stawowa bierze
udział w pochłanianiu oraz dystrybucji sił oddziaływujących na staw. Znaczy to, że chrząstka
stawowa pełni funkcje ścisłe mechaniczną.

Co ważne, tkanka ta jest w stanie skutecznie spełniać swoją funkcje dzięki unikalnej
budowie oraz wyjątkowym cechom swoich poszczególnych komponentów składowych.
Chrząstka stawowa składa się z komórek zwanych chondrocytami, które zawieszone są w
macierzy zewntrzkomórkowej. Macierz ta składa się z trzech podstawowych części (faz):

I. Fazy stałej, składającej się z sieci kolagenowej oraz agregatów proteoglikanowych.

Sieć kolagenowa z kolagenem typu II jako dominującym typem kolagenu
odpowiedzialna jest za nadanie tkance wytrzymałości na rozciąganie. Natomiast
proteoglikany, jako bialka silnie glikozylowane oraz zawierające bardzo duża ilość
negatywnie naładowanych łańcuchów siarczanowych przyciągają oraz zatrzymują w
macierzy znaczne ilości cząsteczek wody.

II. Fazy wodnej stanowiąca 70 do 85% masy tkanki.

III. Fazy jonowej z jonami takimi jak Na⁺, Ca²⁺, K⁺, które to neutralizują negatywny
ładunek proteoglikanów.

Chondrocyty to jedyny rodzaj komórek w chrząstce stawowej i jako jedyne składniki tkanki
posiadające aktywność metaboliczną zdolne są do aktywnej kontroli składu oraz struktury
macierzy zewntrzkomórkowej. Dzięki temu chondrocyty kontrolują procesy produkcji nowej
macierzy a także jej degradacji. Oba te procesy mogą zainicjowane za pośrednictwem sygnałów
chemicznych, biologicznych oraz sygnałów mechanicznych.
Co ważne, aby zachować chrząstkę stawową w stanie pełnej użyteczności oraz stanie nienaruszonym, ściśły balans pomiędzy procesem degradacji oraz syntezą nowej macierzy zewnętrzkomórkowej musi zostać zachowany. Dlatego też, jakiekolwiek przedłużające się zakłócenie z podwyższonym poziomem degradacji, prowadzi do destrukcji tkanki oraz zanieczyszczenie chorób chrząstki stawowej.

Kondycja chrząstki stawowej bardzo silnie zależy od obecności obciążenia mechanicznego działającego na strukturę tkanki. Istnieje wiele dowodów naukowych oraz raportów pokazujących, iż obecność sił mechanicznych jest absolutnie krytyczna dla zachowania zdrowej tkanki chrząstki stawowej. Wykazano, że brak obciążenia na chrząstkę stawową lub jego znaczna redukcja prowadzi do postępującej degradacji oraz niszczenia tej tkanki.

Podczas ruchu stawów chrząstka jest poddawana obciążeniu mechanicznemu które powoduje jej kompresję. Zmiany w chrząstce, wywołane kompresją mechaniczną obejmują deformacje macierzy zewnętrzkomórkowej wraz z zawieszonymi w niej chondrocytami, przepływ i ruch płynu tkankowego, zmiany w ciśnieniu osmotycznym oraz lokalnej koncentracji jonów. Niemniej jednak, różne aktywności fizyczne a co za tym idzie różne siły mechaniczne działające na chrząstkę stawową, będą prowadziły do odmiennych odpowiedzi tkanki. I tak dla przykładu; w ludzkim stawie kolanowym skala nacisku waży się pomiędzy 1 a 6 MPa (w zależności od aktywności stawu). Nie mniej jednak, wartości te mogą ulec dwukrotnemu, a nawet trzykrotnemu zwiększeniu w sytuacjach patologii stawu takich jak po usunięciu łękołki kolanowej. Co ważne, raporty naukowe wskazują, że obciążenie mechaniczne chrząstki stawowej o wartościach pozostających w zakresie wartości fizjologicznych są związane aktywacją procesów ochronnych i anabolicznych. Z drugiej strony natomiast wykazano, iz zbyt wysokie obciążenie mechaniczne, przekraczające wartości fizjologiczne przez długi okres czasu prowadzi do rozpoczęcia procesów katabolicznych i degradacji tkanki.

Tak specyficzna i wyrafinowana odpowiedź tkanki chrzęstnej na obciążenie mechaniczne oparta jest na unikalnej zdolności chondrocytów do rozpoznawania sygnałów mechanicznych docierających ze środowiska zewnętrznego. Jest to o tyle istotne, ponieważ chondrocyty to jedyne komórki w tkance i to one, jako jedyne kontrolują procesy modelowania i przekształcania macierzy zewnętrzkomórkowej. Chondrocyty rozpoznają i są w stanie odpowiadać na szerokie spektrum sygnałów mechanicznych takich jak napięcie mechaniczne, ciśnienie hydrostacyjne oraz mechaniczne, a także tarcie wywołane przepływem płynu międzytkankowego. Dzieje się tak, ponieważ komórki chrząstkii posiadają serie receptorów, których funkcją jest rozpoznawanie oraz transdukcja zjawisk wywołanych przez obciążenie mechaniczne nałożone na tkankę chrzęstną.

Na przestrzeni kilkunastu ostatnich lat badań, pojawiło się bardzo wiele donoseń naukowych dotyczących oraz opisujących mechanizmy mechanotransdukcji w tkance chrzęstnej oraz samych chondrocytach. Istnieje szereg mechanizmów, za pomocą których chondrocyty odbierają sygnały mechaniczne oraz przetwarzają je na sygnały wewnętrzkomórkowe, co z kolei generuje adekwatną odpowiedź fizjologiczną na poziomie tkanki a następnie całego stawu. Jednakże, nie tylko czynniki mechaniczne odgrywają istotną rolę w regulowaniu homeostazy chrząstki stawowej. Istnieje bardzo wiele czynników wzrostu, które kontrolują ten proces. Co ciekawe, najnowsze doniesienia naukowe wskazują, że istnieje silny związek pomiędzy aktywacją transformującego czynnika wzrostu beta (TGF-β) oraz zachowaniem zdrowej i nienaruszonej chrząstki stawowej. Super-rodzina transformującego czynnika wzrostu składa się z czterech różnych podrodzin i razem zawiera ponad 35 białek o różnych funkcjach regulatorowych. Zawiera się w tym między innymi szereg lizoform samego transformującego czynnika wzrostu beta (TGF-β), białka morfogenetyczne kości (BMP), aktywiny i inne. Czynniki wzrostu rodziny TGF-β poprzez kontrolę kluczowych procesów komórkowych takich jak podziały komórkowe, ich
różnicowanie, migracje oraz apoptozę, odgrywają zasadniczą rolę w rozwoju oraz utrzymaniu homeostazy tkanki chrząstnej.

Nie mniej jednak, już od kilku dekad naukowcy udowadniali, że zaburzone lub nienormalnie zwiększone obciążenie chrząstki stawowej jest jednym z najistotniejszych i decydujących czynników ryzyka dla rozwoju chorób degeneracyjnych stawów takich jak osteoarthroza (OA). Osteoarthroza w literaturze bardzo często określana jest mianem choroby ścieśla związanej z czynnikami mechanicznymi i dostępnych jest bardzo wiele przykładów udowadniających, że normalne obciążenie stawów powoduje zmiany degeneracyjne nie tylko w chrząstce stawowej, ale w innych tkankach tworzących staw. Jednym z takich przykładów jest uwarzunkowane urazem chirurgiczne usunięcie łączek kolanowej (menisectomia). Intervencja ta powoduje znaczne zmiany biomechaniczne, które to zostały naukowo powiązane ze zmianami degeneracyjnymi w całym stawie kolanowym. Najpowszezone epidemiologiczne pokazują, że u około 48 % pacjentów, którzy przeszli zabieg usunięcia łączek, zostały stwierdzone radiograficzne objawy rozwoju osteoarthroz ni po 21 latach od przebytej operacji.

Jednakże, ostatnie szczegółowe badania dowodzą, że rozwój osteoarthroz u pacjentów po menisectomii lub innych urazach stawu, nie jest jedynie generowany przez zmiany w samej biomechanice stawu. Zostało wykazane, że dodatkowe czynniki takie jak cytokiny pro-zapalne, produkowane podczas urazu stawu, odgrywają istotną rolę w rozwoju choroby zwrodnieniowej stawów. Cytokiny te, produkowane w początkowej fazie po urazie, stymulują zmiany kataboliczne i współuczestniczą w rozwoju choroby. Dodatkowo, odrębne publikacje dowodzą, że zawawansowany wiek jest istotnym czynnikiem ryzyka dla rozwoju osteoarthroz. Dzieje się tak gdy starzenie się chrząstki stawowej oraz innych tkank budujących staw pociąga za sobą zmiany na poziomie komórkowym, które dodatkowo wspomagają oraz uczestniczą w degradacji stawu powowiedzonej czynnikami mechanicznymi.

Celem tej rozprawy doktorskiej było zbadanie roli obciążenia mechanicznego w aktywacji sygnalizacji czynnika wzrostu TGF-β w chrząstce stawowej. Cześć pierwsza (ROZDZIAŁ 2), która jest przeglądem literaturowym omawia i dyskutuje znaczenie oraz motywację odnośnie użycia najbardziej odpowiednich modeli eksperymentalnych podczas badań nad rolą sygnałów mechanicznych w fiziologii chrząstki stawowej. Cześć druga (ROZDZIAŁ 3, 4, 5 i 6), składająca się z artykułów badawczych, opisuje badania nad rolą sygnałów mechanicznych w procesie aktywacji ścieżek sygnałowych czynnika wzrostu TGF-β w tkance chrząstki stawowej. Badania te zostały przeprowadzone w odpowiednich warunkach, które to miały odzwierciedlać klinicznie ukierunkowane warunki patologiczne oraz czynniki destrukcyjne, które mogłyby zaburzać mechanicznie uwarzunkowaną aktywację sygnałingu TGF-β.

W ROZDZIALE 3 dokonano weryfikacji sygnałów mechanicznych w formie dynamicznej mechanicznej kompresji, jako metody aktywacji ochronnej sygnalizacji TGF-β w eksplantach tkanki chrząstnej. Następnie, zbadano rolę dynamicznej mechanicznej kompresji o różnym nasileniu (fizjologicznym oraz patologicznym) w aktywacji destrukcyjnej ścieżki sygnałowej Smad1/5/8P.

Dane z badań epidemiologicznych wskazują, że zaawansowany wiek jest jednym z najistotniejszych czynników ryzyka dla rozwoju choroby zwrodnieniowej stawów. Ponadto, odrębne badania udowadniają, że zmiany komórkowe zachodzące z wiekiem w chondrocytach mają istotny wpływ na zmiany w ścieżce sygnałowej TGF-β. Dlatego też, celem ROZDZIAŁU 4 było zbadanie czy chrząstka stawowa u osobników w podeszłym wieku posiada ograniczoną zdolność do aktywacji ścieżki sygnałowej TGF-β/Smad2/3P za pomocą sygnałów mechanicznych. Jest to o tyle istotne, że aktywna ścieżka sygnałowa TGF-β/Smad2/3P jest krytyczna w ochronie chondrocytów przed procesem końcowego różnicowania oraz finalnego zastąpienia przez kość.
Raporty naukowe dowodzą, że po urazie stawu, chrząstka stawowa narażona jest na niszczące działanie nadmiernego obciążenia mechanicznego połączonego z działaniem katabolicznego stresu wywołanego przez cytokiny pro-zapalne. Mając na uwadze ten fakt, celem ROZDZIAŁU 5 było zbadanie czy czynniki pro-zapalne obecne podczas aplikacji obciążenia mechanicznego są w stanie ograniczyć lub zahamować aktywację ścieżki sygnałowej TGF-β/Smad2/3P za pomocą sygnałów mechanicznych.

Juz od wielu lat doniesienia naukowe wskazywały, że chrząstka stawowa musi być poddana obciążeniu mechanicznemu aby zachować swoją integralność. Jako, że we wcześniejszych rozdziałach tej rozprawy wykazano, że sygnały mechaniczne są wysoce wydajne jako czynnik aktywujący ścieżkę sygnałową TGF-β/Smad2/3P, celem ROZDZIAŁU 6 było udowodnienie czy regularnie obciążana chrząstka stawowa posiada stały i wysoki poziom aktywności ścieżki sygnałowej TGF-β/Smad2/3P. Ponadto, w Rozdziale 6 chciano udowodnić czy obciążenie mechaniczne jest w stanie powtarzalnie przywrócić aktywacje ścieżki sygnałowej TGF-β/Smad2/3P po uprzednim długotrwałym usunięciu jakiegokolwiek obciążenia działającego na tę tkankę.

W ROZDZIALE 7 wyniki opisane w tej rozprawie doktorskiej zostały przedyskutowane i przeanalizowane. Miało to na celu stworzenie szerokiego obrazu rezultatów oraz dać podstawy do zaproponowania nowych rozwiązań dla przyszłych projektów badawczych, w ramach których możliwe będzie dalsze kontynuowanie linii badawczej, rozpoczętej w ramach tej pracy doktorskiej.

SAMENVATTING

Articulair kraakbeen is het weefsel dat gevonden kan worden aan het eind van (lange) beenderen in synoviale gewrichten. De functie van kraakbeen is essentieel voor het menselijk lichaam omdat het (haast) vrijvloeiende beweging van gewrichten mogelijk maakt. Ook helpt articulair kraakbeen bij het absorberen van de krachten die ontstaan in een gewricht bij beweging. Dit laatste is een strikt mechanische functie van kraakbeen, en kraakbeen kan dit doen door diens unieke samenstelling en de unieke eigenschappen van diens bouwstenen. Kraakbeen bestaat uit extracellulaire matrix en slechts een celtype, de chondrocyt. Deze chondrocyten zijn de enige metabool active component van kraakbeen en zijn verantwoordelijk voor het onderhoud van de extracellulaire matrix. Chondrocyten reguleren de aanmaak en afbraak van de matrix onder invloed van chemische, biologische en mechanische signalen. Hierbij is het erg belangrijk voor gezond kraakbeen dat er een balans is tussen deze afbraak en opbouw. Al langere tijd is het bekend dat belasting cruciaal is voor het behoud van gezond kraakbeen, maar ook dat het overbelasten van het kraakbeen nadelige gevolgen heeft en gekoppeld is aan de ontwikkeling van artrose. De resultaten die wij beschrijven in deze dissertatie bevestigen niet alleen het belang van belasting voor gezond kraakbeen, maar laten ook zien waarom belasting zo belangrijk is.

Zo laten de experimenten in HOOFDSTUK 3 zien dat als kraakbeen (zowel normaal als excessief) belast werd dit leidde tot activering van de voor kraakbeen beschermende TGFβ/Smad2/3P signalering. Ook verlaagde belasting van kraakbeen de voor articulair kraakbeen nadelige Smad1/5 signalering. Deze laatste observatie laat zien dat excessieve mechanische compressie niet in staat is om TGFβ signalering te veranderen van Smad2/3 naar Smad1/5. Echter, mogelijk kunnen andere factoren zoals leeftijd, ontsteking en een andere soort belasting
TGFβ signalering toch zodanig beïnvloeden dat dit tot TGFβ geïnduceerde terminale differentiatie van chondrocyten en artroseachtige veranderingen leidt.

Daarom hebben we in HOOFDSTUK 4 gekeken of leeftijd invloed heeft op TGFβ activering en signalering in belast kraakbeen. Dit hebben we gedaan door immunohistochimisch de TGFβ signaalmultiples te bekijken en te kijken naar bijbehorende gen expressie. De uitkomst was dat oud kraakbeen veel minder goed in staat was om Smad2/3P signalering te activeren na belasting dan jong kraakbeen. Ook vonden we in oud kraakbeen in reactie op belasting geen verhoogde expressie van andere groeifactoren waarvan gedacht wordt dat ze belangrijk zijn voor gezond kraakbeen. Een mogelijke reden voor het gebrek aan mechanische TGFβ activering in ouder kraakbeen is dat de matrix stijver is geworden door de veroudering waardoor TGFβ minder makkelijk geactiveerd kan worden, een andere mogelijke reden is dat de receptor voor TGFβ, ALKS, verlaagd is bij veroudering zoals al eerder beschreven is. Naast veroudering is het ook al lang bekend dat abnormale belasting van kraakbeen een van de belangrijkste risicofactoren voor het ontwikkelen van artrose is. Recent onderzoek hebben echter laten zien dat de ontwikkeling van artrose in patiënten met een meniscectomie niet puur mechanismisch van aard is. Additionele factoren, zoals pro-inflammatoire cytokine die worden gemaakt na het ontstaan van weefselontsteking, bleken belangrijk te zijn voor de ontwikkeling van kraakbeenschade, mogelijk omdat ze de afbraak van kraakbeen stimuleren.

In HOOFDSTUK 5 hebben we daarom onderzocht wat de impact van deze pro-inflammatoire cytokine op de mechanisch gemedieerde TGFβ signalering is. Om dit te kunnen onderzoeken hebben we stukjes kraakbeen blootgesteld aan belasting in de aanwezigheid van interleukine 1β of een geconditioneerd medium gemaakt met behulp van het synovium van artrose patiënten. Vervolgens hebben we gekeken naar de activering van TGFβ signalering op het niveau van genexpressie. Daarnaast hebben we ook naar de expressie van TGFβ receptoren gekeken. Uit onze experimenten bleek dat de pro-inflammatoire factoren de activering van Smad2/3P signalering verminderden. Ook verlaagden deze factoren de expressie van TGFβ, de essentiële receptor voor TGFβ signalering, maar alleen in combinatie met overbelasting van het kraakbeen. Naast overbelasting is het ook al langer bekend dat te weinig belasting schadelijk is voor kraakbeen. Zo hebben bijvoorbeeld patiënten met een dwarslaesie een sneller en groter verlies van kraakbeen dan artrose patiënten.

In HOOFDSTUK 6 beschrijven we dat dit mogelijk komt doordat het weghalen van belasting leidt tot verlies van beschermende TGFβ/Smad2/3P signalering. Belasting bleek echter in staat dit verlies snel te herstellen en zorgde ook voor meer ALKS en minder ALK1 receptor expressie. Ook was een belangrijke uitkomst van deze studie dat belasting collagen type 10 expressie sterk verlaagt maar geen invloed heeft op het glycosaminoglycan gehalte van het kraakbeen. Deze observaties laten zien dat in vivo kraakbeen continue wordt blootgesteld aan actieve TGFβ signalering, en brachten ons tot een model waarin deze continue activiteit van TGFβ het phenotype van de chondrocyte beschermt tegen hypertrofie en zo goed is voor kraakbeen homeostase. Er bestaat geen twijfel dat de resultaten beschreven in deze dissertatie, en de conclusies die daar uit getrokken kunnen worden, een verklaring bieden voor hoe belasting leidt tot verhoogde TGFβ signalering en hoe dit zorgt voor kraakbeen onderhoud. Ook laten onze resultaten zien hoe risicofactoren van artrose zoals ontsteking en veroudering hierop ingrijpen. Het nog verder uitdopen van deze bevindingen, zoals het achterhalen van het precieze moleculaire mechanisme dat hier aan ten grondslag ligt, kan helpen om in de toekomst artrose te bestrijden.
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LIST OF PUBLICATIONS


2. van Caam A, Madej W, Garcia de Vinuesa A, Goumans MJ, Ten Dijke P, Blaney Davidson E, van der Kraan P. TGFβ1-induced SMAD2/3 and SMAD3 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity. Arthritis Research & Therapy 2017


CURRICULUM VITAE

Wojciech Maciej Madej was born in Stalowa Wola, Poland on 21st December 1986 where he grew up in big and loving family. In 2005, he finished his high school in class with profile oriented on biology, chemistry and physics. His interests and curiosity related to nature and science motivated him to start studies in Biology on the best and the oldest university in Poland- Jagiellonian University in Kraków. Beside the ground education he was always interested in applied science. This led him to focus his education on cell and medically oriented biology.

Already on first year of his master studies, he started to work in the department of Cell Biology and Imaging, in a group of dr Anna Maria Osyczka. This work resulted in master thesis where he has investigated the ability of bioglass composite biomaterials to improve osteogenesis of human mesenchymal stem cells. Additionally, during his master’s program, he spent 6 months in a Centre for Biomaterials and Tissue Engineering at the University of Sheffield (UK) where he was investigating the properties of mechanical loading as regulator of extracellular matrix production in human mesenchymal stem cells. After defending his master’s thesis and obtaining specialization in Cell Biology, he got the opportunity to further develop his interest in tissue engineering and applied biology in the Netherlands. In 2010, he joined research group of prof. Pieter Buma in Orthopaedic Research Laboratory in Nijmegen, where he started his PhD project. In collaboration with prof. Peter van der Kraan from Experimental Rheumatology they have developed a new research line in order to investigate the role of mechanical signals in articular cartilage biology. This fruitful work and joint effort resulted in this PhD dissertation. During his PhD program Wojciech Madej had many chances to share the results of his research with a prominent audience on the international conferences like ORS (Orthopaedic Research Society), OARSI (Osteoarthritis Research Society International) and EORS (European Orthopaedic Research Society). He is also a Recipient of 2016 OARSI Young Investigators Award (2016 Amsterdam), an award for the highest scored abstract. In 2017, Wojciech moved back to Gdynia in Poland, where he took up the position of Senior Research and Development Specialist in polish company Biovico. He is a leader of a project that aims to develop a new product as an injectable biomaterial for surface cartilage defects repair.