TGFβ signaling in cartilage health and disease
A tale for young and old

Arjan P.M. van Caam
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TGFβ signaling in cartilage health and disease
A tale for young and old

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For want of an ALK5 the TGFβ signal was lost.
For want of a TGFβ signal the pSmad3 was lost.
For want of a pSmad3 the chondrocyte was lost.
For want of a chondrocyte the matrix was lost.
For want of a matrix the cartilage was lost.
For want of a cartilage the joint was lost.
And all for the want of an ALK5.

after: “For want of a nail”, English proverb
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1. General introduction

The TGFβ-family in cartilage health and disease

(to be submitted for publication)
**Introduction**

Joint pathologies can greatly negatively impact the quality of life of patients. In this chapter we will discuss part of the biology behind the most common joint disease: osteoarthritis (OA). We will start by discussing the basic biology behind cartilage, the tissue OA affects most. Next, we describe cartilage pathophysiology in OA. Subsequently, we will discuss the role of the transforming growth factor β (TGFβ)-family in regulation of cartilage homeostasis by explaining how this family signals and how this signaling impacts cartilage. We end by listing several TGFβ-family members and describing their importance in cartilage biology.

**Osteoarthritis**

OA is the world’s most common joint disease, *e.g.* approximately 30% of all men and women older than 60 have radiographic knee OA [1]. Patients suffer from loss of joint function and pain, which can greatly reduce their quality of life. According to the Osteoarthritis research society international, OA is defined as follows:

“Osteoarthritis is a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness. (OARSI 2015)”

which illustrates both the multifactorial origins and multifactorial outcomes of OA; all (synovial) joint tissues can initiate and be affected by the disease. However, end stage disease is always characterized by cartilage degeneration. Unfortunately, cartilage has poor regenerative capacity, making loss almost always irreversible. Up till now, no disease modifying treatment or cure has been developed [2], leaving total joint replacement as the only option for severely affected patients. Risk factors of OA include genetic predisposition, race, sex, increased body mass, and age [3, 4]. In view of the expected rise in life expectancy and average body weight in western societies, the incidence of OA is expected to rise in the future [3, 4]. Because of this expected increase in OA incidence and the lack of a cure, it is of great importance to either develop such a cure or to learn how to prevent the onset of disease. Both aims can greatly benefit from a better understanding of joint homeostasis and OA pathophysiology. Because cartilage degeneration is the final and irreversible hallmark of OA pathology, a better understanding of this tissue may well be critical to combat this disease.
Articular (Hyaline) Cartilage

Articular cartilage is a type of hyaline cartilage, and is the connective tissue that can be found on the long ends of bones in diarthrodial joints [5, 6]. It combines two of its main characteristics: (visco) elasticity and a lubricated smooth surface to facilitate diarthrodial joint function by working as a shock absorber and by lowering the joint’s friction coefficient. Articular cartilage is of mesodermal origin and, rather uniquely for a tissue, is not innervated nor does it contain blood vessels to supply oxygen or nutrients [5, 6]. Hyaline cartilage contains only one cell type: the chondrocyte. However, chondrocytes make up only a small part of hyaline cartilage (≤ 2% volume), most of this tissue consists of the extracellular matrix (ECM) (98% volume) and water (up to 80% of wet weight) [5, 7]. This ECM is a highly organized network of collagen proteins, hyaluronan molecules, and proteoglycans.

Collagens are the most abundant proteins in the human body and provide it with its shape [8]. In cartilage, over 90% of all collagen is collagen type II [9]. Collagen type II is a fibrillar collagen which forms (long) fibrils consisting of multiple subunits of three collagen proteins arranged in a triple helix [8]. Notably, these fibrils are distributed throughout the ECM and provide (tensile) strength and shape. These fibrils can be formed in association with other collagens like collagen type IX and collagen type XI [8, 10]. This association regulates fibril diameter, strength, (thermo)stability, and the interaction with other (bio)molecules [8]. In addition to these collagens, also network-forming collagens like collagen type IV, VI and X can be found in cartilage (the latter only around hypertrophic-like chondrocytes, see: chondrocyte homeostasis)[9]. These collagens are found in close proximity to chondrocytes and form a network instead of fibers, which helps compartmentalize biomolecules and processes to the pericellular region.

Hyaluronan is a glycosaminoglycan (GAG), which is a large unbranched polysaccharide consisting of repeating disaccharide units [11]. Unlike other GAGs, hyaluronan is not sulfated, nor is it covalently attached to a proteoglycan. A single hyaluronan molecule can contain as much as 25 000 disaccharide repeats and can weigh as much as 10 million Dalton [12]. Due to this size, production of hyaluronan does not occur inside the cell but trough the cell membrane by hyaluronan synthases [12]. Hyaluronan is very hydrophilic and forms spontaneously aggregating networks of randomly coiled molecules [13]. This network forms a scaffold to which other biomolecules such as proteoglycans can bind. Furthermore, this network helps to regulate the water content of cartilage by “trapping” water molecules. Hyaluronan is also a signalling molecule; for example, via the CD44 receptor high molecular mass hyaluronan is anti-angiogenic, whereas small hyaluronan fragments are pro-inflammatory and pro-angiogenic [14, 15].

Proteoglycans are proteins glycosylated with sulfated GAGs. Proteoglycans are classified based on their size in large proteoglycans and small leucine-rich proteoglycans (SLRPs) [16]. Examples of large proteoglycans in cartilage are perlecan, versican and aggrecan,
examples of small leucine-rich proteoglycans are biglycan, decorin, asporin and fibromodulin. Aggrecan is the most abundant large proteoglycan in cartilage and is responsible for 90% of all proteoglycan mass in this tissue [17]. Aggrecan is heavily glycosylated with sulfated GAGs and forms aggregates with hyaluronan via link protein. These aggregates carry a static charge density due to the negative charge of the GAG chains and therefore attract water, resulting in a high osmotic (swelling) pressure [17]. This swelling pressure is needed to counteract compressive forces and helps restore tissue shape after compression. Additionally, this swelling pressure is further increased by the electrostatic repulsion between the sulfated GAG chains, which makes these molecules occupy as much space as possible [17]. SLRPs are less important for generating this swelling pressure but are important for the stabilization and formation of collagen fibrils during growth [18]. Furthermore, SLRPs modulate growth factor signaling by regulating the bioavailability of these factors by binding them, for example biglycan and asporin bind BMP2, BMP4 and TGFβ [18].

The organization and composition of cartilage ECM is not static but dynamic and subject to change based on external input like mechanical force. Chondrocytes, as the only cell type present in cartilage, are responsible for the biological non-physical aspects of this change. These cells can produce large amounts of the ECM building blocks and ECM-strengthening enzymes such as collagen cross linking enzymes. Furthermore, chondrocytes can also produce matrix degrading enzymes like matrix metallopeptidases (MMP) or ADAM metallopeptidases with thrombospondin type 1 motif (ADAMTS), which are needed for remodeling of the existing matrix. Together, the anabolic and catabolic production of chondrocytes function in tandem to help maintain a healthy and functional cartilage ECM.

**The zones of articular cartilage**

Cartilage is not a homogeneous tissue and its organization is a classic example of form fits function: its form differs throughout the different zones that characterize articular cartilage in order to fulfill the specific demands required for the function of each zone. The main determinants of cartilage organization are the forces to which this tissue is exposed to: osmotic swelling pressure from the tissue itself combined with sheer stresses and hydrostatic pressure generated by joint movement.

The uppermost zone of cartilage is the lamina splendens. This layer is only a few micrometer (± 4-8 µm) thick and contains no cells [19]. Its matrix is characterized by collagen fibrils oriented in parallel to the cartilage surface [19, 20] and by high levels of hyaluronan and of the lubricant proteoglycan 4 (PRG4) [21], but this zone contains relatively little other proteoglycans. These characteristics provide a very smooth surface with a low friction coefficient, which lowers shear stress on the cartilage surface during joint movement.
Below the lamina splendens lies the superficial or tangential zone which represents 10 - 20% of articular cartilage [6]. This zone contains a relatively high amount of cells, which have a flattened morphology and are oriented in parallel to the cartilage surface [6]. These cells produce high levels of proteoglycan 4 and hyaluronan. The collagen fibrils in this layer are also oriented in parallel to the cartilage surface and are tightly packed to provide tensile strength [20]. This orientation helps counteract the swelling pressure of tissue below, and helps absorb compressive force and sheer stresses. The superficial zone contains relatively high levels (w/w) of the proteoglycans biglycan and decorin, but relatively low levels (w/w) of aggrecan compared to the lower zones of cartilage [22]. The high levels of hyaluronan in this zone help to retain water in the deeper zones below by limiting outflow. This helps retain water in the deeper cartilage layers, which provides better shock absorption capacity because enclosed fluids like water are (considered) almost physically incompressible. Therefore, cartilage with a higher water content will be more difficult to compress than dehydrated cartilage.

Beneath the superficial zone lies the middle zone, which represents 40-60% of the articular cartilage [6]. Chondrocytes are rounded but sparse. The collagen fibers are oriented isotropically to the cartilage surface [20], which allows for efficient collapse of the tissue upon compression to dissipate the energy of the impact. The collapse of this layer is accompanied by displacement and loss of water. The more this layer is compacted, the more resistance to further collapse is increased [5]. This resistance to further compression is due to an increase in electrostatic repulsion between the proteoglycans and also to an increase in hyaluronan viscosity because of an increase in concentration of this molecule [23].

Underneath the middle zone lies the deep zone (± 30%). In the deep zone, chondrocytes are organized in columns placed between collagen fibrils oriented peripendicular to the cartilage surface [6, 20]. Furthermore, this zone contains relatively the highest levels of aggrecan of all zones [5]. The high levels of aggrecan restrict water flow and...
thereby increase the compressive stiffness of cartilage. The collagen fibers are oriented to withstand the swelling pressure generated by these proteoglycans and to withstand shear stresses generated by compression of the layers above. Part of the deep zone, below the tidemark, contains calcium salts in the matrix and is thus called the calcified zone [6]. The calcified zone forms the interface between bone and cartilage and anchors cartilage to the bone. This zone contains hypertrophic-like chondrocytes which resemble chondrocytes undergoing endochondral ossification (see: chondrocyte homeostasis) [24].

**Osteoarthritic cartilage**

During OA, cartilage homeostasis is disturbed, resulting in the degradation and loss of this tissue. This disturbed homeostasis is reflected in many changes on macroscopic, microscopic and molecular level.

Macroscopically, cartilage thinning can be seen, often combined with loss of cartilage integrity: in early stage OA this loss of integrity is characterized by surface roughening, in late stage OA whole parts of the tissue can be lost.

Microscopically, fibrillations, fissures and ruptures (e.g. at the tidemark) can be observed. Furthermore, especially in the superficial zone, loss of chondrocytes by cell death occurs. However, sometimes proliferation of the remaining cells can be seen, resulting in very characteristic cell clusters not observed in healthy cartilage. Part of the chondrocytes, especially in the deep zone, become hypertrophic-like and are no longer able to maintain a healthy cartilage ECM [24]. In the deep zone also ingrowth of blood vessels occurs, facilitating cartilage resorption by chondro/osteoclasts and the formation of bone.

On a molecular level, the ECM is severely impacted by OA. Collagen fiber organization is disrupted by enzymatic digestion of collagen fibrils [7]. This results in a loss of tensile stiffness and tissue ruptures due to loss of cartilage’s ability to deal with shear stresses and swelling pressure [7]. The core protein of proteoglycans is also enzymatically degraded, resulting in e.g. smaller aggrecan aggregates and more free aggrecan not attached to hyaluronan. This degradation of proteoglycans results in a reduced GAG content for OA cartilage and therefore in a more water permeable ECM and a lower osmotic pressure. Together, these changes result in a reduced compressive stiffness of cartilage [7]. The degradation of aggrecan and collagen fibers is mediated in part by MMP and ADAMTS enzymes. Prime examples are MMP3, MMP9 and MMP13 for collagen degradation [25] and MMP3, MMP13, ADAMTS4 and ADAMTS5 for aggrecan degradation [25, 26]. In the joint, these enzymes are produced in inactive form by both synoviocytes and chondrocytes, and both production and activation of these enzymes is increased under OA conditions, for example by cell stress and reactive oxygen species respectively [25]. Remarkably, in contrast to this increase in production of cartilage degrading enzymes, also the anabolic production of chondrocytes is increased in OA; up to 5 times more
proteins and sulfated GAGs are produced [27, 28]. However, this increased production is evidently not sufficient to maintain a healthy cartilage matrix, illustrating a severe imbalance in matrix maintenance in OA cartilage and indicating a disturbed chondrocyte homeostasis.

Figure 2. Deleterious changes in osteoarthritic cartilage. On the left healthy cartilage is depicted, on the right osteoarthritic cartilage with the deleterious changes that occur listed. The loss of proteoglycans is not depicted.

Chondrocyte homeostasis

Chondrocyte homeostasis is a key determinant of cartilage homeostasis. Without chondrocytes articular cartilage rapidly degrades. This is illustrated by the observation that induction of chondrocyte apoptosis \textit{in vivo} by monosodium iodoacetate leads to rapid onset and development of cartilage degradation [29]. Exemplary for disturbed chondrocyte homeostasis in OA is the presence of proliferating chondrocytes and the appearance of hypertrophic-like chondrocytes throughout the cartilage, which are chondrocytes with a phenotype that resembles that of hypertrophic chondrocytes in the epiphyseal plate [24].

In the epiphyseal plate endochondral ossification occurs in order to facilitate the growth of long bones. In this process, first cartilage is made which is then gradually replaced by bone [30]. During endochondral ossification, chondrocytes undergo a developmental program that goes from rest to proliferation to maturation to hypertrophy and finally either
into cell death via apoptosis (terminal differentiation) or (rarely) further differentiation into an osteoblast phenotype [30]. The role of the hypertrophic stage in this process is to prepare the replacement of cartilage by bone. To enable this replacement, hypertrophic chondrocytes modify their surrounding ECM and secrete factors that stimulate bone formation. The ECM is altered by hypertrophic chondrocytes via the production of MMP13 and collagen type X and via the stimulation of matrix calcification by secretion of matrix vesicles containing calcium salts [31]. Hypertrophic chondrocytes also produce alkaline phosphatase, an enzyme needed for this matrix calcification but also for the growth of hydroxyapatite crystals present in bone [32]. Bone formation is further stimulated by secretion of various growth factors, like vascular endothelial growth factor (VEGF), which stimulate angiogenesis and members of the Wnt family which stimulate osteoblasts formation [33]. Based on these previously mentioned characteristics hypertrophic chondrocytes can be distinguished from normal (articular) chondrocytes but this can also be done based on their size; hypertrophic chondrocytes are much larger than normal chondrocytes. During OA, chondrocytes resembling these hypertrophic chondrocytes, hence their name hypertrophic-like, start to appear in articular cartilage; large chondrocytes that produce MMP13, collagen type X, VEGF and alkaline phosphatase [24]. If they are truly hypertrophic and also induce matrix calcification and undergo further differentiation is unknown yet.

Many factors regulate chondrocyte homeostasis. Chondrocytes are highly mechanosensitive cells, that recognize hydrostatic pressure (generated by joint movement) via integrins, their cell skeleton and mechanosensitive ion channels [34]. In a similar way these cells can recognize tensile strain. In response to these stimuli chondrocytes orientate their cell body to reduce (tensile) strain and modulate the cartilage ECM accordingly. Also pro-inflammatory mediators such as interleukin 1 (IL1) can greatly affect chondrocyte homeostasis, for example by induction of nitric oxide [35] and reactive oxygen species [36] which block proteoglycan synthesis and induce cell stress which can result in apoptosis. Furthermore, pro-inflammatory cytokines and alarmins like S100A8/A9 induce the production of cartilage degrading MMPs and ADAMTSs [37]. Another key regulator of chondrocyte homeostasis is signaling by growth factors. Growth factors modulate chondrocyte metabolism, differentiation, proliferation, survival and regulate ECM production and turnover. An important group of growth factors which modulate chondrocyte homeostasis on all these levels are those belonging to the transforming growth factor β (TGFβ)-family. For example, this family is crucial for the regulation of chondrocyte hypertrophy.
The TGFβ-family

The TGFβ-family consists of over 30 members, including the TGFβ’s, activins, bone morphogenetic proteins (BMP’s) and growth/differentiation factors (GDF’s). Family members are produced as large pro-proteins which carry a cystine knot motif near their C-terminus [38, 39]. This cystine knot allows for the formation of disulfide bridges, essential for proper protein dimerization and folding, which are required for the characteristic quarternary dimer structure of family members [39].

Signaling by TGFβ-family members is induced via formation of heteromeric complexes between two types of serine threonine-protein kinase receptors, conveniently called type I and type II receptors [40]. There are seven type I receptors (activin receptor-like kinase (ALK) 1 to 7, also known as activin A receptor type II-like I (ACVRL1), activin A receptor type I (ACVR1), bone morphogenetic protein receptor type I A (BMPR1A), activin A receptor type I B (ACVR1B), transforming growth factor β receptor type I (TGFBR1), bone morphogenetic protein receptor type I B (BMPR1B), and activin A receptor, type I C (ACVR1C) respectively) and five type II receptors: transforming growth factor β receptor type II (TGFBR2), bone morphogenetic protein receptor type II (BMPR2), activin A receptor type II A (ACVR2A), activin A receptor type II B (ACVR2B) and anti-mullerian hormone type II receptor (AMHR2) (Table 1). In addition, multiple type III receptors have been characterized which facilitate the interaction between ligand and receptor and can stabilize receptor complexes. For example, betaglycan [41, 42] and endoglin [43] have been identified as co-receptors for TGFβ, and the repulsive guidance molecule (RGM) family members: RGM domain family member A (RGMA), dragon and hemojuvelin are co-receptors for BMP signaling [44]. Also inhibitory (pseudo) receptors have been identified; for example CD109 which mitigates TGFβ signaling [45] and BMP and activin membrane bound inhibitor (BAMBI) which mitigates BMP and activin signaling [46].

Because every growth factor recruits a specific, but not necessarily unique, receptor complex, this choice in receptors allows for cell specific growth factor-sensitivity. To signal, a growth factor binds to a type II receptor and together this complex recruits the type I receptor (e.g. for activins and TGFβs) or the other way around in which first the type I receptors and then the type II receptors are recruited (e.g. for BMPs) [40]. After the receptor complex has been assembled, the auto-phosphorylated type II receptor trans-phosphorylates the type I receptor on multiple sites, activating the kinase domain of the type I receptor. The activated type I receptor subsequently transmits the signal to intracellular signaling proteins, which are recruited via the help of adaptor proteins. Although multiple signaling pathways can be activated, for example the mitogen-activated protein kinase (MAPK) pathway involving TGFβ-activated kinase 1 (TAK1 also known as MAP3K7), the unique and canonical pathway for TGFβ-family signaling is the receptor-SMAD (R-SMAD) pathway.
**General introduction**

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<td>ACVR2A ACVR2B BMPR2</td>
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Table 1. TGFβ-family receptors, their type II receptors and their typical ligands in cartilage. Note that AMHR2 is not listed because no role for this receptor or its ligand anti-müllerian hormone has been established in cartilage. Adapted from [47].

**R-Smad-dependent signaling**

R-SMADs are ±50 kDa sized proteins that upon activation by carboxy-terminal phosphorylation can migrate to the nucleus and function as transcription factors. Two groups of R-SMADs can be distinguished based upon structural and functional homology: one group consisting out of SMAD1, SMAD5 and SMAD9 (also known as SMAD8) and one group containing SMAD2 and SMAD3 [40]. Upon receptor type I activation, these R-SMADs are directly phosphorylated on two serines at their C-terminal SXS motif. Specific ALKs phosphorylate specific SMADs; ALK1, 2, 3 and 6 phosphorylate SMAD1, 5 and 9, whereas ALK4, 5 and 7 phosphorylate SMAD2 and SMAD3. This phosphorylation facilitates complex formation of the R-SMADs with the common SMAD (co-SMAD), SMAD4. Two R-SMADs can associate with one co-SMAD and this complex is subsequently transported to the nucleus. Inside the nucleus, this SMAD complex directly binds DNA, although with low affinity, and recruits transcription factors for a high affinity interaction with DNA. The only exception to this rule is a common splice variant of SMAD2 that contains exon 3 which by itself cannot bind DNA and requires additional transcription factors to bind DNA. The location where SMAD complexes bind DNA is dependent on characteristic SMAD binding regions (SBR) present in the DNA sequence, and these differ for SMAD3/4 and SMAD1/5/9; e.g. GTCTAGAC for SMAD2/3/4 complexes [48] and GCCGnCGC for SMAD1/5/9/4 complexes [49]. Furthermore, the interaction between SMADs and DNA is further regulated by the presence of master regulator transcription factors [50]. Master regulators vary per tissue, and an important master regulator of chondrocyte phenotype is SRY-box9 (Sox9), which for example closely interacts with SMAD3 on the collagen type II promotor [51]. It is important to note that because of this dependency on master regulatory proteins, R-SMADs can regulate expression of different genes in different tissues.
Figure 3. TGFβ-family SMAD-dependent signaling. Ligand binding induces the formation of a receptor complex. This complex recruits R-SMAD2/3 via SMAD anchor for receptor activation (SARA) and/or R-SMAD1/5/9 via endofin. The R-SMADs are subsequently phosphorylated at their C-terminal SXS domain by the type I receptor and a complex is formed with the common SMAD4. This complex translocates to the nucleus where it can bind DNA at SMAD binding regions (SBR) and activate transcription. Important target genes are the inhibitory SMADs: SMAD6 and SMAD7, whose expression provides the cell with a negative feedback mechanism.
Two important target genes of R-SMAD signaling are the inhibitory-SMADs (I-SMAD): SMAD6 and SMAD7 [52, 53]. These I-SMADs function as inhibitors of R-SMAD signaling on multiple levels by inhibiting C-terminal R-SMAD phosphorylation, inhibiting R-SMAD DNA binding, inhibiting complex formation between SMAD4 and R-SMADs and by inducing receptor dephosphorylation and degradation via recruitment of Smurf ubiquitin ligases to the receptor. In this way, SMAD6 predominantly inhibits SMAD 1/5/9 and SMAD7 predominantly inhibits SMAD2/3 signaling, and both I-SMADs provide cells with an important negative feedback mechanism to inactivate R-SMAD signaling.

An (simplified) overview of the SMAD dependent signaling pathway is depicted in figure 3.

**Smad-dependent signaling in chondrocyte and cartilage biology**

SMAD signaling is essential for the formation and maintenance of healthy cartilage. Both *in vivo* and *in vitro* experiments point towards essential but distinct roles of SMAD2/3 versus SMAD1/5/9 signaling in chondrocyte biology.

In humans, genetic variation in SMAD3 is associated with hip and knee OA and total burden of the disease [54, 55]. Dominant nonsense, missense or frameshift mutations in SMAD3 lead to aneurysms-OA syndrome, a severe condition characterized by cardiovascular anomalies and early onset of OA in multiple joints (mean age of diagnosis = 42 year) [56, 57]. Mice with a SMAD3 null mutation (SMAD3Δexon8/Δexon8) develop normal articular cartilage, but this cartilage starts to degrade rapidly one month after birth, resulting in severe OA [58]. This rapid degradation is linked to an increased amount of hypertrophic chondrocytes present in both articular and epiphyseal cartilage [58]. However, SMAD3 null mice also have a greatly diminished life span and suffer from severe systemic effects such as growth retardation, spontaneous tumorigenesis and impaired T-cell immunity which can affect cartilage biology indirectly [58-60]. To circumvent these systemic effects, chondrocyte specific SMAD3-null mice (SMAD3Δexon2+3/Δexon2+3) have been made using Col2-Cre and floxed SMAD3 [61, 62]. These mice also display increased chondrocyte hypertrophy and accelerated cartilage degeneration, confirming the direct role of SMAD3 in these processes [61]. Furthermore, *in vitro* studies with overexpression or knockout of SMAD3 also support a role for SMAD3 in inhibiting chondrocyte terminal differentiation by showing an inhibitory or stimulating effect on chondrocyte hypertrophy respectively [63-66]. The anti-hypertrophic effect of SMAD3 is attributed to its interaction with core-binding factor subunit α 1 (CBFA1), also known as runt-related transcription factor 2 (RUNX2) [61, 67, 68]. RUNX2 is a potent regulator of chondrocyte maturation. For example, overexpression of RUNX2 in the murine teratoma-derived ATDC5 chondrocyte-like cell line induces chondrocyte hypertrophy, whereas inhibition of RUNX2 achieves the opposite [61, 68-70]. SMAD3 counteracts RUNX2 function by directly binding this
transcription factor and recruiting silencing histone class II deacetylases to RUNX2 responsive genes [67] such as *MMP13*, *collagen type 10 a1* and *alkaline phosphatase*. The potency of SMAD3 in preventing chondrocyte hypertrophy is demonstrated *in vivo* by the observation that heterozygotic *SMAD3* null mice (*SMAD3<sup>wt/Δexon8</sup>*) do not have (reported) accelerated cartilage degeneration, whereas *e.g.* T-cell biology is still negatively affected, indicating that having only one genetic copy of *SMAD3* is enough to rescue the chondrocyte phenotype [58, 60].

The role of SMAD2 in cartilage biology is less clear than that of SMAD3. In humans, there is no known association between genetic variation in *SMAD2* and OA. SMAD2 knockout animals or hypomorphs are not viable and die early during embryogenesis (< embryonic day (E) 9 [71-74]), before the formation of articular joints [75], and are thus not usable to study the role of SMAD2 in articular cartilage. Animals with only 1 genetic copy of *SMAD2* (*SMAD2<sup>+/-</sup>*) can be viable [71, 76], but no cartilage phenotype has been reported. Cartilage-specific SMAD2 knockout mice have recently been studied [77], and such mice have shortened growth plates during embryogenesis due to a shortened proliferative zone and elongated hypertrophic zone. This last observation indicates a similar anti-hypertrophic effect of SMAD2 as SMAD3. Unfortunately, the role of SMAD2 in mature cartilage was not studied in this model. An *in vitro* study seems to further support a role for SMAD2 similar to that of SMAD3, because overexpression of a dominant negative variant of SMAD2 enhances, whereas overexpression of wildtype SMAD2 protects against chondrocyte hypertrophy, although less potent than SMAD3 [63].

SMAD1 and SMAD5 seem to have an important but interchangeable role in cartilage biology. In contrast, SMAD9 seems to be of little importance for cartilage formation or maintenance. In humans, no association has been reported between genetic variation in *SMAD1*, *SMAD5* or *SMAD9* and OA, possibly due to redundant roles in cartilage. Like SMAD2 knockout mice, SMAD1 [78] or SMAD5 [79] knockout mice die before the formation of articular joints. Knockout of SMAD9 is not embryonically lethal yet does not seem to affect (epiphyseal) cartilage biology [80]. To study SMAD1 and SMAD5 in chondrogenesis, cartilage specific knockouts have been made using *Col2-cre* and floxed SMAD1 and SMAD5 [80]. Cartilage specific removal of either SMAD1 or SMAD5 does not result in a phenotype. Furthermore, crossing these cartilage specific knockout mice with whole body SMAD9 knockout mice does not result in a phenotype either. Only when both SMAD1 and SMAD5 are ablated, severe chondrodysplasia can be observed [80]. Additional removal of SMAD9 does not add much to this severe phenotype. Together, these observations indicate that SMAD1 and SMAD5 have an important, but redundant, role in cartilage formation whereas the role of SMAD9 seems limited [80]. The severe chondrodysplasia is in part due to a lack of hypertrophic chondrocytes in the growth plates, suggesting that SMAD1/5 signaling regulates chondrocyte maturation. Unfortunately, (inducible) SMAD1/5 knockout mice have not been used yet to study chondrocyte hypertrophy in mature articular cartilage or OA, but *in vitro*
studies support a stimulatory role of SMAD1/5 in chondrocyte maturation. For example, overexpression of SMAD6, an inhibitor of SMAD1/5/9, results in inhibited chondrogenesis in the ATDC5 cell line [81] and delayed chondrocyte hypertrophy [82]. The stimulatory effects of SMAD1/5 on chondrocyte maturation are most likely due to a stimulation of RUNX2 function. Such a stimulatory role of SMAD1/5 on RUNX2 function has been described to be essential for BMP2-induced osteoblast differentiation [83-86]. Furthermore, in pre-hypertrophic chondrocytes SMAD1/5-RUNX2 interaction induces the activation of a reporter construct based on the Col10a1 promoter, expression of which is an important phenotypic marker of hypertrophy [68, 87]. How exactly SMAD1 and 5 stimulate RUNX2 function is yet unclear, but possibly this stimulation occurs indirectly via induction of jumonji domain containing 3 (JMJD3) expression. JMJD3 is a histone demethylase targeting H3K27me3, which enables transcription of otherwise silenced genes. Silencing of JMJD3 lowers RUNX2 dependent gene expression, and during osteoblast differentiation both SMAD1 and SMAD5 regulate JMJD3 expression [88]. Therefore both SMAD1 and SMAD5 can regulate RUNX2 activity indirectly via JMJD3, but a more direct mechanism is not excluded.

Smad-independent signaling

TGFβ-family members can also activate other signaling pathways than the R-SMAD pathways. Prime examples are the MAPK pathways involving TAK1 or extracellular signal-regulated kinase 1 and 2 (ERK1/2, also known as MAPK3/1), Phosphoinositide 3-kinase (PI3Ks)-dependent signaling and signaling via the GTPase ras homolog family member A (RhoA) [89]. The SMAD independent signaling pathways are depicted in figure 4. It is important to note that these pathways are not unique for TGFβ-family signaling but are also important signaling mediators of many other growth factors and cytokines as well.

Activation of MAPKs by TGFβ-family members relies on adaptor proteins, e.g. SHC adaptor protein 1 (Shc1) for ERK [90] and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) for TAK1 [91, 92]. Shc1 binds, and is directly phosphorylated by, type I receptors [90]. This phosphorylation of Shc1 triggers a MAPK signaling pathway via Grb2/SOS, Ras, Raf, and MAP2K1 resulting in activation of ERK1/2 [90]. TRAF6 binds both TAK1 and type I and II receptors, and becomes activated upon receptor type I – type II complex formation via oligomerization and auto-ubiquitination but independently of type I receptor kinase activity [92]. TRAF6 subsequently poly-ubiquitinates TAK1, which recruits TGFβ activated kinase 1/MAP3K7 binding protein 1 (TAB1), and together poly-ubiquitination and TAB1 allow TAK1 to auto-phosphorylate and become active [92]. Ultimately, activation of MAPK signaling by TGFβ-family members results in activation of JNK and p38 kinases which directly regulate gene expression by modulating transcription factors such as: Jun proto-oncogene (c-JUN), MYC proto-oncogene (c-MYC), activating transcription factor 2 (ATF2), and CCAAT/enhancer-binding protein β (C/EBP-β)[93].
Figure 4. TGFβ-family SMAD-independent signaling. Ligand binding induces the formation of a receptor complex. This complex can initiate SMAD-independent signaling via activation of adaptor proteins e.g. SHC and TRAF6 or direct activation of signaling kinases (PI3K, RhoA). This activation results in initiation of signaling cascades, culminating in the activation of ERK, MTOR, ROCK, p38MAPKs, JNKs and NFκb. These pathways induce transcription and translation and regulate diverse cellular processes. Enzymes are indicated by a yellow star, transcription factors by a hexagonal shape. 4EBP1 = Eukaryotic translation initiation factor 4E-binding protein 1, AKT = RAC-alpha serine/threonine-protein kinase, AP-1 = Activator protein 1, ATF2 = Cyclic AMP-dependent transcription factor ATF-2, CEBPβ = CCAAT/enhancer-binding protein β, ERK = Mitogen-activated protein kinase 3, GRB2 = Growth factor receptor-bound protein 2, GSK3β = Glycogen synthase kinase-3 β, HSP27 = Heat shock protein beta-1, IkBa = NF-kappa-B inhibitor alpha, IKK = Inhibitor of nuclear factor kappa-B kinase, JNK = c-Jun N-terminal kinase, MAP2K = Dual specificity mitogen-activated protein kinase 2, MAPKAPK = MAP kinase-activated protein kinase, MEF2 = Myocyte-specific enhancer factor 2A, MKK = Dual specificity mitogen-activated protein kinase kinase, MTOR = Serine/threonine-protein kinase mTOR, MTORC = MTOR complex, cMyc = Myc proto-oncogene protein, NEMO = NF-kappa-B essential modulator, NFκb = nuclear factor kappa-light-chain-enhancer of activated B cells, p38 MAPK = p38 mitogen-activated protein kinases, PI3K = Phosphatidylinositol 3-kinase, PIP3 = Phosphatidylinositol (3,4,5)-trisphosphate, Raf = RAF proto-oncogene
serine/threonine-protein kinase, RAS = GTPase HRas, RhoA = Transforming protein RhoA, ROCK = Rho-associated protein kinase 1, S6K1 = Ribosomal protein S6 kinase beta-1, SHC = SHC-transforming protein 1, SOS = Son of sevenless homolog 1, SP-1 = Sp1 transcription factor, TAB = TGF-beta-activated kinase 1 and MAP3K7-binding protein 1, TAK1 = Mitogen-activated protein kinase kinase kinase 7, TCFs = ternary complex factors, TRAF6 = TNF receptor-associated factor 6.

Both ERK and TAK1 contribute to TGFβ-family signaling in chondrocytes. Functional inhibition of ERK1 in ATDC5 cells via inhibition of its activating kinase MAP2K1 with the inhibitor U0126 reduces TGFβ-induced aggrecan expression [94]. Functional inhibition of ERK1 in rat chondrocytes with the use of the MAP2K1 inhibitor PD98059 reduces TGFβ-induced proliferation and aggrecan and collagen type II expression [95, 96]. Furthermore, in both human and bovine primary chondrocytes, use of PD98059 lowers TGFβ-induced mRNA and protein expression of the metallopeptidase inhibitor TIMP3 [97], indicating that ERK activity is a cartilage-protective component of TGFβ signaling in mature chondrocytes. In contrast, during TGFβ-induced chondrogenesis, use of PD98059 increases Col2a1 and Sox9 expression and proteoglycan synthesis in rat, chicken and human mesenchymal stem cells [98-100], indicating that ERK1/2 is an inhibitory component of TGFβ signaling during TGFβ-induced chondrogenesis.

Functional inhibition of TAK1 using a dominant negative form of TAK1 greatly diminishes the ability of TGFβ and BMP2 to induce collagen type II expression in rabbit articular chondrocytes [101]. In Col2-Cre; TAK1<sup>6/6</sup> mice, TAK1 deletion in collagen type II expressing cells during embryogenesis results in runting and severe chondrodysplasia due to reduced chondrocyte proliferation and delayed maturation [102], and these mice die shortly before [102] or after birth [103]. This severe phenotype resembles the phenotype of mice lacking BMP receptor / SMAD1/5 signaling. Indeed these mice show impaired BMP-induced R-SMAD and p38 MAPK/JNK/ERK signaling [102, 103], confirming that TAK1 is an important component of BMP signaling in cartilage. Postnatal deletion of TAK1 using inducible Col2a1-Cre<sup>ERT2</sup>;TAK1<sup>6/6</sup> mice also results in growth retardation and these mice have profoundly decreased proteoglycan and collagen type II production in their articular cartilage [104]. This last observation shows that the importance of TAK1 in cartilage biology is not limited to embryogenesis.

A possible role of TAK1 signaling in chondrocytes is the regulation of Sox9 expression. In chondrocytes, deletion of TAK1 inhibits endogenous and BMP2-induced Sox9 mRNA and protein expression, whereas overexpression of TAK1 enhances Sox9 levels [104]. This observation is reflected in vivo: both articular and epiphyseal chondrocytes from Col2a1-Cre<sup>ERT2</sup>;TAK1<sup>6/6</sup> mice show lower Sox9 protein expression after deletion of TAK1 compared to control animals [104]. The induction of Sox9 expression by TAK1 involves binding of activating transcription factor 2 (ATF2), a downstream target of TAK1 via p38 MAPK, to the Sox9 promoter [104]. Furthermore, p38 MAPK signaling has also been shown to stabilize Sox9
mRNA in human chondrocytes, further strengthening the importance of this MAPK pathway in Sox9 regulation[105].

In addition to MAPK signaling, both TGFβ and BMP signaling can also activate PI3K signaling but it is unclear how and to what extent this occurs in chondrocytes [106-109]. In case of TGFβ, this activation requires receptor type II presence and receptor type I kinase activity [107, 109]. Because PI3Ks activation kinetics closely resemble SMAD activation kinetics [107], it has been suggested that PI3Ks are a direct target of type I receptors [107, 108]. Possibly this activation runs via Ras [110]. Activation of PI3Ks leads to phosphorylation and activation of AKT serine/threonine kinase 1/2/3 (AKT1/2/3). During endochondral ossification, AKT signaling is an inhibitor of chondrocyte hypertrophy. In forelimb explant cultures of mice expressing an inducible constitutively-active form of AKT1 (myrAKT1-MER, a fusion protein of myristoylated AKT with a modified estrogen receptor that becomes active upon presence of 4-hydroxytamoxifen), activation of myrAKT1-MER blocks Runx2 expression and chondrocyte hypertrophy, whereas inhibition of PI3K activity with LY294002 enhances Runx2 expression and hypertrophy [111]. Furthermore, mice overexpressing constitutively-active AKT1 (myrAKT) have decreased chondrocyte hypertrophy and ECM calcification in their limbs, but show enhanced ECM deposition, resulting in enlarged bones [112]. In contrast, AKT1−/− mice develop short limbs, but not because of excessive chondrocyte hypertrophy but because of a lack of growth plate calcification [113], showing that AKT1 signaling is essential for ECM calcification in cartilage.

An important mediator of PI3K/AKT1 signaling in chondrocytes is mechanistic target of rapamycin (MTOR) [112]. In cartilage, MTOR is upregulated in OA [114] and is an inhibitor of protective autophagy [114-116]. In vivo, intra-articular or intra-peritoneal injection of rapamycin, i.e. an inhibitor of MTOR, increases autophagy and reduces cartilage damage in the DMM model of OA [115, 116]. Furthermore, cartilage specific MTOR knockout mice (Col2-rt-TA-Cre; MTORfl/fl) are protected against DMM induced OA [114] confirming that MTOR signaling is deleterious in OA conditions. However, the cartilage-beneficial effects of AKT1 during endochondral ossification have also been attributed to MTOR [112], indicating that PI3K/AKT1/MTOR signaling outcome is context, e.g. chondrocyte maturation state, dependent.

TGFβ-family members can also signal via the GTPase RhoA [93], but also here it is unclear how and to what extent this occurs in chondrocytes. A well known role for RhoA signaling in various cell types is the formation of focal adhesions and actin stress fibers [117]. In primary chondrocytes, formation of such stress fibers via RhoA inhibits Sox9 expression, and this is possibly one of the reasons why chondrocytes dedifferentiate in vitro [118]. Loss of Sox9 expression is also observed in ATDC5 cells after overexpression of RhoA, but this can be blocked by inhibition of Rho Associated coiled-coil containing protein kinase 1 (ROCK1) downstream of RhoA [119, 120]. In primary human chondrocytes, ROCK inhibition induces Sox9, Col2a1 and Acan mRNA expression [105], while in primary murine chondrocytes, ROCK inhibition enhances the output of a Sox9-responsive luciferase assay along with Col2
and Acan mRNA expression [120]. Therefore, these studies also indicate a negative effect of RhoA/ROCK signaling on chondrocyte phenotype. However, in murine mesenchymal cells during chondrogenic differentiation, ROCK1 inhibition negatively affects Sox9 function [120], suggesting that cellular context has a large impact on RhoA/ROCK1 signaling outcome. Finally, overexpression of RhoA has also been reported to block hypertrophic differentiation of ATDC5 cells [121, 122], but this observation could very well be due to less chondrogenesis overall, due to increased proliferation and loss of Sox9 expression.

### Interaction between the Smad-dependent and Smad-independent signaling pathways

Despite their names, the SMAD-dependent and SMAD-independent pathways are not truly distinct signaling pathways; in the cell, signaling of both pathways is integrated, and their interaction can greatly affect TGFβ-family signaling outcome. SMAD-independent pathways can interact with SMAD dependent signaling by post translational modification of the linker region of SMAD proteins [123]. The linker region is a regulatory domain located in the middle of the SMAD protein, and post-translational modification of this region regulates nuclear entry, SMAD-protein interactions and SMAD turnover, and thus greatly affects SMAD function [123]. However, the relative importance of these SMAD linker modifications in cartilage biology is not well understood yet and warrants further research.

The reverse also holds true; SMAD signaling can inhibit above mentioned non-canonical pathways either directly or indirectly. An example of direct inhibition is via competition for co-factors, e.g. SMAD3 and NFκB (a transcription factor downstream of TAK1) can compete for their common co-transcription factor CREB binding protein (CBP) in endothelial cells [124]. An example of indirect inhibition is via induction of negative regulators of transcription factors, e.g. SMAD3 is a potent inducer of JunB, a negative regulator of the transcription factor complex AP-1 (a transcription factor downstream of TAK1 which in turn can negatively regulate SMAD signaling) [125]. The outcome of TGFβ-family signaling is thus an integration of both SMAD-dependent and SMAD-independent signaling, and not the result of the activation of a single pathway.
The TGFβ-family in cartilage maintenance

Via these aforementioned pathways, the TGFβ-family members play key roles in the maintenance of articular cartilage. Prime examples of TGFβ-family member with such a role are TGFβ1, TGFβ2 and TGFβ3, BMP2 and BMP7.

TGFβ1, TGFβ2 and TGFβ3

In mammals, three TGFβ’s exist; TGFβ1, TGFβ2, and TGFβ3, encoded by three different genes: TGFB1, TGFB2 and TGFB3, and these homologs (and not isoforms) share a high percentage of amino acid identity (71-79%). All three TGFβ’s are produced in inactive form as homodimers (or more rarely as heterodimers [126]) bound by latency associated peptide (LAP) (forming small latency complex) and latent TGFβ binding protein (LTBP) (forming large latency associated complex) [127]. This production in inactive form separates secretion from activity, an important concept in TGFβ biology.

The importance of TGFβ in cartilage biology was first observed in 1985 when two proteins were extracted from bone that were capable of inducing chondrogenesis in rat embryonic mesenchymal cells, and therefore called cartilage inducing factor A and B [128]. Later research revealed that these proteins were identical to TGFβ1 and TGFβ2 [129, 130]. All three forms of TGFβ are produced by chondrocytes [131-133] and a large amount (60-200 ng/ml) of inactive TGFβ is bound to the ECM of cartilage [134, 135]. Chondrocytes also express the TGFβ type I receptors ALK1 and ALK5, the type II receptor TGFBR2, and coreceptors such as endoglin and betaglycan [136]. Genetic variation in neither TGFB1, TGFB2 nor TGFB3 is (yet) associated with OA, nor is genetic variation in TGFBR1 (= ALK5), ACVRL1 (= ALK1), TGFBR2, ENG (= endoglin) or TGFBR3 (= betaglycan). However a gain of function variation in asporin (ASPN), a protein which limits TGFβ bioavailability by sequestering it, is associated with OA development [137].

Because TGFβ is produced in inactive form, activation is a crucial step in TGFβ signaling. For this, the non covalent bond between LAP and TGFβ has to be disrupted. This can occur enzymatically by degradation of LAP by e.g. MMP3 [138], or non enzymatically by a conformational change in LAPs tertiary structure. Shearing stress [139] or chemical modification of LAP by reactive oxygen species [140] can induce such a conformational change. Recently, compressive force was also shown to possibly be an important physiological activator of ECM-bound TGFβ because this activates SMAD2/3 signaling [141].

In chondrocytes, TGFβ induces both SMAD2/3 and SMAD1/5/9-dependent signaling via ALK5 and ALK1 respectively and SMAD-independent signaling [142, 143]. Which pathway is activated by TGFβ depends on receptor level expression, co-receptor expression, i.e. endoglin enhances signaling via ALK1, and dose of TGFβ; in chondrocytes a low dose of TGFβ
predominantly signals via pSMAD2/3, whereas at high dosages pSMAD1/5 signaling becomes more pronounced. Importantly, both pathways have been described to antagonize each other in chondrocytes [142, 143].

TGFβ signaling is associated with cartilage ECM production and maintenance. In mice, a single intra-articular injection of rhTGFβ1 or rhTGFβ2 increases proteoglycan synthesis twofold after four days as measured by 35S-sulfate incorporation in patellar articular cartilage [144, 145]. Also in vitro, TGFβ enhances proteoglycan production in both chondrocytes and cartilage explants on both mRNA and protein level [146-149], together with production of other ECM components like collagen type II a 1 (Col2a1) [149, 150], cartilage oligomeric matrix protein [151, 152], perlecan [153], fibronectin [154] and hyaluronan [155]. Furthermore, TGFβ is a potent inducer of lubricin (PRG4) in chondrocytes [156], a key lubricating component of synovial fluid. However, in contrast to the previously mentioned studies, also inhibitory effects of TGFβ on chondrocyte proliferation, proteoglycan synthesis and collagen type II production have been described both in vitro and in vivo, [150, 157-162]. Possibly, different receptor signaling plays a role in these apparently contradictory observations.

TGFβ signaling also has important anti-inflammatory effects in cartilage. TGFβ1 counteracts pro-inflammatory IL-1 signaling in vivo [163-165] and in vitro [166] and helps cartilage proteoglycan content recover after inflammation induced depletion [131, 167]. How TGFβ exerts its anti-inflammatory effects is not fully elucidated yet, but in primary rabbit articular chondrocytes, both TGFβ1 and TGFβ3 down-regulate IL-1 receptor (IL1R1) expression approximately 50% on protein and mRNA level [166, 168]. In macrophages, TGFβ signaling can also stabilize NFκB inhibitor alpha (NFKBIA, also known as IκBα), which is a potent inhibitor of NFκB, but this mechanism has not been described for chondrocytes yet [169]. Another, already mentioned, possible mechanism is the competition between SMAD3 and NFκB for co-activating transcription factors like CREB binding protein, as has been described in endothelial cells [124]. Additionally, many inflammatory genes counteracted by TGFβ contain both SMAD3 and NFκb binding sites, leaving room for epigenetic regulation as mechanism. Notably, the anti-inflammatory effect of TGFβ is lost with advancing age [164], possibly due to age-related loss of ALK5 expression [131].

An especially important role for TGFβ signaling in chondrocytes lies in regulation of chondrocyte hypertrophy. TGFβ-induced pSMAD2/3 signaling via ALK5 blocks hypertrophy and terminal differentiation of chondrocytes [58, 63, 65, 143, 170]. In contrast, TGFβ-induced pSMAD1/5 via ALK1 is associated with inhibition of pSMAD2/3 signaling and chondrocyte hypertrophy [142, 143]. Both effects of TGFβ on hypertrophy can be observed in vivo. Removal of TGFβ signaling in cartilage of two weeks old Col2-CreER; Tgfbr2lox/lox mice results in an OA-like phenotype within 6 months via Runx2 mediated MMP13 and ADAMTSS expression [171, 172]. Furthermore, expression of a kinase-deficient dominant negative form of Tgfbr2 in mice induces chondrocyte hypertrophy and osteoarthritis [172]. In contrast, removal of TGFβ
signaling in the hypertrophic chondrocytes of Col10a1-cre; Tgfbr2<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice delays terminal differentiation in the epiphyseal plate [173], and removal of TGFβ signaling in cartilage of eight weeks old AgcCreERT2<sup>−/−</sup>; Tgfbr2<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice results in less hypertrophic chondrocytes, after 12 months [174]. Because in both the Col2-CreER; Tgfbr2<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> and AgcCreERT2<sup>−/−</sup>; Tgfbr2<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> models efficient removal of Tgfbr2 was demonstrated, their different outcome illustrates that age of model onset and (thus) chondrocyte maturation status greatly affect the outcome and impact of TGFβ signaling in chondrocytes.

In line with the observation that chondrocyte maturation affects TGFβ signaling, the ability of TGFβ to induce two such opposing pathways has been postulated as an important driver of age-related development of OA; an age-related change in receptor expression towards relatively more ALK1 expression would change protective signaling into deleterious signaling and lead to OA. Indeed, in ageing mice, the expression of TGFβ receptors changes towards relatively higher ALK1 expression, and this change in ratio correlates with MMP13 expression and OA development [143]. Furthermore, a similar change can be observed in guinea pigs [175], in which an age-related loss of TGFβ-ALK5 signaling occurs while ALK1 signaling remains stable. The importance of a changing ALK1/ALK5 ratio in ageing as cause for OA is supported by computational modeling; especially TGFβ signaling via ALK1 can explain MMP13 expression and cartilage damage in aged cartilage [176].

Besides in ageing cartilage, also in OA cartilage TGFβ signaling can have detrimental effects. AgcCreERT2<sup>−/−</sup>; Tgfbr2<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> mice are protected against mild DMM-induced cartilage damage as measured 8 weeks after model induction. Also systemic use of the ALK4/5/7 inhibitor SB-505124 lessens cartilage damage 2 months post surgery in ACLT [177]. Possibly the ability of TGFβ to induce proteases and protease activators plays a role in these deleterious effects. TGFβ can induce both MMP3 and HtrA serine peptidase 1 (HTRA1) expression in chondrocytes, both enzymes with the capacity to degrade the ECM [178]. HTRA1 is known to degrade the pericellular matrix which activates the discoidin domain receptor tyrosine kinase 2 (DDR2) receptor and deleterious signaling in the chondrocyte [179]. Of note, HTRA1 can also modulate TGFβ-family signaling for example by cleaving the extracellular domain of TGFβ-family type II and type III receptors, but also by cleaving of LAP.

In conclusion, TGFβ signaling has a wide range of effects in cartilage, and its outcome, both good and bad, is context dependent. A better understanding of how chondrocyte context regulates TGFβ signaling would greatly help approaches that seek to separate the good from the bad in diseases like OA.

**BMP2 and BMP7**

Chondrocytes can produce several BMPs, including BMP2, BMP4, BMP6 and BMP7 (OP1) [136, 180], and chondrocytes express the BMP type I receptors ALK1, ALK2, ALK3 and ALK6,
and the type II receptors: BMPR2, ACVR2A and ACVR2B. Genetic variation in neither of these factors is (yet) associated with OA. Like the TGFβs, BMPs are produced as a dimer but not only as homodimers; heterodimers of BMP2/6, BMP2/7 and BMP4/7 have been described both in vitro and in vivo [181]. In contrast to TGFβ, BMP activity is not thought to be controlled via reassembly of a BMP with its prodomain to form a latency complex [181]; BMPs do reassemble with their prodomain to facilitate ECM binding, but this does not seem to confer latency because it does not impair receptor type I and II binding [127, 181, 182]. Instead, antagonistic scavenger proteins, e.g. gremlin, noggin and sclerostin, and decoy receptors like BAMBI, play an important role in modulating BMP activity, all of which are produced by chondrocytes. Additionally, BMPs can antagonize each other via competition for receptor type II binding [183].

BMP2 mRNA expression can be detected in both healthy and arthritic cartilage and does not seem to be affected by ageing [184]. BMP2 protein can be detected in chondrocytes exposed to inflammatory conditions and near cartilage lesions [185, 186]. Matrix-bound BMP2 levels have not been reported, but BMP2 binds the proteoglycan perlecan, which would locate it to the pericellular matrix of chondrocytes. To signal, BMP2 can use ALK2, ALK3 and ALK6, and the type II receptors: BMPR2, ACVR2A and ACVR2B to induce SMAD1/5 phosphorylation and SMAD-independent signaling [181].

In cartilage, BMP2 signaling induces ECM production; ex vivo in juvenile cartilage, BMP2 induces proteoglycan content and col2a1 production [187], while in vivo intra-articular injection of BMP2 in murine knee joints enhances proteoglycan synthesis 2.5-fold (i.e. 35S-incorporation) in patellar cartilage within 2 days [145]. Furthermore, intra articular overexpression of BMP2 in the murine knee joint using adenoviruses also enhances cartilage proteoglycan content [188]. Although BMP2 expression is induced in inflammatory conditions [186, 189], it, in contrast to TGFβ, does not have an anti-inflammatory effect in chondrocytes, and BMP2 effects are inhibited by IL1 but not by TNFα [190, 191]. In spite of this, BMP2 does facilitate proteoglycan recovery after inflammation-induced depletion [188]. However, prolonged exposure of cartilage to high levels of BMP2 does induce increased aggrecan turnover as measured by VIDPEN and NITEGE neoepitope staining, indicating that BMP2 can induce metalloproteinase and aggrecanase activity [188].

In vitro in mesenchymal stromal cells and chondrocyte-like cell lines, and in vivo in epiphyseal chondrocytes, BMP2 is a well known inducer of chondrocyte maturation and hypertrophy [192]. However, in mice with cartilage specific, tamoxifen inducible, overexpression of BMP2 (Col2 rTA; TRE-BMP2) increased hypertrophy is not observed in articular cartilage after 6 weeks of prolonged exposure to tamoxifen [193]. Furthermore, in the DMM model of experimental OA, the presence of excessive BMP2 signaling does not affect cartilage damage but does induce very extensive osteophyte formation [193]. These observations might indicate that in vivo, articular cartilage is not very sensitive for BMP2 signaling compared to osteophyte-forming tissues like periosteeum. Possibly, signaling of
hyaluronan, an important structural component of cartilage, via CD44 plays a role in this, as it has been demonstrated that this inhibits BMP2-induced pSMAD1/5 signaling in the murine bone marrow-derived ST2 stromal cell line [194]. Alternatively, GDF5 has recently been shown to be a context-dependent inhibitor of BMP2 signaling via a yet unknown mechanism possibly involving receptor competition [195], and GDF5 is present in (im)mature cartilage [196-198].

BMP7 mRNA and protein expression can be detected in both immature and mature articular cartilage, but its levels decline with advancing age [199, 200]. This loss in BMP7 expression is linked to increased methylation of its promoter [201]. Immunohistochemically, mature BMP7 can predominantly be detected in chondrocytes of the superficial zone, whereas its pro-form is mainly detected in deep zone chondrocytes [199, 202]. The total amount of mature BMP7 present in the cartilage ECM is estimated at 50 ng/g dry weight, but this amount decreases approximately 5-fold in OA cartilage [199, 203]. Whether this loss of mature BMP7 is due to a loss in mRNA expression is unclear; both increased and decreased BMP7 mRNA expression have been reported for OA cartilage [199, 203]. Inflammatory conditions like IL1 have also been described to induce BMP7 mRNA expression in chondrocytes, and a high dose of IL1 leads to activation of pro-form BMP7 into active form [204]. To signal, BMP7 can use ALK2, ALK3 and ALK6, and the type II receptors: BMPR2, ACVR2A and ACVR2B to induce SMAD1/5 phosphorylation and SMAD-independent signaling [181]. In contrast to BMP2, a positive interaction of CD44 with BMP7 signaling has been described; Without CD44, chondrocytes are not able to induce pSMAD1/5 in response to BMP7 [205-207]. Notably, ageing lowers the BMP7 response of chondrocytes. This loss has been linked to oxidative stress [208], but also an age-related decrease in expression of its type2 receptor Bmpr2 can possibly explain this loss [136].

In chondrocytes, BMP7 signaling induces ECM production. Both proteoglycan and Col2a1 production are induced in vitro in juvenile and adult human primary chondrocytes [209] and ex vivo in juvenile bovine explants [187]. Furthermore, inhibition of endogenous BMP7 production with antisense oligonucleotides in cartilage explants lowers aggrecan production [210]. In vitro, BMP7 also induces the expression of cartilage oligomeric matrix protein [152] and hyaluronan synthase 2, resulting in more hyaluronan production by chondrocytes, facilitating deposition of aggrecan in the ECM [211]. BMP7 can counteract an IL1- or LPS-induced inhibition of proteoglycan synthesis in chondrocytes [212-214], and partly inhibit IL1-induced MMP13 expression, especially in the presence of IGF1 [215]. However, one study also reports that BMP7 signaling is inhibited by IL1 via down regulation of ALK2, ALK3 and SMAD1 expression [216].

The positive effects of BMP7 in vitro are reflected in vivo; conditional deletion of BMP7 from limb mesenchym in Prx-Cre; Bmp7^flx/flx mice results in proteoglycan loss (at 8 weeks old) and enhanced MMP13 expression (at 24 weeks old) [217]. Of note, joint and cartilage formation were not detectably affected in these mice but BMP7 deletion did result
in synovial inflammation, making it difficult to exclude indirect effects. Administration of BMP7 is beneficial for cartilage in OA [218, 219]; Osteoarthritic cartilage is still responsive to BMP7 [198], and weekly injections of BMP7 diminish cartilage damage in the rabbit ACLT model of OA [220]. Furthermore, BMP7 increases the quantity and quality of cartilage repair tissue in rabbits with a full thickness cartilage defect [221]. Remarkably, although BMP7 can induce ectopic bone formation [222], excessive osteophyte formation was not observed in these studies, nor was excessive osteophyte formation observed in humans after a single injection of BMP7 in a Phase 1 study done in symptomatic knee OA patients [223].

Although BMP7 induces SMAD1/5 phosphorylation, its signaling is not associated with chondrocyte hypertrophy. Bovine articular chondrocytes do not undergo hypertrophy when cultured with BMP7 [224] and BMP7 blocks hypertrophy in the ATDC5 cell line [225]. In long bone formation *ex vivo*, BMP7 does stimulate the formation of pre-hypertrophic chondrocytes in the epiphyseal plate but blocks the transition towards hypertrophy [226]. This anti-hypertrophic effect of BMP7 has been contributed to its ability to induce the transcription factor BAPX-1/NKX-3.2, a potent modulator of chondrocyte hypertrophy [227]. However, BMP7 does induce alkaline phosphatase expression and osteogenesis in primary fetal chondrocytes [228], and chick sternum chondrocytes do undergo hypertrophy when cultured with BMP7 [229], indicating that BMP7 can induce chondrocyte hypertrophy, but like TGFβ in a context dependent manner.

**Activin A, BMP9, BMP4, BMP6 and GDF5**

Next to these well studied TGFβ-family members, several other family members have been ascribed a role in cartilage biology, but relatively little is known about their role and importance. This includes Activin A, BMP9, BMP4, BMP6 and GDF5.

Activin A signals via ACVR2A/ACVR2B and ALK4/ALK7 to induce SMAD2/3 [230]. Expression of the Activin A subunit Inhibin β A (*Inhba*) is upregulated in OA cartilage, and so is activin A [231, 232]. In OA conditions, activin A can possibly inhibit IL1 induced ADAMTS4/5 activity [232], but expression of its inhibitor follistatin is also profoundly upregulated during OA [233, 234]. Furthermore, a lack of response of adult cartilage tissue to activin A has been observed in multiple studies [152, 209, 235]; for example, activin A slightly induces proteoglycan synthesis (*i.e.*$^{35}$S-incorporation) in immature (1-3 months old) but not in mature (> 18 months old) bovine articular cartilage explants [235]. These observations indicate that activin A is not involved in the maintenance of mature healthy cartilage, but can possibly play a role during OA.

Regarding the role in cartilage of the inhibitory counterparts of the activins; the inhibins, very little is known; only one study reports that inhibin inhibits both proteoglycan synthesis (*i.e.*$^{35}$S-incorporation) and DNA synthesis in bovine chondrocytes [235].
BMP9, also known as GDF-2, signals via ACVR2A/ACVR2B and BMPR2 via ALK1 and ALK2 to induce SMAD1/5 and SMAD-independent signaling. BMP9 production could not be detected in chondrocytes [180] but BMP9 circulates in high levels in blood [236]. In juvenile bovine cartilage, BMP9 enhances the production of proteoglycans and does so more potently than BMP2 [187]. In bovine chondrocytes, BMP9 enhances proteoglycan and collagen type 2 production [237]. However, BMP9 can also induce ECM mineralization when chondrocytes are seeded in biodegradable PGA scaffolds [237]. Additionally, also in mesenchymal progenitor cells, BMP9 is a potent inducer of chondrogenesis accompanied by hypertrophy [238]. Therefore, BMP9 possibly has a detrimental effect on chondrocyte homeostasis.

BMP4 signals via ALK2 and ALK3 to induce pSMAD1/5 and SMAD-independent signaling. BMP4 is best known for its positive effect on chondrogenesis of stem cells and during embryogenesis. BMP4 has been used to enhance the repair of full thickness cartilage defects by chondroprogenitors like MSCs in vivo, and has been described to outperform BMP7 in such a setting [239]. However, very little is known regarding its role in mature articular cartilage. BMP4 is expressed in mature articular chondrocytes, and its expression is possibly increased during OA near cartilage defects just like BMP2 [240]. In bovine chondrocytes and cartilage explants, BMP4 enhances proteoglycan synthesis, collagen type 2 production and proliferation [235], and BMP4 has similar effects in human chondrocytes cultured in alginate [213]. However, if BMP4 is of any importance for the homeostasis of mature articular cartilage cannot be concluded from this limited evidence.

BMP6 also signals via ALK2 and ALK3 to induce pSMAD1/5. BMP6 is important for chondrogenesis during embryogenesis, and is required for adipose-derived stromal cells to differentiate towards chondrocytes, but, like BMP4, very little is known regarding its role in mature articular cartilage. In humans, BMP6 mRNA and protein are expressed in both healthy and OA articular cartilage and expression is not affected by OA [180]. In human chondrocytes, BMP6 induces proteoglycan synthesis and collagen type 2 production [213, 241], but with advancing age, chondrocyte response to BMP6 decreases [241]. However, in the epiphyseal plate, BMP6 is especially expressed in the hypertrophic zone [242] where it has a stimulatory role on chondrocyte maturation. Furthermore, a pro-hypertrophic effect of BMP6 has also been observed in ATDC5 cells [243]. These studies indicate that BMP6 signaling could be detrimental for articular cartilage like BMP9.

GDF5 is a very important initiator of joint formation and patterning. The importance of GDF5 in cartilage biology is illustrated by the observations that mutations in GDF5 can lead to chondrodysplasias and that genetic variation in GDF5, resulting in less GDF5 expression, is associated with OA development [244]. However, regarding the role of GDF5 in mature cartilage relatively little is known. In chondrocytes, GDF5 predominantly signals via ALK6 [228] but can also signal via ALK2 and ALK3 [195] to induce SMAD1/5 phosphorylation and SMAD-independent signaling [245]. GDF5 is expressed in mature cartilage, and its expression is not affected by OA [180, 197, 198]. In cartilage, GDF5 induces proteoglycan synthesis [197, 228]
and COMP expression [152] but not collagen type 2 synthesis or chondrocyte proliferation [197, 198]. Notably, OA chondrocytes do not respond consistently to GDF5 [245], \textit{i.e.} large variation can be observed between OA patients on gene expression of targets like Acan and Sox9 after addition of GDF5. Possibly, a yet undiscovered co-receptor plays a role in this; such a co-receptor has been postulated to explain how GDF5 can function as a downstream BMP2 antagonist in ATDC5 cells even though it uses the same type I and type II receptors [195]. Identification of such a co-receptor would greatly help in understanding GDF5 signalling in chondrocytes and how it differs from BMP signaling using the same receptors.

**Conclusion**

In conclusion, members of the TGFβ-family are crucial for the homeostasis of chondrocytes, and thus of cartilage, via both SMAD-dependent and SMAD-independent signaling, although both pathways are more integrated than their names suggest. Cellular context plays a large role in TGFβ-family signaling, greatly affecting its outcome, which is illustrated by \textit{e.g.} the differential effects of TGFβ and BMP7 in immature versus mature chondrocytes. A better understanding of how cellular context regulates TGFβ-family signaling would greatly help in separating the beneficial effects of family members from the detrimental effects, making more efficient cartilage repair approaches or OA treatment possible; a challenging but rewarding task for future research.

**Aim and outline of this thesis**

As mentioned, OA is the world’s most common joint disease, and currently no disease-modifying anti-OA drugs are available. This lack fuels the search for a curative OA treatment. To aid in the development of such a treatment, a better understanding of the disease process is of great importance. In part, such comprehension comes from a good understanding of how tissue homeostasis is regulated. Another part of this understanding can be gained by investigating how risk factors affect tissue homeostasis and contribute to disease development. In view of its importance in cartilage biology, the TGFβ-family is a prime target for studies in both directions. Therefore, the aims of this thesis were to (1) further characterize the importance of TGFβ-family signaling in cartilage homeostasis and (2) to show how ageing, the main risk factor for OA, impacts this signaling.

To begin, in chapter two, we wanted to know the extent of TGFβ-family signaling in healthy cartilage, \textit{i.e.} if normal healthy cartilage is continuously exposed to TGFβ-family signaling. We approached this question by isolating cartilage explants from bovine metacarpophalangeal joints (MCP) and analyzing TGFβ-family signaling immediately after
isolation and at later time points by investigating pSmad2 levels and corresponding gene expression. Next we analyzed in this chapter if loading, a process to which cartilage is daily exposed, plays a role in TGFβ-family signaling. Therefore we repeatedly loaded and unloaded cartilage explants using unconfined, dynamic mechanical compression and studying the outcome on gene expression and pSMAD2 levels. These experiments taught us that loading is an important activator of SMAD2/3 signaling in cartilage, and therefore we sought to find its physiological role. For this, we regularly compressed cartilage explants for up to two weeks, and investigated the effect of this regular compression on GAG content and chondrocyte hypertrophy.

After we established the importance of loading in activation of pSmad2/3 signaling in cartilage, we realized that ageing might negatively affect this process, because in previous studies of our lab we identified age-related changes in expression of TGFβ-family receptors in mice [125,137]. However, in these studies, ageing and OA development occurred concurrently, making it difficult to separate cause and effect. Therefore we sought to study age-related changes in TGFβ-family receptors and members in a model in which both OA and ageing could be separated. Chapter three describes that with the use of bovine MCP joints we were able to separate these processes. Bovine MCP joints can be obtained from animals of different ages and osteoarthritic cartilage can be excluded from analysis by visual inspection, which allowed us to study only ageing. Of these joints we obtained a piece of cartilage of a standardized location and isolated mRNA to measure gene expression and made tissue sections to study histology. Furthermore, we also incubated cartilage explants of animals of different ages with TGFβ and BMP9 to study if cartilage of a different age responds differently to these growth factors.

Together with this study we also investigated if ageing negatively affects loading-induced pSMAD2/3 directly in chapter four. We did this by loading cartilage pieces of young and old animals and analyzing how age impacted the outcome. Because we noticed that old cartilage is stiffer and more difficult to compress we used two forces: normal (i.e. 3 MPa) and excessive (i.e. 12 MPa). First we analyzed if (excessive) loading affects cartilage integrity by looking for tears and fissures using histology. Next we analyzed mRNA and analyzed gene expression 2 and 6 h after compression. Furthermore, we also investigated Smad2 phosphorylation and nuclear accumulation after compression by immunohistochemistry.

Because the previous chapters established the importance of loading in SMAD2/3 signaling in chondrocyte biology, we also wanted to further characterize the effects of ALK1 / Smad1/5/9 signaling on chondrocyte biology. To do this, we stimulated in chapter five primary bovine chondrocytes with BMP9, a high affinity ALK1 ligand, and investigated Smad1/5 phosphorylation and downstream gene expression. Furthermore, we cultured primary chondrocytes for a week with BMP9 and analyzed the effects hereof on chondrocyte hypertrophy using qPCR. Additionally, we were interested in the interaction of BMP9 with TGFβ and therefore stimulated chondrocytes with a combination of both growth factors, and
investigated their resulting interaction on SMAD2/3 and SMAD1/5/9 phosphorylation, gene expression and chondrocyte hypertrophy.

In view of the fact that TGFβ signaling has been linked to many pathophysiological aspects of OA but not yet to this disease’s main clinical outcome: pain, we investigated in **chapter six** if TGFβ signaling plays a role in induction of nerve growth factor (NGF), an important pain stimulus in OA. For this, we stimulated chondrocyte cell lines, primary chondrocytes and cartilage explants with various dosages of TGFβ and measured NGF gene expression. Furthermore, we also stimulated chondrocytes with IL1β, an inflammatory cytokine and well known inducer of NGF in cartilage, to compare the effects of TGFβ and IL1β. Because TGFβ signaling in chondrocyte biology is seen as a double-edged sword, we tried in **chapter seven**, to modulate TGFβ signaling with the use of small molecule inhibitors to direct TGFβ signaling away from its for cartilage potentially deleterious pSMAD1/5 signaling towards beneficial pSMAD2/3 signaling. We approached this by using an ALK5 kinase inhibitor, *i.e.* SB-505124, an ALK1 kinase inhibitor, *i.e.* LDN-193189, and a TAK1 kinase inhibitor, (5Z)-7-oxozeaenol, to inhibit both SMAD-dependent and SMAD-independent signaling. We added these inhibitors to both primary chondrocytes and cartilage explants and analyzed SMAD phosphorylation and gene expression.

Finally, in **chapter eight** a summary of the results described in this thesis is provided, together with some considerations regarding the implications of the described findings for cartilage biology and osteoarthritis.
References


2. Unloading results in rapid loss of TGFβ signaling in articular cartilage: role of loading-induced TGFβ signaling in maintenance of articular chondrocyte phenotype?

Madej W & van Caam A, Blaney Davidson E, Buma P, van der Kraan PM.

Abstract

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

Method
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 TGFβ 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 TGFβ on proteoglycan level and chondrocyte phenotype (Col10a1 mRNA expression) was analyzed.

Results
Unloading of articular cartilage results in rapid loss of TGFβ signaling while subsequent compressive loading swiftly restored this. Loading and exogenous TGFβ enhanced expression of TGFβ1 and ALK5. Unloading of explants for 2 weeks resulted in proteoglycan loss and increased Col10a1 expression. Both loading and exogenous TGFβ inhibited elevated Col10a1 expression but not proteoglycan loss.

Conclusion
Our data might imply that in vivo regular physiological loading of articular cartilage leads to enduring TGFβ signaling and TGFβ-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.
Introduction

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 (TGFβ) is stored in high amounts (up to ~300 ng/g of all isoforms) in the articular cartilage matrix but in a latent form [12]. TGFβ signaling has been shown to be essential for the preservation of articular cartilage. Loss of the intracellular TGFβ signaling molecule Smad3 or functional loss of the TGFβ 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 TGFβ signaling in chondrocyte-like cells [19,20]. Recently, we described that compressive in vitro loading of bovine articular cartilage explants rapidly induces TGFβ signaling [21]. Based on these observations we hypothesized that under normal in vivo conditions articular cartilage is always subject to active TGFβ 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 TGFβ signaling was lost when articular cartilage was taken out of its natural environment and if the changes in TGFβ 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 Col10a1, and if induced changes could be prevented by loading or exogenously added TGFβ. Our data indicate that unloading results in rapid loss of TGFβ 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 TGFβ.
Material and methods

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®, UK) 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.

Effect of unloading on TGFβ signaling in articular cartilage (Fig. 1A)

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). 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.

Fig. 1. Schematic representation of experiments conducted in this study. (A) The effect of unloading on TGFβ 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 TGFβ 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.
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.

**Effect of repeated physiological mechanical compression on TGFβ 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. 1B). 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.

**Effect of loading on glycosaminoglycan (GAG) content and Col10a1 expression (Fig. 1C)**

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 rhIGF-1 (PeproTech, NJ, USA) or 10 ng/ml rhTGFβ1 (Biolegend, CA, USA) or combination of 20 ng/ml of rhIGF-1 + 10 ng/ml rhTGFβ1 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 unloaded condition for 14 days in DMEM/F-12 medium supplemented with 10 % FBS or 1 ng/ml rhTGFβ1 or 10 ng/ml rhTGFβ1 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.

**Dynamic mechanical compression of articular cartilage explants**
To compress cartilage, a BOSE® ElectroForce® BioDynamicTM 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.

**Gene expression analysis**
Samples were homogenized using a micro dismembrator (B. Braun Biotech International, Melsungen, Germany). Total RNA was isolated using RNeasy Fibrous tissue kits (Qiagen Inc., Valenzia, CA, USA) according to manufacturers 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 I) (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).

**Immunohistochemical 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 h 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 Smad2P (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 dimethylaminoazobenzene (DAB) reagent.
Unloading results in rapid loss of TGFβ signaling in articular cartilage: role of loading-induced TGFβ signaling in maintenance of articular chondrocyte phenotype?

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Table I. Template, efficiency and sequence of the primers used in this study. P is product size in basepairs (bp) and E is efficiency in percentage (%).

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).

Statistical analysis
All quantitative data analysis were expressed as a Tukey box blot with mean showed 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 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. Unpaired one tailed t-test was used to estimate the effect of
time on Pai1 expression levels. One way ANOVA with Tukey’s post-test was used to estimate the effect of addition of TGFβ1 or Activin A on Smad7, Pai1 and Tgfb1 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).

Results

Unloading results in loss of TGFβ signaling

To investigate if unloading results in loss of TGFβ 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 TGFβ 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 Pai1 (a marker for active TGFβ signaling) expression when compared to fresh tissue (Supplementary Fig. 2).

To determine whether this rapid drop in Smad2/3 signaling was reflected in TGFβ signaling-dependent gene expression, expression of the known ALK5/Smad3 responsive genes Smad7 [24], Pai1 [25] and Alk5 (also known as Tgfbr1)[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 TGFβ signaling did not result in a more severe loss of gene expression, indicating that no residual TGFβ 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).
Unloading results in rapid loss of TGFβ signaling in articular cartilage: role of loading-induced TGFβ signaling in maintenance of articular chondrocyte phenotype?

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 TGFβ-responsive genes: Alk5, Smad7 and Pai1 24 h and 48 h after unloading compared to fresh samples. Tukey box plot, + = mean, N = 7, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. (C) The effect of the ALK4/5/7 blocker SB-505124 on relative gene expression of Alk5, Smad7 and Pai1 24 h and 48 h 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.

Reloading repeatedly induces TGFβ signaling

Next, we evaluated if compressive reloading could restore Smad2P signaling and TGFβ-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).
Fig. 3. Repeatable restoration of active Smad2/3 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 TGFβ-responsive genes: Alk5, Smad7 and Pari 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 TGFβ-signaling in the absence or presence of SB-505124. (E) Relative gene expression of Alk1 in (repeatedly) dynamically compressed (3 MPa) cartilage compared to unloaded controls 48 h after compression. Tukey box plot, + = mean, N = 8, ***P ≤ 0.001.
Two hours after the first 30 min of loading, gene expression of \textit{Alk5}, \textit{Smad7} and \textit{Pai1} was significantly induced, indicating that loading restores TGFβ signaling (Fig. 3B). 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 \textit{Smad7} and \textit{Pai1} was strongly elevated. We could confirm our earlier observations [21] that the loading-induced expression of \textit{Smad7} can be fully blocked by the ALK4/5/7 inhibitor SB-505124 (Fig. 3C), indicating that compression-induced \textit{Smad7} expression indeed runs via active Smad2/3P.

Notably, SB-505124 did not inhibit all compression-induced gene expression. For example, a loading-induced \(\sim 4\)-fold increase in \textit{Bmp2} expression (Supplementary Fig. 3) was unaffected. Because \textit{Bmp2} expression was also not responsive to exogenously added TGFβ, regulation of this gene is most likely TGFβ-independent. This observation thus shows that SB-505124 does not affect compression-induced gene expression that is induced independently of TGFβ. In Fig. 2D a schematic representation of the effects of loading and unloading is depicted showing the repeated mechano-responsiveness of TGFβ signaling in cartilage. Finally, we were also able to confirm our earlier observations that loading reduces \textit{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: TGFβ and Activin-A. Incubation of bovine explants with exogenously added TGFβ 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 \textit{Alk5}, \textit{Smad7} or \textit{Pai1} although it was bioactive (Supplementary Fig. 4).

**Loading-induced TGFβ signaling blocks chondrocyte hypertrophy**

Subsequently, the potential physiological relevance of the loading-induced TGFβ signaling was investigated. We postulated that TGFβ 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 TGFβ was completely ineffective and even lowered IGF1 effects on GAG content (Fig. 4B). We conclude that it is unlikely that TGFβ plays a direct role in maintenance of GAG content in articular cartilage.

Apart from GAG loss, culturing of bovine explants in the absence of loading resulted in strongly increased expression of \textit{Col10a1}, an accepted marker for early hypertrophic differentiation of chondrocytes [27]. The increase in \textit{Col10a1} expression was not affected by addition of 10% fetal calf serum, underling that serum factors are not able to inhibit hypertrophic differentiation of articular chondrocytes (Fig. 4C). In contrast, addition of 1 ng/ml TGFβ fully blocked induction of \textit{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.

**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 TGFβ1 or subjected to dynamic mechanical compression (3 MPa). Tukey box plot, + = mean, N = 8, ***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 TGFβ1, or a combination of IGF1 and TGFβ1. Tukey box plot, N = 9, *P ≤ 0.05, ***P ≤ 0.001. (C) Expression of the hypertrophy marker Col10a1 after 2 weeks ex vivo culture in serum free medium or in the presence of 10% FCS, 1 or 10 ng/ml TGFβ1, 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 Col10a1 expression after 2 weeks ex vivo culture. Tukey box plot, + = mean, N = 6. **P ≤ 0.01, ***P ≤ 0.001.
Discussion

This study is the first to demonstrate that removal of articular cartilage from in its in vivo situation results in rapid loss of TGFβ signaling and that subsequent compressive loading can repeatedly restore this signaling. This suggests that the absence of loading will result in the loss of TGFβ signaling in articular cartilage. The consequence of this is reduced expression of TGFβ1 and the TGFβ 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 TGFβ prevent the increase in Col10a1 expression but not of proteoglycan loss.

Our data demonstrate the repeated mechano-responsiveness of TGFβ 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 TGFβ signaling by compressive mechanical loading: Articular cartilage contains high amounts of TGFβ (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 TGFβ1 associated with LAP, but unfolding of LAP by mechanical force (40 pN) can release active TGFβ [28,29]. Mechanical force (compressive loading) is thus able to release active TGFβ, and this has been shown in multiple systems [30,31]. However, upon unloading, all active TGFβ 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 TGFβ in cartilage.

We hypothesize that this loading-released TGFβ will bind to its receptors, but will also rapidly bind the ECM becoming unavailable again [32]. In the absence of mechanical force TGFβ signaling will rapidly diminish. The loading-induced TGFβ signaling will induce synthesis of TGFβ1 [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 TGFβ-dependent Smad2/3 signaling and decreasing Smad1/5/8 signaling (Fig. 5). Chondrocyte terminal differentiation is stimulated by Smad1/5/8 activation and inhibited by 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 TGFβ 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 TGFβ 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]. The observation that
the biological consequence of the loading/TGFβ 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 TGFβ and compressive loading inhibited the up-regulation of the early hypertrophy marker Col10a1, suggesting that loading-induced TGFβ 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 TGFβ signaling can be blocked by this inhibitor, in line with our previous work [21]. Our results are in line with earlier observations that TGFβ, 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 TGFβ signaling.

Fig. 5. Proposed hypothetical model for compression-mediated protection of articular cartilage integrity. Compression of articular cartilage leads to release of active TGFβ from the ECM. This active TGFβ 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 TGFβ1, which after production will bind to the ECM, returning the system to its original state.
Our study has a number of limitations. We used expression of *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 TGFβ, 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 mRNA expression 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 TGFβ 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 shown to be able to activate TGFβ [31]. Moreover, our data indicate that short physiological compressive loading once every day will be sufficient to maintain TGFβ-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 TGFβ. 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 TGFβ type II receptor TGFBR2 [41] to show the importance of TGFβ 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 TGFβ 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 TGFβ 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.

**Acknowledgment**

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Supplementary data

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

**Supplementary Figure 2:** Active TGFβ-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 Pai1 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 TGFβ 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, Pai1 or Tgfb1 expression in bovine cartilage explants. (A) Relative gene expression of Smad7, Pai1 and Tgfb1 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 Pai1 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.

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 manufacturers protocol (Roche Diagnostics GmbH, Germany). Tukey box plot, + = mean, N = 5, *P ≤ 0.05.
Supplementary Materials and Methods

Dynamic mechanical compression of articular cartilage explants
Cartilage samples that were to be mechanically stimulated were placed into bioreactor chamber of a BOSE® ElectroForce® BioDynamicTM bioreactor (5160 BioDynamic System) equipped with a 50lbf load-cell (BOSE Bose Corp. ElectroForce Systems Group, MN, USA). The chamber was filled with 70 ml of DMEM/F12 medium. First, a preset compression force of 5 N (0.3 MPa) was applied which guaranteed 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. All compressions were performed for 30 min (1800 cycles). The compression procedure was performed in an incubator under standard culture conditions (37°C, 5% CO₂ and 95% humidity). All explants that served as unloaded controls were also placed in the bioreactor incubator but in a separate well. After the compression procedure all the samples were placed back into a regular culture incubator. At desired time intervals samples were flash frozen in liquid nitrogen and stored at -80°C.

Gene expression analysis
First deep frozen articular cartilage samples were homogenized using a micro dismembrator (B. Braun Biotech International, Melsungen, Germany) at 1500 RPM for 1 minute. Total RNA was isolated from the homogenate using an RNeasy Mini Kit (Qiagen Inc., Valenzia, CA, USA). The homogenate was dissolved in RLT buffer containing 1 % (v/v) of 2-mercaptoethanol solution and transferred to 1.5 ml tubes. Subsequently, the capsules of microdismembrator were rinsed with 650 µl of ultra pure H₂O. 10 µl of proteinase K (Qiagen Inc., Valenzia, CA, USA) was added and samples were incubated at 55°C for 10 min. Afterwards tubes were centrifuged for 3 min with 12 000 RPM and supernatant was transferred to a new tube. Thereafter, 450 µl of 100 % ethanol was added and samples were mixed. Samples were brought on RNAesy column and centrifuged for 1 min with 10 000 RPM. Flow was drained and column was washed first with RW1 buffer and then twice with RPE buffer, each time by centrifugation for 1 min with 10 000 RPM. Finally, RNA was eluted with 30 µl of ultra pure RNAse free H₂O by centrifugation for 1 min with 10 000 RPM. Isolated mRNA was transcribed into cDNA using M-MLV reverse transcriptase. To perform the reverse transcriptase (RT) reaction; 1.9 µl ultra pure water, 2.4 µl 10 x DNAse buffer, 2.0 µl 0.1 M DTT, 0.8 µl 25 mM dNTP, 0.4 µg oligo dT primer, 1 µl 200 U/µl M-MLV reverse transcriptase (all Life Technologies, USA) and 0.5 µl 40 U/µl RNAsin (Promega, the Netherlands) was added, and samples were incubated for 5 min at 25 °C, 60 min at 39 °C, and 5 min at 95 °C using a thermocycler. The obtained cDNA was diluted 10x in ultra pure water, and gene expression was measured using 0.25 µM of validated cDNA-specific primers (see Table 1) (Biolegio, the Netherlands) in a quantitative real time polymerase chain reaction (qPCR) using SYBR green master mix.
(Applied Biosystems, Darmstadt, Germany). The following protocol was used: after 10 min at 95 °C, 40 cycles of 15 sec 95 °C and 1 min 60 °C each were run. Hereafter a melting curve was made to verify gene specific amplification. For calculations of the -ΔCt, 2 reference genes were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S14 (RPS14).

All used primers are listed in Table 1.

**Immunohistochemical analysis**

All samples for immunohistochemical staining were fixed overnight in 4% phosphate buffered formalin (pH 7.0) at 4 °C. First the specimens were dehydrated in a tissue processing apparatus (Pathos, Milestone Medical Inc.) and embedded in paraffin. Later 6 µm thick sections were cut and mounted on Superfrost™ Plus Microscope Slides (Thermo Scientific, Waltham, USA). Sections were deparaffinised and washed with phosphate-buffered saline (pH 7.0). For antigen unmasking, sections were incubated in citrate buffer (0.1 M sodium citrate and 0.1 M citric acid) for 2 h at RT. Endogenous peroxidase was inhibited by incubation for 30 min in solution of 1% hydrogen peroxidase in methanol. Then, sections were blocked with 5% normal goat serum. Afterwards, sections and incubated overnight at 4°C with specific primary antibodies against C-terminally phosphorylated SMAD2P (rabbit pAb anti Phospho-Smad2 (Ser465/467) (1:100) (Cell Signalling Technology, Danvers, Massachusetts, USA) [39, 40]. After washing with phosphate-buffered saline, sections were incubated for 30 min at room temperature with the biotin-labeled secondary antibody (Dako, Glostrup, Denmark). This was followed by a biotin–streptavidin detection system used according to the manufacturers’ protocol (Vector Laboratories, Baiklin Game, California, USA). Staining was visualized using dimethylaminoazobenzene (DAB) reagent, dehydrated and mounted with Permount (Fisher Chemicals, New Jersey, USA).

**Spectrophotometric analysis of cartilage glycosaminoglycan content**

First explants were thawed and any excessive liquid was removed. Then cartilage explants were weighed and subsequently digested overnight at 60°C with papain mixture (consisting of 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 to get rid of residue. Next, the digest was diluted 20 times in ultra pure water. Subsequently, 200 µl of the DMB solution (40 mM glycine, 40 mM NaCl, 0.012 N HCl, 45 µM 1,9 dimethylmethylene blue, pH 3.0) was added to 40 µl of diluted digest, and sulphated glycosaminoglycan content was immediately determined by spectrophotometric analysis by measuring absorbance at λ = 595 nm using a 96-well plate reader (Biorad, CA, USA). Chondroitin sulfate type A (Sigma–Aldrich, St. Louis, MO, USA) from shark cartilage was used to create a standard curve [41].
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Cell viability analysis (XTT assay)
Immediately before use, the XTT labeling reagent and electron-coupling reagent (both from Roche Diagnostics GmbH, Germany) were thawed in a waterbath (37°C). Later each component was mixed carefully to obtain clear solutions. Subsequently XTT labeling and electron coupling reagents were mixed in ratio 50:1 respectively. Afterwards, 100 µl of XTT labeling mixture was added to 96 well plate with articular cartilage explants (one 4 mm Ø explant per well) in 100 µl of non-phenol red DMEM/F12 medium (Gibco®, UK). Explants were incubated with XTT labeling mixture for up to 5 hours. At the 2 hours time point, a 100 µl of XTT mixture was aspirated and transferred to a separate 96 well plate. The spectrofotometrical absorbance of samples was measured by microplate reader under 480 nm wave length. The reference wavelength was 750 nm. After this measurement, the 100 µl was added back to the explants. To obtain a reliable estimate of XTT mixture conversion by metabolically active cells, measurements were repeated after 3, 4 and 5 hours. After subtraction of background XTT conversion, the area under the curve was calculated for each explant. Next DNA content of samples was measured (see below), and measured XTT activity was corrected for DNA content. Obtained values were used for statistical analysis.

DNA content analysis with Quant-iT™ PicoGreen® dsDNA Assay Kit
First, articular cartilage explants (4 mm Ø) were digested overnight (O/N) at 60°C with papain mixture (consisted of 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 used for measurement. Subsequently, 1 X TE (Tris-EDTA buffer) (Invitrogen, OR, USA) buffer was prepared. dsDNA standards (Invitrogen, OR, USA) were diluted in TE buffer to obtain dilution series from 2000 ng/ml to 31.25 ng/ml for standard curve. Afterwards, articular cartilage digests of experimental samples were diluted in TE buffer in proportion 1:10. After transferring of all standards and experimental samples to 96 well plate in duplos, 100 µl of Quant-iT PicoGreen® reagent (200 X diluted in TE buffer) (Invitrogen, OR, USA) was added to 100 µl of sample or standard. Samples were mixed well and incubated for 2 to 5 minutes at room temperature, protected from light. Samples fluorescence was measured with fluorescence microplate reader (excitation 480 nm and emission 520 nm). To minimize the photobleaching effect the measurement times were constant for all samples.

Statistical analysis
All quantitative data analysis were expressed as a Tukey box blot with mean showed as “+” and outliers showed as “•”. The sample size was always mentioned in the figure legend. First 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.

Unpaired one tailed t-test was used to estimate the effect of time on Pai1 expression levels. One way ANOVA with Tukey’s post test was used to estimate the effect of addition of TGFβ1 or Activin A on Smad7, Pai1 and Tgfb1 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).
Unloading results in rapid loss of TGFβ signaling in articular cartilage: role of loading-induced TGFβ signaling in maintenance of articular chondrocyte phenotype?

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3.
Expression of TGFβ-family signalling components in ageing cartilage: age-related loss of TGFβ and BMP receptors


Osteoarthritis Cartilage. 2016 Jul;24(7):1235-45
Abstract

Objective
Ageing is the main risk factor for osteoarthritis (OA). We investigated if expression of transforming growth factor β (TGFβ)-family components, a family which is crucial for the maintenance of healthy articular cartilage, is altered during ageing in cartilage. Moreover, we investigated the functional significance of selected age-related changes.

Design
Age-related changes in expression of TGFβ-family members were analysed by quantitative PCR in healthy articular cartilage obtained from 42 cows (age: ¼–10 years). To obtain functional insight of selected changes, cartilage explants were stimulated with TGFβ1 or bone morphogenetic protein (BMP) 9, and TGFβ1 and BMP response genes were measured.

Results
Age-related cartilage thinning and loss of collagen type 2α1 expression (~256-fold) was observed, validating our data set for studying ageing in cartilage. Expression of the TGFβ-family type I receptors; bAlk2, bAlk3, bAlk4 and bAlk5 dropped significantly with advancing age, whereas bAlk1 expression did not. Of the type II receptors, expression of bBmpr2 decreased significantly. Type III receptor expression was unaffected by ageing. Expression of the ligands bTgfb1 and bGdf5 also decreased with age. In explants, an age-related decrease in TGFβ1-response was observed for the pSmad3-dependent gene bSerpine1 (P = 0.016). In contrast, ageing did not affect BMP9 signalling, an Alk1 ligand, as measured by expression of the pSmad1/5 dependent gene bId1.

Conclusions
Ageing negatively affects both the TGFβ-ALK5 and BMP-BMPR signalling routes, and aged chondrocytes display a lowered pSmad3-dependent response to TGFβ1. Because pSmad3 signalling is essential for cartilage homeostasis, we propose that this change contributes to OA development.
Introduction

Osteoarthritis (OA) is the world’s most common joint disease, and cartilage degradation is its main hallmark [1]. Eventually, OA leads to pain and disability, greatly affecting quality of life of patients. Many risk factors have been identified for OA including: obesity, female gender, and occupational risks, but its main risk factor is (old) age [2, 3]. To better understand why ageing is a risk factor for OA, research has centred on age–related changes in cartilage, as this is the main tissue affected by this pathology.

During ageing, many changes accumulate in cartilage. On a macroscopic level, progressive thinning of the cartilage occurs, most prominently at load-bearing areas [4, 5, 6, 7]. On a more detailed level, in the extracellular matrix (ECM), structure, size and expression of the essential structural proteoglycan aggrecan change during ageing [8]. Furthermore, age-related accumulation of advanced glycation end products (AGEs) occurs in collagen fibres [9, 10]. Together, these changes adversely affect the mechanical properties of the cartilage matrix, as they lead to a loss of water retaining capacity and matrix stiffening respectively. On a cellular level, up to 40% of chondrocytes are lost during ageing [11]. Possibly, this loss occurs because ageing chondrocytes produce less of and/or respond less to essential anabolic growth factors like insulin-like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2), transforming growth factor β (TGFβ) and bone morphogenetic protein 7 (BMP7) [12, 13, 14, 15].

Both TGFβ and BMP7 belong to the TGFβ-family. Canonical signalling by members of this family induces receptor-regulated Smad (R-Smad) activation via phosphorylation, which is crucial for chondrocyte homeostasis [16]. Phosphorylated Smad3 (pSmad3) is chondroprotective and inhibits many processes deleterious for cartilage, including matrix metalloprotease 13 (MMP13) production and cellular hypertrophy [17, 18]. Phosphorylated Smad1/5 (pSmad1/5) is associated with ECM production, e.g., glycosaminoglycans and collagen type II expression, but, in contrast to pSmad3, also with MMP13 production and deleterious chondrocyte hypertrophy [16, 19].

Previously, we have demonstrated in murine cartilage that TGFβ-family signalling is affected by ageing [13, 20, 21]. However, in these experiments in mice, ageing and OA were concomitant. Because OA chondrocytes are profoundly different from ageing chondrocytes, e.g., in anabolic activity [22, 23], and both processes possibly interfere, we wanted to further study cartilage ageing in a system in which we could observe age-related changes independently of an OA process. To achieve this, we investigated ageing in non-OA cartilage of the bovine metacarpophalangeal (MCP) joint. Bovine material can be obtained in a wide age range, and examination of the cartilage allows for inclusion of only healthy joints with an intact cartilage surface.

In this study we investigated the impact of ageing on expression of receptors and ligands of the TGFβ-family. We were predominantly interested in expression of TGFβ and
bone morphogenetic protein (BMP) receptors, as especially little is known of the latter in the context of ageing. We show that in old cartilage the Smad2/3-inducing receptors bAcvr1b and bTgfbr1 are profoundly lower expressed than in young cartilage, just as the BMP-receptors bBmpr1 and bBmpr2. Furthermore, we show that reduction of Tgfbr1 expression is reflected in a reduced response to TGFβ1. Overall, we conclude that changes in TGFβ-family signalling occur before onset of OA symptoms but could predispose cartilage to this disease.

Materials and methods

Sample collection
Bovine MCP joints were obtained from a local abattoir within 3 h post mortem. Joints were opened and the cartilage surface was examined for signs of OA. Only joints showing an intact cartilage surface were included in this study. Subsequently, a ∼0.5 cm² full thickness cartilage slice was obtained from the middle of the medial condyle adjacent to the intertrochlear notch of the metacarpal bone. This cartilage sample was shortly rinsed in saline and flash frozen in liquid nitrogen for later use. In total 42 female cows were included with ages ranging from 9 months old up to 10 years old (see Supplementary Fig. 1).

Detection of gene expression
A ∼0.5 cm² cartilage slice was homogenized using a Mikro-dismembrator (B.Braun, Germany) for 1 min at 2,000 × g. Subsequently, RNA was isolated according to manufacturers protocol. Next, 8 μl of sample was treated with 1 μl DNAse (Life Technologies, USA) for 10 min at room temperature to remove DNA contamination. Hereafter, DNAse was inactivated at 65°C with 1 μl 25 mM EDTA (Life Technologies, USA) for 10 min. To perform reverse transcriptase (RT) reaction; 1.9 μl ultra pure water, 2.4 μl 10× DNAse buffer, 2.0 μl 0.1 M DTT, 0.8 μl 25 mM dNTP, 0.4 μg oligo dT primer, 1 μl 200 U/μl M-MLV RT (all Life Technologies, USA) and 0.5 μl 40 U/μl RNAseIn (Promega, the Netherlands) was added, and samples were incubated for 5 min at 25°C, 60 min at 39°C, and 5 min at 95°C using a thermocycler. The obtained cDNA was diluted 10× in ultra pure water, and gene expression was measured using 0.25 μM of validated cDNA-specific primers (see Table 1) (Biolegio, the Netherlands) in a quantitative real time polymerase chain reaction (qPCR) using SYBR green master mix (Applied Biosystems, USA). The following protocol was used: after 10 min at 95°C, 40 cycles of 15 s 95°C and 1 min 60°C each were run. A melting curve was made to verify gene specific amplification. For calculations of the −ΔCt, the average of the following four reference genes was used: bovine (b) glyceraldehyde 3-phosphate dehydrogenase (bGapdh), ribosomal protein S14 (bRps14), ribosomal protein L22 (bRpl22) and beta glucuronidase (bGusb). These reference genes correlate highly in our samples, showing that ageing does not affect their interrelation (see Supplementary Fig. 2).
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

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Table I. Template, efficiency and sequence of the primers used in this study

**Immunohistochemistry (IHC)**

Samples were fixed overnight in phosphate buffered formalin (10%). Four animals were used per age-group. Hereafter, the samples were dehydrated in a tissue processing apparatus (Pathos, Milestone Medical Inc.) and embedded in paraffin. Six μm thick sections were cut and deparaffinised. Subsequently, IHC was performed: first, sections were treated with citrate buffer (0.1 M sodium citrate and 0.1 M citric acid) for 2 h at room temperature for antigen unmasking. Next, endogenous peroxidase was inactivated by incubation with 1% hydrogen peroxidase in methanol for 30 min. Then, sections were blocked with 5% normal rabbit serum. Afterwards, sections were incubated overnight at 4°C with goat polyclonal TGFβ RI Antibody (V-22) 1:200 (v/v) (sc-398-G Santa Cruz Biotechnology, USA) or goat polyclonal ALK-1 Antibody (D-20) 1:200 (v/v) (sc-19546 Santa Cruz Biotechnology, USA) [20]. Hereafter, sections were washed with PBS (pH 7.0) and incubated for 30 min with a biotin-labelled rabbit anti goat antibody (Dako, Glodstrup, Denmark). Next streptavidin-linked horseradish peroxidase was added (Vector Laboratories, Baiklin Game, California, USA), and staining was visualised using dimethylaminoazobenzene. To quantify IHC, a 0-3 scoring was used.
(½ values allowed) by a scorer using blinded sections (as recommended by [24]). A value of three represented strongest staining in contrast to a value of 0, which represented the weakest staining (see Supplementary Fig. 3). Four animals were used per age group, and per animal four sections were scored for each of the three cartilage zones: surface, middle and deep.

**Ex vivo stimulation of explants**
MCP joints were obtained from 22 animals aged 0.5 up to 11 years old and opened under aseptic conditions. With the use of a biopsy punch, ~7 mm² explants were made. Subsequently, these explants were incubated for 24 h in standard cell culture conditions in DMEM/F12 1:1 (Gibco, USA) containing penicillin/streptomycin and pyruvate but no FCS. After 24 h, explants were stimulated with 1 ng/ml rhTGFβ1 or 5 ng/ml rhBMP9 (both R&D systems, USA) for 24 h. Per condition, four pooled explants were used, except for the unstimulated control for which two times four pooled explants were used. After stimulation, mRNA was isolated using the aforementioned protocol.

**Statistics**
Gene expression of individual animals is plotted with a solid line depicting the best fit regression analysis and 95% confidence interval (CI) as dotted line. For every analysis, data was checked for normality using the Shapiro–Wilk test. A Kruskal–Wallis test with Dunn’s multiple comparison post hoc test was used to analyse the scoring of IHC. All statistics were conducted using GraphPad Prism v 5.0.

**Results**

**Bovine cartilage as a valid model for age-related changes in cartilage**
To validate our bovine model and chosen age range (¾-10 years old) as a model for ageing cartilage, we first characterized well known markers of ageing cartilage in our data set. Macroscopically, with advancing age, thinning of the cartilage was observed and cartilage colour changed from purple towards yellow. Microscopically, aged cartilage showed decreased cellularity compared to young cartilage (for quantification of cell number and thickness see [25]), and a clear tidemark could be detected in old cartilage using a hematoxilin staining (Fig. 1A). Closer examination showed that this tidemark actually consisted out of multiple tidemarks (Fig. 1B). Furthermore, in young cartilage the osteochondral junction was less well defined, as shown by the close interaction of bone marrow and blood vessels with the cartilage, and absence of a tidemark. Moreover, the superficial zone of old cartilage looks irregular when looking at the lamina splendens as visualized by Safranin O/Fast green staining (Fig. 1A). On gene expression, a very profound ~250-fold drop in bovine collagen type 2.
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

Alpha 1 (bCol2a1) expression was observed ($P < 0.0001$, $R^2 = 0.63$) when comparing young vs old (Fig. 1C). In contrast, aggrecan (bAcan) expression did not drop extensively (~2-fold) ($P < 0.01$, $R^2 = 0.16$), and expression of matrix gla protein (bMgp) was significantly (~8 fold) higher in old cartilage vs young cartilage, showing that not of every gene expression decreases with ageing. Because these observations on macroscopic, microscopic and gene expression level reflect well known markers of ageing cartilage [4, 5, 7, 11, 26, 27], we concluded that our data set is a valid model for studying ageing processes in articular cartilage.

**Fig. 1.** Age-related changes in bovine cartilage. (A) Safranin O/Fast green and Hematoxilin/Eosin staining of articular cartilage obtained from a 2 years old (left) or 10 years old cow (right). Pictures are representative of three animals each. 100× Magnification, scale bar = 100 μm. Thickness and cellularity are reduced and a clear tidemark is visible (arrow) in 10 year old cartilage. Note that the surface is intact. (B) 800× Magnification, scale bar = 12.5 μm. Multiple tidemarks (stars) in healthy 10 year old cartilage. No tidemark is visible between non-calcified and calcified cartilage in 2 year old cartilage. (C) Relative gene expression of bovine collagen type 2 alpha 1 (bCol2a1), aggrecan (bAcan) and matrix Gla protein (bMgp) in ageing cartilage as determined by qPCR. Regression analysis (solid line) with 95% CI (dotted line) depicted. Expression of bCol2a1 decreases whereas expression of bMgp increases during ageing.
**Figure 2**

**A**
- *bAcvrl1 (ALK1)*
- *bAcvrl1 (ALK2)*
- *bBmpr1a (ALK3)*

Relative gene expression (ΔCt) plotted against age (years).

**B**
- *bTgfbr1 (ALK5)*
- *bAcvr1b (ALK4)*

Relative gene expression (ΔCt) plotted against age (years).

**C**

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Images of superficial, middle, and deep zones under different ages.

**D**

Bar charts showing relative gene expression (arbitrary score) for Acvrl1 (ALK1) and Tgfbr1 (ALK5) across different zones and ages.

- Acvrl1 (ALK1)
  - Superficial zone
  - Middle zone
  - Deep zone

- Tgfbr1 (ALK5)
  - Superficial zone
  - Middle zone
  - Deep zone
Fig. 2. Age-related drop in TGFβ-family receptor expression in healthy cartilage. (A) Relative gene expression of the Smad1/5 phosphorylating receptors: activin A receptor like type 1 (bAcvrl1), activin A receptor type I (bAcvr1) and bone morphogenetic protein receptor type IA (bBmpr1a), in ageing cartilage as determined by qPCR. Regression analysis (solid line) with 95% CI (dotted line) depicted. Expression of bBmpr1a clearly decreases with advancing age. (B) Relative gene expression of the Smad2/3 phosphorylating receptors: transforming growth factor beta receptor I (bTgfbr1) and activin A receptor type IB (bAcvr1b) in ageing cartilage as determined by qPCR. Regression analysis (solid line) with 95% CI (dotted line) depicted. Expression of bTgfbr1 and bAcvr1b clearly decreases with age. (C) IHC of bAcvrl1 (left) and bTgfbr1 (right) in 1 year and 5 years old animals in the three zones of articular cartilage. Pictures are representative of four animals each. 1000× Magnification, scale bar = 10 μm. During ageing, bTgfbr1 expression decreases below the detection threshold and unstained cells start to appear (arrows). (D) Scoring of Acvrl1 and Tgfbr1 staining in the superficial, middle and deep zone of 1 and 5 year old bovine cartilage using a 0–3 scale (see Supplementary Fig. 3). Four animals were scored in quadruple for each group for each zone. Error bars represent 95% CI. Statistics were calculated using a Kruskal–Wallis test; * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001.

Expression of TGFβ-family type I receptors during ageing

Previously we demonstrated decreased levels of phosphorylated Smad2 in aged murine cartilage compared to young [13] and linked this observation to altered expression of TGFβ-family-type I receptors; the activin receptor-like kinase (ALK) receptors [13, 20]. Therefore, we first measured gene expression of the four Smad1/5 phosphorylating receptors: bAcvrl1 (=ALK1), bAcvr1 (=ALK2), bBmpr1 (=ALK3) and bBmpr1b (=ALK6) (Fig. 2A). bAcvrl1 expression was unaffected by ageing. bAcvr1 expression did drop ~3-fold but, in view of the relatively high p-value (P = 0.02, R² = 0.13), not very convincingly. In contrast bBmpr1 expression clearly diminished ~4-fold during ageing (P = 0.001, R² = 0.24). bBmpr1b expression was not reliably detected, despite the use of multiple primer sets. Subsequently, we measured the three Smad2/3 phoshorylating receptors: bAcvrl1b (=ALK4), bTgfbr1 (=ALK5) and bAcvr1c (=ALK7) (Fig. 2B). Of these three receptors, expression of bAcvrl1b and bTgfbr1 was readily detected. In contrast, we were unable to reliably detect bAcvr1c expression, again despite the use of multiple primer sets. Expression of both bAcvrl1b and bTgfbr1 decreased ~8-fold and ~4-fold respectively (P = 0.0009, R² = 0.30 for bAcvrl1b and P = 0.0004 R² = 0.29 for bTgfbr1) with advancing age. Next we validated part of this gene expression data by immunohistochemically staining the corresponding proteins (Fig. 2C) and quantifying this (Fig. 2D). bAcvrl1 and bTgfbr1 were selected to relate this study to our previous results obtained in mice [20]. Staining for bTgfbr1 clearly showed abundant positive cells in young animals, but with advancing age Tgfbr1 staining decreased in the superficial, middle and deep zone of the cartilage. This was in contrast with the bAcvrl1 staining, which was only reduced in the deep zone. Taken together, our data show a profound decrease in expression of the Smad2/3 phosphorylating receptors bAcvrl1b and bTgfbr1 with advancing age, whereas of the Smad1/5 phosphorylating receptors bBmpr1 expression was clearly diminished but bAcvrl1 expression was not.
Expression of TGFβ-family type II and type III receptors in ageing cartilage

Next to receptor type I expression, the presence of type II receptors is necessary for TGFβ-family signalling and therefore we measured expression of these receptors [28]. Furthermore, because several ligands of the TGFβ-family can use multiple type I receptors (e.g., TGFβ1), we measured expression of the type III receptors *Endoglin* (*bEng*) and *Betaglycan* (*bTgfbr3*) because these receptors can direct receptor choice of such a ligand (Fig. 3). With advancing age, gene expression of the type II receptor *bBmpr2* clearly decreased $\sim$6-fold ($P < 0.0001$, $R^2 = 0.38$) (Fig. 3). In contrast, *bTgfbr2* expression was not significantly decreased with age ($P = 0.0517$, $R^2 = 0.10$) (Fig. 3). Expression of both *bEndoglin* and *bTgfbr3* did not change during ageing.

![Fig. 3. Age-related changes in TGFβ-family type II and type III receptors in healthy cartilage. Relative gene expression of bone morphogenetic protein receptor type II (*bBmpr2*), transforming growth factor beta receptor II (*bTgfbr2*), endoglin (*bEng*), and transforming growth factor beta receptor III (*bTgfbr3*) in ageing cartilage as determined by qPCR. Regression analysis (solid line) with 95% CI (dotted line) depicted. Only expression of *bBmpr2* clearly decreases with advancing age.](image)

Decreased expression of TGFβ1 and GDF5 in ageing cartilage

In addition to the changes we observed on receptor expression, we also looked into the expression of ligands. As TGFβ is crucial for healthy articular cartilage, we investigated
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

Expression of the three TGFβ isoforms: TGFβ1, TGFβ2 and TGFβ3. Of these three TGFβ isoforms, only expression of bTgfb1 was significantly ∼3-fold (P = 0.0002, R² = 0.32) less expressed in old cartilage compared to young cartilage (Fig. 4). In contrast, expression of both

**Fig. 4.** Age-related drop in TGFβ-family ligands. Relative gene expression of transforming growth factor beta 1 (bTgfb1), transforming growth factor beta 2 (bTgfb2), transforming growth factor beta 3 (bTgfb3), growth differentiation factor 5 (bGdf5), bone morphogenetic protein 2 (bBmp2) and bone morphogenetic protein 7 (bBmp7) in ageing cartilage as determined by qPCR. Regression analysis (solid line) with 95% CI (dotted line) depicted. Expression of both bTgfb1 and bGdf5 clearly decreases with advancing age.
Tgfb2 and Tgfb3 was not lowered with advancing age. Next, we measured bGdf5 expression, because reduced expression of this growth factor has been identified as a risk factor for OA [29]. bGdf5 expression significantly ($P = 0.0090, R^2 = 0.10$) decreased $\sim$2-fold with advancing age. Additionally, we measured bBmp2 and bBmp7 expression in view of their importance for cartilage biology. bBmp2 expression was not affected by age, whereas bBmp7 expression was remarkably low (undetected) in most samples, This made it difficult to draw a clear conclusion regarding bBmp7 expression. Taken together our data shows that expression of at least two important growth factors for cartilage homeostasis decreases with advancing age.

**Functional implications of altered receptor expression in ageing cartilage**

In view of the changes we observed in receptor expression, we wanted to investigate the functional implications of these changes. We focused on the TGFβ receptors Acvrl1 (ALK1) and Tgfbr1 (ALK5) because of TGFβ’s importance in cartilage biology. First, we analysed our data set for clues of decreased TGFβ signalling. Previously, we have identified expression of bSmad7 as an indicator of TGFβ signalling in bovine explants [30]. When analysing bSmad7, we observed a clear $\sim$4-fold reduction in expression with advancing age ($P = 0.0089, R^2 = 0.17$) (Fig. 5A). To prove that aged cartilage indeed is less responsive to TGFβ, we stimulated cartilage explants of 22 animals (aged 0.5–11 years old) *ex vivo* with TGFβ1 for 24 h and measured bSmad7 expression. However, TGFβ-induced bSmad7 expression was not significantly different between young and old animals (Fig. 5C). A possible reason for this is that TGFβ1 can induce bSmad7 expression *via* both bTgfbr1 and bAcvrl1 [31,32] and bAcvrl1 expression was unaffected by ageing. Therefore, we subsequently measured bSerpine1 expression, a gene which is induced by TGFβ in a specifically pSmad3-dependent way [33], and thus a good indicator of only bTgfbr1 signalling. Although bSerpine1 expression did not change with age (Fig. 5B), old cartilage clearly responded less to TGFβ1 than young cartilage, because age-dependent bSerpine1 expression in response to TGFβ1 is characterized by a negative regression coefficient ($P = 0.0157$) (Fig. 5D). This negative coefficient reflects a $\sim$3-fold lower response to TGFβ1 of 10 year old cartilage compared to 6 months old cartilage. Finally, we wanted to investigate if Acvrl1 functionality did not decrease with age. Therefore we stimulated explants with BMP9, a high affinity Acvrl1 ligand [34]. BMP9 stimulation induced bId1 expression to a similar extent ($\sim$4-fold) in both young and old animals showing that Acvrl1 (ALK1) function was not diminished (Fig. 5E). Taken together, these results indicate that, in ageing chondrocytes, loss of bTgfbr1 expression indeed has functional consequences for cellular response to TGFβ1.
Ageing affects cartilage on multiple levels, including chondrocyte homeostasis. In this paper, we investigated the impact of ageing on the expression of TGFβ-family signalling components in healthy articular cartilage. With the use of bovine cartilage we were able to separate ageing from OA, two processes that are often concomitant and interfering. We report that ageing decreases Smad2/3 phosphorylating receptors and that this is reflected in a lowered Smad3-dependent TGFβ1 response.

In chondrocyte biology, ageing and (early) OA can have diametrically opposed effects. For example, ageing negatively affects production of essential matrix components like glycosaminoglycans and collagen type 2a1, whereas in early OA expression of both matrix components is markedly upregulated [22, 23]. Another example is that while cell division is as good as absent in (mature) ageing cartilage, proliferation and clonal expansion of chondrocytes are common during OA [35, 36]. These opposite effects of ageing and OA on chondrocyte biology are most likely a reflection of antagonistic regulation of gene expression. Therefore, it is difficult to study the effects of ageing on chondrocyte gene expression in joints with concurrent OA, and ideally both processes should be studied separately. In mice, we found this separation difficult to do, as e.g., in our hands the widely used C57BL/6 strain spontaneously developed OA as young as 8 months old, leaving little room to study ageing separately. However, in the bovine MCP joint we were able to study ageing without concomitant OA.

Ageing affects cartilage on a macroscopic, microscopic and cellular level. We were able to identify many previously defined features of ageing cartilage in our bovine data set, including progressive thinning of the cartilage, loss of superficial zone organization, tidemark duplications and profound loss of $Col2a1$ expression [4, 5, 7, 11, 26, 27]. Therefore using bovine cartilage to study ageing is a valuable tool to replace healthy human cartilage, which is often very difficult to obtain reliably.

Phosphorylated Smad3 (pSmad3) is an essential transcription factor for the maintenance of healthy cartilage. Mice lacking Smad3 show rapid and profound degeneration of their articular cartilage, a process in which chondrocyte hypertrophy plays a major role [17]. Chondrocyte hypertrophy is characterized by elevated production of matrix degrading enzymes like MMP13, and eventually loss of cell viability [37, 38]. Phosphorylated Smad3 effectively inhibits chondrocyte hypertrophy via its interaction with, and subsequent inhibition of, the transcription factor runt-related transcription factor 2 (RUNX2) [16, 39]. RUNX2 is regarded as the master regulator of chondrocyte maturation, as chondrocyte hypertrophy is blocked in cells lacking RUNX2 [40]. We show that, compared to young chondrocytes, aged chondrocytes express less of the Smad2/3 phosphorylating receptors $bAcrv1b$ and $bTgfbr1$. Furthermore, this decreased $bTgfbr1$ expression is coupled to diminished pSmad3-
dependent TGFβ1 signalling (bSerpine1 expression). The direct dependency of TGFβ-induced Serpine1 expression on pSmad3 has been well established [33, 41, 42], but a dependency of Serpine1 on pSmad2 has not clearly been established. Therefore, and because we did not measure pSmad2 levels directly, we cannot conclude from our experiments if pSmad2 signalling was also diminished. However, we do think this is likely [31, 32, 43]. Overall, taken the importance of pSmad3 in cartilage biology into account, our observations suggest that ageing chondrocytes are more prone to become hypertrophic, and give a possible reason for age as the main risk factor of OA.

A diminished response to TGFβ1 with advancing age was suggested by our previous studies showing age-related loss of TGFβ type I and type II receptors in vivo in murine cartilage [13, 20]. Here we actually confirm that a reduced response is indeed the case. Previously, the effects of TGFβ on chondrocytes of different ages have been studied using chondrocytes cultured in monolayer, but this has yielded variable results on a common parameter as proteoglycan production [14, 44, 45]. With increasing age, both increased and decreased proteoglycan production have been described in response to TGFβ [14, 44, 45], highlighting the difficulty of studying cellular response of aged cells in vitro. Because the outcome of TGFβ signalling is very dependent on cellular context, e.g., which phase of the cell cycle cells are in or ECM composition [46], we think that using explants with cells in their native environment is a more suitable approach to study TGFβ signalling in aged cells compared to the use of matrix deprived monolayer chondrocytes. Of note, we have previously also shown that compared to young cartilage, aged bovine cartilage has a diminished capacity to induce Smad2/3 signalling in response to mechanical stimulation [25]. This indicates that the receptor changes we observe also have functional consequences when cells are not directly stimulated with an exogenous ligand.

Although we chose to focus on age-related changes in TGFβ signalling, BMP signalling is also relevant for cartilage homeostasis, for example in regulation of proteoglycan content. Locally produced BMP2 and BMP7 have been shown to be important for maintenance of cartilage GAG content [47, 48]. Both BMP2 and BMP7 use Bmpr2 as type II receptor and share Bmpr1 as type I receptor. Remarkably, we observed profound downregulation of both receptors (∼4-fold in bBmpr1 expression, ∼6-fold bBmpr2) with advancing age, and are the first to report so. Possibly this decrease results in lower BMP2 and BMP7 signalling in elder cartilage, resulting in less proteoglycan maintenance. However, although we did observe age-related downregulation of BMP receptors, we only observed a ∼2 fold drop in aggrecan expression. This indicates that the relative importance of BMP signalling in aggrecan production is maybe limited. Additionally, Bmpr1 is also the receptor used by GDF5. Notably we also observed that GDF5 expression itself decreases with ageing. Reduced GDF5 expression is associated with OA, because a SNP lowering expression of this growth factor was the first genetic risk factor to be identified for OA [29]. Our results indicate that the
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage. GDF5/BMP7/BMPR1 axis is profoundly impacted by ageing, and warrants further study into this signalling route.

However, BMP signalling can also be detrimental for cartilage [19]. Both phosphorylated Smad1 or Smad5 have been shown to enhance RUNX2 function, and can via this way induce chondrocyte hypertrophy [49, 50, 51]. In view of this, it is relevant that we did not observe an age-related decrease in Acvr1 expression or function. Previously we have suggested that a shift in balance between Acvr1 and Tgfbr1 receptors could be a cause of OA, and that Acvr1 is involved in chondrocyte Mmp13 expression [20]. In our samples we also observed this shift, because Acvr1 expression remained stable over time whereas Tgfbr1 expression diminished. Therefore we can confirm this shift as a robust age-related effect in cartilage that occurs across species. In contrast to previous papers, which studied cartilage ageing in mice, we did not observe a loss of Tgfb2, Tgfb3, or Tgfbr2 expression [13, 20]. Possibly, this difference is due to a species-specific effect, or interference of an OA-related effect like inflammation. Additionally, it could imply an additional level of regulation of these factors on protein level, as we did not study this.

Our study does have limitations. First of all, we did not use human material and therefore species specific observations cannot be excluded. However, we observed many changes documented for human cartilage in bovine cartilage [4, 5, 7, 11, 26, 27], indicating that similar processes happen in both species. Secondly, in all cases we predominantly analysed mRNA expression and did not study the translation of this signal into protein. Nonetheless, with the use of mRNA we were able to detect many age-related changes in TGFβ-family signalling that have been confirmed on protein level in other species, like the change in Acvr1/Tgfbr1 ratio [20]. Finally, we macroscopically selected healthy cartilage. This doesn’t fully exclude microscopic changes, thus making it possible that very early OA samples entered our sample pool.

In conclusion, ageing bovine cartilage displays many characteristics of ageing human cartilage, making it a valuable tool to supplement rare human material. The separation of ageing and OA allowed us to study ageing independently of OA, which revealed that expression of TGFβ and BMP receptors decreases with advancing age. The decrease in expression of the TGFβ-receptor Tgfbr1 resulted in a lowered phosphorylated Smad3-dependent response of older cartilage to TGFβ1. Due to the importance of phosphorylated Smad3 in cartilage biology, reduction of Smad3 activation during ageing could explain the close relation between ageing and OA development, highlighting ageing as the main risk factor for the development of this disease.
Supplementary data

Supplementary Figure 1. Distribution of the in total 42 animals over different age groups. On the lower right the standardized sample spot is depicted in the bovine MCP joint seen from the ventral towards the dorsal side. AC = articular cartilage, IN = intratrochlear notch, T = tendon. Female cows are already fertile at 1 years old (adolescent), and fully skeletally mature at 2-3 years old (adult). Cows keep gaining weight up to an age of 5 years old (middle age).

Supplementary Figure 2. Correlation of reference genes showing their interrelation. RPS = ribosomal protein S14, RPL = ribosomal protein L22 and GusB = glucuronidase, beta.
Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.

### Supplementary Figure 3.

Scoring system used to quantify Acvrl1 (ALK1) and Tgfbr1 (ALK5) expression in bovine cartilage.

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<td><img src="image8.png" alt="Image" /></td>
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References

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

19. van der Kraan, P.M., Blaney Davidson, E.N., and van den Berg, W.B. Bone morphogenetic proteins and articular cartilage: to serve and protect or a wolf in sheep clothing’s?. Osteoarthritis Cartilage. 2010; 18: 735–741


Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage.
4.

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

Madej W, van Caam A, Davidson EN, Hannink G, Buma P, van der Kraan PM.

Osteoarthritis Cartilage. 2016 Jan;24(1):146-57
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 (IH) 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 (Col2a1) and fibronectin (Fn1) 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 (OA).
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-Tgfb1 [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 (ECM) as well as the chondrocytes. In articular cartilage the size, structure and sulfation characteristics of aggrecan in the 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 TGFβ [18,19].

Particularly TGFβ is an essential anabolic growth factor in articular cartilage as it prevents deleterious chondrocyte terminal differentiation [20]. Importantly, TGFβ can signal via two different type I receptors, i.e. ALK5 and ALK1, in chondrocytes, being able to induce opposing effects in cartilage. There is evidence that TGFβ action in restriction of cartilage terminal differentiation is limited to TGFβ signaling via the type I receptor-TGFBR1 (ALK-5), 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 TGFBR1 (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.
Materials and methods

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 Tables 1 and 2). Joints were obtained from the local abattoir within 3 h post mortem. Full cartilage thickness (young-986 ± 34 μm, aged-725 ± 42 μm thick) explants (without sub-chondral bone) were isolated with a 4 mm biopsy punch (Kai-medical, Japan). After isolation, all specimens were equilibrated for 48 h in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco®, UK) (1 ml medium per one 4 mm Ø explant) 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) in standard culture conditions (37°C, 5% CO₂ and 95% humidity). No serum was added to the medium.

Histological analysis
Full thickness osteochondral biopsies were isolated from MCP, with a diamond coated blade saw. Biopsies were fixed overnight at 4°C in 10% phosphate buffered formalin (pH 7) and decalcified for 1 week in 10% formic acid at room temperature. Specimens were dehydrated with a tissue processing apparatus (Pathos, Milestone Medical Inc.). After embedding in paraffin, sections of 7 μm were cut, than stained with Mayer’s hematoxylin and visualized under a standard light microscope.

Dynamic mechanical compression of cartilage explants
Explant from stimulation groups were subjected to force controlled, sinusoidal, unconfined, dynamic mechanical compression with 3 or 12 MPa for 30 min, with 1 Hz, exactly like described before [6]. Full detailed description is included in Supplementary materials.

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.
Expression of TGFβ-family signalling components in ageing cartilage: age-related loss of TGFβ and BMP receptors.

**Total mRNA isolation and quantitative RT-PCR (Q-PCR)**

mRNA isolation and Q-PCR were performed exactly like described before [6]. Full detailed description is included in Supplementary materials. Primers used are included in Table I.

<table>
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<th>Gene symbol</th>
<th>Full gene name</th>
<th>Ref seq</th>
<th>P (bp)</th>
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**Table I.** Primers list. Only primers with efficiency between 93% and 105% were used.

**Immunohistochemical (IH) analysis**

At 2 h after compression, samples were fixed overnight 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].

**Computational scoring of Smad2P IH**

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).

**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 Tables 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).

**Results**

**Age-related changes in articular cartilage structure**
Histological comparison of articular cartilage of 2 year 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 sub-chondral 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).
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 E, respectively) when compared to corresponding unloaded controls (Fig. 2A and D, 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. 2J). Analysis of strain profiles showed that young cartilage was able to deform significantly more, starting from 1550 second ($P = 0.04$) compared to aged cartilage when subjected to 3 MPa mechanical compression (Fig. 2J). 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. 2J).
Fig. 2. Effect of dynamic mechanical compression on young and old articular cartilage. (A–F) Representative cross sections of articular cartilage stained with Safranin O and Fast Green. Intact articular cartilage specimens were dynamically compressed with 3 MPa or 12 MPa. Compression was performed as a sine wave with frequency of 1 Hz for 30 min (1800 cycles). (A, B, C) 1 year old cartilage: (A) unloaded, (B) 3 MPa compressed and (C) 12 MPa compressed cartilage, (D, E, F) 10 years old cartilage: (D) Unloaded, (E) 3 MPa compressed and (F) 12 MPa compressed cartilage. (G,H,I) Cross section of 10 years old cartilage stained with Picro Sirius Red and examined under the polarized light: (G) Unloaded, (H) 3 MPa compressed and (I) 12 MPa compressed cartilage. Arrows indicate the cartilage surface discontinuity. (J) Articular cartilage strain during dynamic mechanical compression with 3 or 12 MPa. Blue lines represent averaged strain of cartilage isolated from 1, 2 and 3 years old individuals. Red lines represent averaged strain of cartilage isolated from 6 and 10 years old individuals. Grey shadows represent the curve’s 95% CI.
Ageing affects the influence of dynamic mechanical compression on the expression of articular cartilage ECM 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 h after compression a significantly diverse (3.9-fold, 2^{2.0Ct}, P = 0.048) regulation of bCol2a1 expression with up-regulation in young and down-regulation in aged in cartilage by 12 MPa compression was found (Fig. 3B). Analysis of the bFn1 expression at 6 h 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^{1.1Ct}, P = 0.001), with up-regulation in young cartilage and down-regulation in aged cartilage. Remarkably, at the 6 h 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^{1.8Ct}, P = 0.002 for 3 MPa and 2.3-fold, 2^{1.2Ct}, P = 0.025 for 12 MPa compressed cartilage) (Fig. 3D).

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.

At 2 h time point, a potent and significant (P = 0.001 for all cases) up-regulation of bTgfb1 expression was measured in both age and stimulation groups (Fig. 4A). At 6 h 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 h 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^{5.6Ct}, P < 0.0001) (Fig. 4B). At 6 h 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^{1.3Ct}, P < 0.0001 for 3 MPa and 48.1-fold, 2^{5.6Ct}, P < 0.0001 for 12 MPa compressed group) was identified in cartilage of different age. At 2 h 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^{1.6Ct}, P = 0.045) and 12 MPa (4.2-fold, 2^{1.1Ct}, P = 0.011) (Fig. 4C).
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 bHspg2 (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 bHspg2 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. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. 
Expression of TGFB-family signalling components in ageing cartilage: age-related loss of TGFB and BMP receptors.

Fig. 4. Influence of dynamic mechanical compression on the expression of key tissue physiology mediators in cartilage of different age. The influence of 3 and 12 MPa dynamic mechanical compression on relative expression of (A) bTgfb1, (B) bCtgf and (C) bBmp2 in young and aged articular cartilage. Mechanical compression potently induced bTgfb1 expression in young and aged cartilage (A). However, mechanical compression had significantly different effect on bCtgf and bBmp2 expression regulation in cartilage of different age (B and C). 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.
The up-regulation of $bbBmp2$ expression in young cartilage was still present at 6 h 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 $bbBmp2$ was observed. Thus, significantly different regulation of $bbBmp2$ in cartilage of different age was present in 3 MPa (2.2-fold, $2^{1.3Ct}$, $P = 0.048$) and in 12 MPa group (5.2-fold, $2^{2.4Ct}$, $P < 0.0001$).

**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. 5Ab, e, h) when compared to unloaded control (Fig. 5Aa, 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).

**Ageing reduces mechanically-induced activation of Smad3P signaling reporter genes**

To investigate if the reduction of mechanically-induced TGFβ 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 TGFB1 (ALK-5) 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, $2^{1.3Ct}$, $P = 0.002$) $bTgfbr1$ expression levels in aged cartilage than in young cartilage (Fig. 6A).
Expression of TGFβ-family signalling components in ageing cartilage: age-related loss of TGFβ and BMP receptors.

**Fig. 5.** Influence of dynamic mechanical compression on Smad2 activation in cartilage of different age. Clear induction of Smad2P staining was observed in young cartilage compressed with 3 and 12 MPa when compared to unloaded controls (Ab, e, h to Aa, d, g), (Ac, f, i to Aa, d, g). Clearly lower level of induction of Smad2P by compression was noticed in old cartilage compressed with 3 MPa and 12 MPa when compared to young cartilage (Ak, n to Ab, e, h) and (Al, o to Ac, f, i). Scoring of IH staining showed significantly lower load-induced Smad2P staining in aged 3 MPa and 12 MPa compressed cartilage when compared to young cartilage compressed with the same pressure (B). Smad2P staining in young compressed cartilage was most likely restricted to the cell nuclei (Ca, b) whereas in old compressed cartilage staining was more predominantly present in the cell cytoplasm (Cc, d). Scoring of nuclear IH staining showed significant reduction in Smad2P nuclear staining in aged cartilage (D). Arrows indicate the Smad2P staining detected by threshold used for computational scoring of IH. Young cartilage: 1 year old; aged cartilage: 10 years old.
At 2 h 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, 2^{5.9Ct}, 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, 2^{2.6Ct}, 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, 2^{1.9Ct}, P = 0.015) (Fig. 6B). At 6 h 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). However, only in 12 MPa compressed cartilage, a significantly different levels of bSerpine1 up-regulation levels between young and aged cartilage were observed (5.9-fold, 2^{2.6Ct}, 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 h after both levels of mechanical compression (22-fold 2^{4.5Ct}, P < 0.0001 for 3 MPa and 25-fold 2^{4.7Ct}, 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 vs aged cartilage (17-fold, 2^{4.1Ct}, P < 0.0001 for 3 MPa and 10-fold, 2^{3.3Ct}, P < 0.0001 for 12 MPa stimulation group). At 6 h time point the age-related differences were not 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).
Fig. 6. Basal expression of Smad2/3 activating receptor (*Tgfbr1*) and influence of dynamic mechanical compression on relative expression of Smad3P reporter genes in cartilage of different age. (A) Basal expression of *bTgfbr1* in cartilage of different age. The influence of 3 and 12 MPa dynamic mechanical compression on relative expression of (B) *bSerpine1*, (C) *bJunB* and (D) *Smad7* in young and aged articular cartilage. Significantly higher *bSerpine1* up-regulations were observed in young compressed cartilage than in aged (B). Moreover, only in young compressed cartilage the up-regulation of *bJunB* was detected (C). In 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.
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 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 TGFβ1 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 (ALK4 and ALK7) 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 TGFβ1 growth factor and subsequent Smad2/3P signaling activation. Moreover, a reduced synthesis of TGFβ 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 \textit{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]. Moreover, according to the Encyclopedia of DNA Elements data (ENCODE, http://genome.ucsc.edu/, release date of the genome assembly-20.01.2015), two CpG islands are present in the \textit{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 \textit{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 \textit{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 \textit{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 tissue to mechanical signals. Age-related alteration in cartilage
mechanotransduction can point to mechanisms of age-related articular cartilage diseases like
OA.
Supplementary Materials and methods

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Supplementary Table 1. Experimental setup for the gene expression analysis

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

Dynamic mechanical compression of articular cartilage explants.

After 48 hours equilibration in standard culture conditions, cartilage explants were randomly distributed to two stimulation groups (3 MPa - physiological or 12 MPa- excessive compression) and corresponding unloaded control groups. Explant from stimulation groups were subjected to sinusoidal, unconfined, dynamic mechanical compression using BOSE® ElectroForce® BioDynamic™ bioreactor (5160 BioDynamic System) equipped with a 50 lbf load-cell (BOSE, Bose Corp. ElectroForce Systems Group, MN, USA) exactly like described before [6], however in 60 ml of (DMEM/F12) medium (for details see the Supplementary Fig.1). The compression duration was 30 min (1800 cycles) with a frequency 1Hz and it was performed under standard culture conditions (37°C, 5% CO₂ and 95% humidity). After the compression all the samples were placed back into the culture wells and back to the culture incubator for 2 or 6 h. At required time intervals samples were flash frozen in liquid nitrogen and stored at -80°C.
**Total mRNA isolation and quantitative RT-PCR (Q-PCR).**

Deep frozen articular cartilage samples were homogenized with a micro-dismembrator (B. Braun Biotech International, Melsungen, Germany) at 1500 RPM for 1 minute. The homogenate of one 4 mm Ø cartilage explant was dissolved in 300 µl of RLT buffer containing 1 % (v/v) 2-mercaptoethanol. Total RNA was isolated using an RNeasy Mini Kit (Qiagen Inc., Valenzia, CA, USA) according to the manufacturer’s protocol. Isolated mRNA was transcribed into cDNA using M-MLV reverse transcriptase. Obtained cDNA was used in quantitative PCR (qPCR) analysis, carried out with the StepOnePlus Real-Time PCR System (Applied Biosystems, Germany) using SYBR-green according to the manufacturer’s protocol. Validated primers were used and are listed in Table1. Thereafter, all Ct values for the genes of interest were first corrected for Ct values of *glyceraldehyde 3-phosphate dehydrogenase* (bovine Gapdh) to obtain ΔCt values. Finally, all the ΔCt values of the experimental groups (compressed cartilage) were corrected for the values of appropriate controls (unloaded cartilage) to obtain ΔΔCt values.

**Supplementary Fig. 1.** Schematic representation of Bose 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 on 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 consist of N = 18 measured values (6 different areas of the section measured in 3 different individuals).
Expression of TGFβ-family signalling components in ageing cartilage: age-related loss of TGFβ and BMP receptors.

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.
References


Expression of TGFβ-family signalling components in ageing cartilage: age-related loss of TGFβ and BMP receptors.

The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1


Osteoarthritis Cartilage. 2015 Jun;23(6):985-95
Abstract

Objective
In osteoarthritic cartilage, expression of the receptor ALK1 correlates with markers of deleterious chondrocyte hypertrophy. Recently, bone morphogenetic protein 9 (BMP9) was identified as a high affinity ligand for ALK1. Therefore, we studied if BMP9 signaling results in expression of hypertrophy markers in chondrocytes. Furthermore, because transforming growth factorβ1 (TGFβ1) is a well known anti-hypertrophic factor, the interaction between BMP9 and TGFβ1 signaling was also studied.

Design
Primary chondrocytes were isolated from bovine cartilage and stimulated with BMP9 and/or TGFβ1 to measure intracellular signaling via pSmads with the use of Western blot. Expression of Smad-responsive genes or hypertrophy-marker genes was measured using qPCR. To confirm observations on TGFβ/Smad3 responsive genes, a Smad3-dependent CAGA12-luc transcriptional reporter assay was performed in the chondrocyte G6 cell line.

Results
In primary chondrocytes, BMP9 potently induced phosphorylation of Smad1/5 and Smad2 to a lesser extent. BMP9-induced Smad1/5 phosphorylation was rapidly (2 h) reflected in gene expression, whereas Smad2 phosphorylation was not. Remarkably, BMP9 and TGFβ1 dose-dependently synergized on Smad2 phosphorylation, and showed an additive effect on expression of Smad3-dependent genes like bSerpine1 after 24 h. The activation of the TGFβ/Smad3 signaling cascade was confirmed using the CAGA12-luc transcriptional reporter. BMP9 selectively induced bAlpl and bColX expression, which are considered early markers of cellular hypertrophy, but this was potently antagonized by addition of a low dose of TGFβ1.

Conclusions
This study shows that in vitro in chondrocytes, BMP9 potently induces pSmad1/5 and a chondrocyte hypertrophy-like state, which is potently blocked by TGFβ1. This observation underlines the importance of TGFβ1 in maintenance of chondrocyte phenotype.
Introduction

Osteoarthritis (OA) is the most common joint disease, characterized by degeneration of articular cartilage. This loss of articular cartilage is the result of an imbalance between matrix synthesis and degradation. Chondrocytes play a crucial role in balancing both processes with their capacity to produce both matrix molecules, like collagens, and catabolic proteolytic enzymes such as Matrix Metalloproteases (MMPs). During OA, a subpopulation of chondrocytes differentiates towards a hypertrophy-like phenotype, characterized by increased production of catabolic enzymes such as MMP13 [1–3]. As a consequence, these chondrocytes actively break down their surrounding matrix, resulting in pathology.

Signaling by members of the Transforming Growth Factor β (TGFβ)-superfamily, which include TGFβs, Activins and Bone morphogenetic proteins (BMPs), regulate matrix synthesis, matrix degradation and cellular differentiation in chondrocytes [4]. The TGFβ-superfamily members signal via Activin receptor-Like Kinase (ALK) receptors [5]. There are seven ALKs, which phosphorylate specific sets of receptor-Smads: ALK1/2/3 and 6 phosphorylate Smad1/5 (pSmad1/5), whereas ALK4/5 and 7 phosphorylate Smad2/3 (pSmad2/3). Both pSmad2 and pSmad3, but predominantly pSmad3, have been shown to be essential for chondrogenesis and maintenance of chondrocyte phenotype by potently blocking hypertrophy [6–8], whereas pSmad1 and pSmad5 are required for chondrocyte hypertrophy and terminal differentiation [9–12].

In human osteoarthritic cartilage, ALK1 expression is positively correlated with expression of MMP13, the main cartilage degrading enzyme, and of COL10A1, a marker of chondrocyte terminal differentiation [13]. Recently, BMP9 was identified as a high affinity ALK1 ligand [14,15]. BMP9 is produced in the liver and is present in human, bovine and murine serum in high amounts of up to 10 ng/ml [16–18], responsible for over 60% of all BMP activity found in serum [15]. In endothelial cells, BMP9 potently induces pSmad1/5 and downstream signaling [14,15]. Furthermore, in mesenchymal stem cells, BMP9 is a potent inducer of chondrocyte hypertrophy, and has been shown to induce osteogenic differentiation [19,20]. Combined, these observations suggest a pro-hypertrophic effect of BMP9 in chondrocytes. However, it has been reported that BMP9 is also able to induce pSmad2 and subsequent downstream effects in endothelial cells [21,22]. Furthermore, a synergistic action of BMP9 with TGFβ has been described in endothelial cells, in which BMP9 enhances TGFβ induced Smad2 phosphorylation and Smad3-mediated transcriptional responses [23].

In view of the opposing functions of pSmad1/5 and pSmad2/3 in chondrocyte differentiation, we wondered what the effect of BMP9 signaling on chondrocytes would be. Therefore, the aim of this study was to investigate BMP9 induced Smad phosphorylation and downstream gene expression, and to unravel if BMP9 signaling results in induction of a hypertrophy-like phenotype in chondrocytes. Furthermore, because of the reported synergy with TGFβ, we also studied the interaction of BMP9 with TGFβ.
We show that in primary chondrocytes, BMP9 indeed induces both phosphorylation of Smad1/5 and Smad2. Additionally, we demonstrate that BMP9 can synergize with TGFβ on Smad2 phosphorylation and expression of certain Smad3-dependent target genes. Furthermore, we report that BMP9 induces a hypertrophy-like state in chondrocyte as characterized by Alkaline phosphatase (Alpl) and Collagen 10a1 (Col10a1) expression, which is potently counteracted by TGFβ1. Our results underline the idea that pSmad1/5 and pSmad2/3 have opposing roles in regulation of chondrocyte differentiation.

**Materials and methods**

**Chondrocyte culture**

Primary bovine chondrocytes were isolated from the metacarpophalangeal joint of cows post mortem. Cartilage slices were incubated overnight in 1.5 mg/ml Collagenase B (Roche Diagnostics, Germany) in DMEM/F12 (Gibco, UK) supplied with 50 mg/L gentamicin (Centrafarm Services, the Netherlands) and 100 mg/L pyruvate (Gibco UK) in a ratio of 1 g cartilage per 10 ml medium. Subsequently, chondrocyte suspension was spun down at 1500 rpm for 10 min, washed three times using saline (Aguettant, France) and resuspended in DMEM/F12 containing 10% fetal calf serum (FCS) (Thermo Scientific UK). Chondrocytes were seeded at a density of $1 \times 10^5$ cells per cm$^2$ in 6 wells plates (Greiner Bio-one International, the Netherlands) for protein studies, or in 24 wells plates (Bio-one International, the Netherlands) for mRNA experiments. Cells were cultured for 1 week at 37°C and 5% CO$_2$ before start of experiments. Subsequently, cells were serum starved and stimulated with recombinant hBMP9, hBMP2, hBMP7 (R&D systems, USA), or hTGFβ$_1$ (BioLegend, the Netherlands) or a combination for indicated time periods and dosages. Each experiment was conducted at least three times, each time with cells of a different animal. In each experiment, conditions were tested in duplicate.

**Detection of pSmads using SDS-PAGE and western blot**

Cells were placed on ice and washed twice using ice cold saline. Subsequently, cells were lysed using lysis buffer (Cell signaling, USA) containing protease inhibitor cocktail (complete, Roche Diagnostics, Germany). Cell lysates were sonicated on ice for 10 cycles, of 30 s sonication and 30 s rest, using a Bioruptor (Diagenode, USA). Hereafter, samples were centrifugated at 4°C at 13,500 rpm for 15 min and pellets were discarded. With a BCA-assay (Thermo Scientific, USA), protein concentration was measured. An equal amount of protein was loaded on a 7.5% Bisacrylamide gel, for SDS-PAGE. Next, proteins were transferred to a nitrocellulose membrane using wet transfer (Towbin buffer, 2.5 h 275 mA at 4°C). After overnight incubation at 4°C with 1:1000 polyclonal Rabbit anti P-Smad1/5 (S463/465)/Smad8 (S426/428) (Cell signaling, USA) or P-Smad2 (S465/467) (Cell signaling, USA), membranes were incubated with
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

1:1500 polyclonal Goat anti Rabbit labeled with Horseradish peroxidase (DAKO, Belgium) for 1 h. Hereafter, enhanced chemiluminescence (ECL) using ECL plus kit (GE Healthcare, UK) was used to visualize the proteins. As loading control β-Actin rabbit mAb (13E5) (Cell signaling) or Gapdh mouse mAb (1G5) (Sigma Aldrich, Germany) was used, both 1:1500.

**Detection of gene expression**

RNA was isolated using TRI-reagent (Sigma–Aldrich, Germany) by following manufacturer’s protocol. After this procedure, RNA was dissolved in ultra pure water and RNA concentration was measured using a NanoDrop photospectrometer (Thermo Scientific, USA). Subsequently, 1 μg of RNA dissolved in 8 μl water was treated with 1 μl DNAse (Life Technologies, USA) for 10 min at room temperature, and incubated at 65°C with 1 μl 25 mM EDTA (Life Technologies, USA) for 10 min. To perform reverse transcriptase (RT) reaction; 1.9 μl ultra pure water, 2.4 μl 10 x DNAse buffer, 2.0 μl 0.1 M DTT, 0.8 μl 25 mM dNTP, 0.4 μg oligo dT primer, 1 μl 200 U/μl M-MLV Reverse transcriptase (all Life Technologies, USA) and 0.5 μl 40 U/μl RNAsin (Promega, the Netherlands) was added, and samples were incubated for 5 min at 25°C, 60 min at 39°C, and 5 min at 95°C using a thermo cycler. Gene expression was measured using 0.5 μM of validated cDNA-specific primers (see Table I) (Biolegio, the Netherlands) in a quantitative real time polymerase chain reaction (qPCR) using SYBR green master mix (Applied Biosystems). The following protocol was used: after 10 min at 95°C, 40 cycles of 15 s 95°C and 1 min 60°C each were run. For calculations of the −ΔCt, two reference genes were used: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S14 (RPS14).

**CAGA<sub>12</sub>-luciferase transcriptional reporter assay**

The CAGA<sub>12</sub>-luciferase reporter construct produces luciferase in response to Smad3-Smad4 activation [24]. For this assay a human chondrocyte derived cell line (G6) was used. Cells were transduced with an adenovirus containing the CAGA<sub>12</sub>-luciferase construct at a multiplicity of infection of 10. Two days after transduction, cells were serum starved for 8 h, and hereafter stimulated with the reported doses of rhTGFβ1 and rhBMP9 for 16 h. Subsequently, cells were lysed and luciferase activity was measured after adding Bright glo™ (Promega) on a luminometer according to manufacturer’s protocol.
Table I. Primer sequences used in this study

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Statistics

All quantitative data are expressed as a mean of multiple repeats ± SD. For every analysis data was checked for normality using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) with Tukey’s multiple comparison post test was used to determine the significance. The statistical analyses were performed with the SPSS 20.0 (IBM, Chicago, USA) statistical software package.

Results

In primary chondrocytes, BMP9 potently induces pSmad1/5 phosphorylation, and Smad2 phosphorylation to a lesser extent

First, expression of the two receptors BMP9 can use; ALK1 and ALK2 [15,25] was measured using qPCR, and detected at 0.11 and 0.23 fold of reference gene value respectively. Subsequently, chondrocytes were stimulated with a dose range of rhBMP9 to investigate its potency in inducing Smad phosphorylation. Concentrations reported [16–18] as physiological (50 pg, 250 pg, 1 ng, and 2.5 ng/ml) as well as excessive (25 ng/ml) were tested. In this range, rhBMP9 dose-dependently induced pSmad1/5 after 1 h, starting from a dose of 50 pg/ml (Fig. 1A). Furthermore, from a dose of 1 ng/ml or higher, BMP9 also induced pSmad2, but not in all donors. Both BMP9-induced Smad1/5 and Smad2 phosphorylation lasted up to at least 2 h after stimulation. Therefore we conclude that in primary chondrocytes, BMP9 can induce phosphorylation of both pSmad1/5 and pSmad2, but that the induction of pSmad2 requires a higher dose of BMP9.
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

Fig. 1. BMP9 induces Smad phosphorylation and downstream gene expression in primary bovine chondrocytes. (A) Primary bovine chondrocytes were stimulated with a dose range of rhBMP9 for 1 and 2 h, and induction of Smad1/5 and Smad2 phosphorylation was analyzed on Western blot using specific antibodies. pSmad1/5 is potently dose-dependently induced by BMP9 from 50 pg/ml, whereas pSmad2 is induced from a dose of 1 ng/ml and higher.
(B) Chondrocytes of three donors were stimulated in duplo with 0.25, 1 and 2.5 ng/ml BMP9 for 2, 6 and 24 h, to study BMP9-induced gene expression downstream pSmad1/5 and pSmad2/3 using qPCR. BMP9 significantly induced expression of bId1 at all time points, but not of bSerpine1. For Western blot, a donor is shown in which BMP9-stimulation resulted in both Smad1/5 and Smad2 phosphorylation. For qPCR data, average ± s.e.m. was plotted, with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey’s post-hoc test (* = \( P < 0.05 \), ** = \( P < 0.01 \) and *** = \( P < 0.001 \)).

**Physiological doses of BMP9 induce expression of bld1, a Smad1/5-dependent gene, but not of Smad3-dependent genes**

To investigate if the observed Smad phosphorylation results in gene transcription, mRNA expression of Smad-dependent genes was measured 2, 6 and 24 h after stimulation with rhBMP9 using qPCR (Fig. 1B). The effect of three doses of BMP9 (0.25, 1 and 2.5 ng/ml) with varying induction of Smad phosphorylation, was measured. Expression of bld1, marker of pSmad1/5 signaling, was profoundly upregulated \( \sim 32 \) fold \( (\sim 5 \Delta C_t) \) at all time points compared to unstimulated samples, but with little difference between dosage. bSerpine1, a classical Smad3-dependent gene [24,26], was measured to study if BMP9 induced pSmad2 was reflected in gene expression, because in adults not many Smad2-dependent genes have been identified [26,27], and pSmad2 and pSmad3 follow a very similar expression pattern, even when induced TGFβ independently [28,29]. In contrast to bld1, expression of bSerpine1 was not induced by BMP9 at any time point. Two other Smad3-dependent genes; bTgfb1 [26] and bAlk5 [30] were also not affected by BMP9. Expression of bSmad7 and bJunb, two genes that have been described as responsive to both pSmad1/5 and pSmad2/3 [31,32] were upregulated by BMP9, but remarkably only at 1 ng/ml or higher. In conclusion, BMP9 potently induced expression of Smad1/5-dependent genes, but not Smad3 specific genes.

**BMP9 synergizes with TGFβ1 on induction of Smad2 phosphorylation**

Because previously a synergistic effect of BMP9 and TGFβ1 on Smad2/3 phosphorylation has been reported [23], we investigated if this synergy also occurs in chondrocytes. To investigate a possible dose-dependency, two doses of rhTGFβ1, 0.1 and 1 ng/ml, and three doses of BMP9, 0.5, 5 and, based on the previously mentioned study [23], 50 ng/ml were used (Fig. 2A). As expected, stimulation of chondrocytes with TGFβ1 led to phosphorylation of Smad2 and Smad1/5. Furthermore, BMP9 strongly induced pSmad1/5 but not pSmad2 in this donor. Most remarkably, BMP9 dose-dependently enhanced TGFβ1-induced Smad2 phosphorylation of both TGFβ1 doses. In addition, inhibition of BMP9-induced pSmad1/5 was observed in the presence of 0.1 ng/ml TGFβ1, but not in the presence of 1 ng/ml TGFβ1. These data suggest that TGFβ1 and BMP9 synergize on the induction of pSmad2 in chondrocytes.
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

**Fig. 1.** Co-stimulation enhanced luciferase production significantly compared to TGFβ1 stimulation alone. For Western blot, a representative donor is shown. For qPCR data, average ± s.e.m. was plotted with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey’s post-hoc test (* = P < 0.05, ** = P < 0.01 and *** = P < 0.001).

**Fig. 2.** BMP9 synergizes with TGFβ1 on pSmad2 induction resulting in enhanced expression of pSmad2/3 responsive genes. (A) Chondrocytes were treated for 1 h with either 0.1 or 1 ng/ml of rhTGFβ1, 0.5, 5 or 50 ng/ml of rhBMP9 or a combination of both growth factors and subsequently pSmad2 and pSmad1/5 were visualized on Western blot using specific antibodies. An inhibitory effect of 0.1 ng/ml on BMP9-induced pSmad1/5 was observed. Furthermore, a synergistic effect of BMP9 on TGFβ1 induced pSmad2 was observed, as signal intensity was increased for co-stimulated samples compared to samples only stimulated with TGFβ1. (B) and (C) Co-stimulation of chondrocytes with BMP9 and TGFβ1 was also investigated on gene expression of Smad-dependent genes 2 h (B) or 24 h (C) after stimulation, by stimulating four donors in duplo. At 2 h after stimulation, no effect on bSerpine1 expression could be detected. bSmad7 and bJunB expression was upregulated more strongly in samples treated with both growth factors compared to single treatment, however not in a synergistic manner. At 24 h after stimulation, combination treatment induced more bSerpine1, bTgfβ1 and bSmad7 expression compared to single treatment. (D) To study if co stimulation of chondrocytes with BMP9 and TGFβ1 results in enhanced Smad responsive biologic activity, the chondrocyte G6 cell line was transduced with the pSmad3-responsive CAGA<sub>3</sub>-luc construct, and treated with either 1 ng/ml TGFβ1, 5 or 50 ng/ml BMP9 and a combination of both for 16 h. Both growth factors synergized, as co-stimulation enhanced luciferase production significantly compared to TGFβ1 stimulation alone. For Western blot, a representative donor is shown. For qPCR data, average ± s.e.m. was plotted with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey’s post-hoc test (* = P < 0.05, ** = P < 0.01 and *** = P < 0.001).
C

**Fig. 2. continued**
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

Co-stimulation of chondrocytes with BMP9 and TGFβ1 results in an additive effect on expression of Smad3-dependent genes

To investigate the observed synergy between BMP9 and TGFβ1 further downstream, mRNA levels of Smad3-dependent genes were measured after 2 and 24 h. A low dose of TGFβ1, 0.1 ng/ml, and high dose of BMP9, 50 ng/ml were used, because these two conditions showed profound synergy on pSmad2 in the previous experiment. Two hour after stimulation, both TGFβ1 and BMP9 induced expression of \( bId1 \), \( \sim 2.8\text{-fold (} \sim 1.5 \Delta C_t \) and \( \sim 16\text{-fold (} \sim 4 \Delta C_t \) respectively, but no additive, synergistic of inhibitory effect, was observed between both growth factors (Fig. 2B). Expression of the Smad3-dependent genes \( bSerpine1 \), \( bTgfb1 \), \( bAlk5 \) was not yet induced by either growth factor alone or in combination. Single stimulation with TGFβ1 or BMP9 both induced bSmad7 expression \( \sim 2.5\text{-fold (} \sim 1.3 \Delta C_t \) and co-stimulation increased the expression \( \sim 6\text{-fold (} \sim 2.5 \Delta C_t \). Furthermore, \( bJunb \) levels were only significantly upregulated \( \sim 4\text{-fold (} \sim 2 \Delta C_t \) after co-stimulation with TGFβ1 and BMP9.

Twenty-four hour after stimulation, \( bId1 \) expression was still upregulated \( \sim 2.8\text{-fold (} \sim 1.5 \Delta C_t \) by BMP9 but no longer by TGFβ1. Again, no additive, synergistic of inhibitory effect was observed on this pSmad1/5 dependent gene (Fig. 2C). In contrast to the 2 h timepoint, Smad3-dependent genes were upregulated after 24 h. Only co-stimulation with TGFβ1 and BMP9 significantly upregulated \( bSerpine1 \) expression 2-fold \( \sim 1 \Delta C_t \), suggesting enhanced pSmad2/3 signaling. Enhanced pSmad2/3 signaling is further indicated by \( bTgfb1 \) expression levels, as co-stimulation induced significantly higher expression of this gene compared to single stimulations; \( \sim 3\text{-fold (} \sim 1.6 \Delta C_t \) vs \( \sim 2\text{-fold (} \sim 1 \Delta C_t \) respectively. Similar to 2 h stimulation, an additive effect of TGFβ1 and BMP9 was observed on \( bSmad7 \); both TGFβ1 and BMP9 induced expression \( \sim 2.5\text{-fold (} \sim 1.3 \Delta C_t \) but co-stimulation induced expression \( 4.6\text{-fold (} \sim 2.2 \Delta C_t \). Therefore, these data show that co-stimulation with TGFβ1 and BMP9 results in an additive effect on pSmad2/3-responsive gene expression, but not on pSmad1/5-responsive genes.

Synergy between BMP9 and TGFβ1 on CAGA\(_{12}\)-luc activity

To further confirm the synergy between BMP9 and TGFβ1 on Smad2/3 phosphorylation, we made use of a Smad3/Smad4-reporter construct: CAGA\(_{12}\)-luc. Due to low transfection rates we were not able to use primary cells and used the human chondrocyte-like G6 cell line. Stimulation with 1 ng/ml TGFβ1 strongly induced luciferase activity \( \sim 7\text{-fold, whereas BMP9 in doses of 5 and 50 ng/ml did not (Fig. 2D). Although co-stimulation of TGFβ1 with 5 ng/ml BMP9 only shows a trend towards enhanced pSmad3 signaling, co-stimulation with 50 ng/ml BMP9 strikingly and significantly enhanced luciferase activity up to \( \sim 10\text{-fold, displaying clear synergy between TGFβ1 and BMP9, and therefore this dose was used in our next experiments.}}


A high dose of BMP2 and BMP7 does not synergize with TGFβ1 on pSmad2 phosphorylation

Now that the synergy between BMP9 and TGFβ1 on pSmad2/3 was confirmed in chondrocytes, we investigated if this is a common BMP characteristic. We compared BMP9 to BMP2 and BMP7 due to their importance in chondrocyte biology [33], and their use of the same type II receptors but different type I receptors as BMP9 [15,34]. Because the synergy between BMP9 and TGFβ1 is best observed at a high dose of BMP9, we chose to use a high dose (250 ng/ml) of BMP2 and BMP7 as well. Both BMPs induced pSmad1/5 comparable to BMP9 (Fig. 3), but in contrast to BMP9, both BMPs lacked a synergistic interaction with TGFβ1 on pSmad2 phosphorylation. This indicates that the synergy with TGFβ1 on pSmad2 is a unique characteristic of BMP9.

![Smad1/5p and Gapdh Western blot](image)

**Fig. 3.** BMP2 and BMP7 do not synergize with TGFβ1 on pSmad2. Chondrocytes were treated for 1 h with 250 ng/ml of BMP2 or 250 ng/ml BMP7, 50 ng/ml BMP9 and 0.1 ng/ml TGFβ, and pSmad1/5 and pSmad2 induction was analyzed on Western blot using specific antibodies. Although both BMPs induced similar pSmad1/5 compared to BMP9, BMP2 and BMP7, unlike BMP9, did not synergize with TGFβ1 on induction of Smad2 phosphorylation. This Western blot depicts a representative experiment.
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

**Fig. 4.** BMP9 induces expression of early hypertrophy-like marker genes, which is potently blocked by a low dose of TGFβ1. Chondrocytes of three donors were treated in duplo with 0.1 ng/ml of rhTGFβ1, 5 or 50 ng/ml of rhBMP9 or a combination of both growth factors for 7 days, and gene expression of markers of chondrocyte hypertrophy and terminal differentiation were measured using qPCR. Stimulation medium was refreshed every 3 days. After 7 days of treatment, expression of both Col10a1 and bAlpl was significantly increased in samples stimulated with 50 ng/ml rhBMP9, but not in samples treated with 50 ng/ml rhBMP9 and 0.1 ng/ml TGFβ1, indicating an inhibitory effect of TGFβ1. This inhibitory effect of TGFβ1 on BMP9 induced signaling is reflected in bId1 expression, as addition of TGFβ1 inhibited BMP9 induced bId1 expression significantly. For qPCR data, average ± s.e.m. was plotted with each dot representing the average of one donor. Statistics were calculated using one-way ANOVA with a Tukey’s post-hoc test.
High dose of BMP9 induces expression of chondrocyte hypertrophy marker genes which is blocked by TGFβ1

Overexpression of BMP9’s constitutively active receptor ALK1 induces expression of chondrocyte hypertrophy markers [13]. Therefore, we analyzed the effect of BMP9 signaling on expression of hypertrophy markers after 1 week. Considering that pSmad3 is a potent blocker of chondrocyte hypertrophy, co-stimulation with TGFβ1 was included (Fig. 4). After 1 week, bId1 expression was highly upregulated ∼49-fold (∼5.6 ΔCt) by 25 ng/ml BMP9. Remarkably, co-stimulation with 0.1 ng/ml TGFβ1 significantly lowered this induction to ∼21-fold (∼4.4 ΔCt). Of the four markers of chondrocyte hypertrophy that were measured, expression of bMMP13 or bSPP1 was not significantly regulated by BMP9, TGFβ1, or the combination of both. In contrast, expression of both bAlpl and bCol10a1, was highly upregulated by 25 ng/ml BMP9; ∼10.6-fold (∼3.4 ΔCt) and ∼4.6-fold (∼2.2 ΔCt) respectively. Strikingly, 0.1 ng/ml TGFβ1 could block BMP9-induced expression of these hypertrophy markers. Notably, matrix mineralization could not yet be observed by either alcian blue or alizarin red staining in our experiments. However, also after addition of two known inducers of matrix mineralization; β-glycerophosphate and ascorbic acid, no mineralization could be observed either. Apart from the inhibition of hypertrophy marker genes, co-stimulation also significantly induced bCol2a1 expression ∼2.1-fold (∼1.1 ΔCt), showing a beneficial effect of simultaneous stimulation with TGFβ1 and BMP9 on matrix production. Based upon these results we conclude that BMP9 induces the expression of certain hypertrophy-like marker genes, which is counteracted by a low dose of TGFβ1.

Discussion

Increasing evidence suggests pivotal, but opposing, roles for pSmad2/3 and pSmad1/5 in chondrocyte differentiation, with a hypertrophy-inducing role for the latter [35–37]. We investigated in vitro in primary chondrocytes the effects of BMP9, a potent pSmad1/5 inducing ligand that circulates in blood. We report that, in primary chondrocytes, BMP9 induces pSmad1/5 and expression of the chondrocyte hypertrophy markers bCol10a1 and bAlpl, but that this effect is potently counteracted by TGFβ1. Possibly, this inhibition is facilitated by the synergy we observed between BMP9 and TGFβ1 on Smad2/3 phosphorylation, a synergy which is unique for BMP9 compared to BMP2 or BMP7.

Previous reports have characterized BMP9 as a potent inducer of pSmad1/5 in a variety of endothelial cell lines [21,22]. We were able to show that also in primary chondrocytes, BMP9 potently induces pSmad1/5. Surprisingly, in some donors, we not only observed Smad1/5 but also Smad2 phosphorylation after stimulation with BMP9. In some, but not all, endothelial cell lines, BMP9-induced pSmad2 has been reported as well [21,22]. BMP9-induced pSmad2 is remarkable, as both type I receptors that BMP9 can bind; ALK1
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

and ALK2, are not known to directly phosphorylate Smad2. Therefore, how BMP9 induces pSmad2 is yet unclear.

After phosphorylation, Smad1/5 and Smad2/3 form complexes with Smad4, the common-Smad, and translocate to the nucleus where these complexes bind DNA and regulate gene transcription [5]. When analyzing BMP9-induced gene expression in primary chondrocytes, we observed rapid and robust up regulation of the pSmad1/5-dependent gene bld1, but not of pSmad3-dependent genes like bSerpine1. This lack of Smad3-dependent gene expression is apparently in contradiction with our observations on Western blot, which suggest pSmad2/3 signaling. However, in both endothelial cells and chondrocytes, inhibition of pSmad3 signaling by ALK1 signaling has previously been reported. In both cell types, over-expression of constitutively active ALK1 reduced pSmad3-dependent transcriptional activity, while not affecting Smad phosphorylation itself [38–40]. Possibly, the rapid induction of Smad7 we found in this study can explain the observed lack of pSmad2/3-responsive gene expression. Smad7 is an inhibitor of receptor-Smad signaling (inhibitory-Smad) with a multitude of actions, including the ability to prevent binding of the pSmad2/3-Smad4 complex to DNA [41,42]. Expression of this inhibitory-Smad was rapidly induced ∼3.2-fold within 2 h of BMP9 stimulation. Additionally, in chondrocytes, adenoviral over-expression of Smad7 has previously been reported to inhibit mSerpine1 transcription [43]. Therefore, in primary chondrocytes, Smad7 might be an important regulator of the crosstalk between the BMP9-induced pSmad1/5 and pSmad2/3 signaling pathways.

The crosstalk between pSmad1/5 and pSmad2/3 signaling controls chondrocyte differentiation (reviewed in [37]). Via an interaction with the Runx2, a transcription factor regarded as the key regulator of endochondral ossification, pSmad1 and 5 can directly enhance terminal differentiation by inducing expression of genes like Col10a1 and Mmp13 [35,44,45]. Prolonged stimulation with BMP9 indeed induced expression of the early hypertrophy marker genes bCol10a1 and bAlpl, but increased bMmp13 expression was not observed. However, bMmp13 expression mainly occurs in late stage hypertrophic chondrocytes [46], a cellular state most likely not achieved in our experimental setting, also indicated by the lack of mineralization in our experiments. Compared to pSmad1/5, pSmad2/3 has an opposite effect on Runx2 activity; it silences Runx2-responsive genes via recruitment of histone deacetylases [36]. In our experiments, BMP9-induced expression of hypertrophy markers was indeed antagonized by a low dose of TGFβ1, a well known inducer of Smad2 and 3 phosphorylation. Possibly, this antagonism is facilitated by the synergy we observed between BMP9 and TGFβ1 on Smad2/3 phosphorylation. In addition to Smad signaling, Smad-independent BMP-signaling via e.g., ERK and TAK1 can play a role in chondrocyte hypertrophy [47,48]. A limitation of our study is that we did not investigate these pathways. However, the Smad-dependent and Smad-independent pathways are closely interwoven by regulation of the former by the latter [49], and we think that Smad signaling is the key effector in BMP-induced chondrocyte
hypertrophy. Nonetheless, additional experiments are needed to reveal whether this actually holds true.

In cartilage, the inhibition of BMP9 induced hypertrophy is possibly an important physiological role of TGFβ1. TGFβ1 itself is produced by chondrocytes, and present in cartilage in high amounts [50]. Although we were unable to detect BMP9 mRNA in chondrocytes, BMP9 is most likely readily available in cartilage in vivo. The liver produces large amounts of BMP9, and BMP9 circulates in levels as high as \(~10\) ng/ml in bovine and \(~12\) ng/ml in human serum and plasma [16–18]. Furthermore, BMP9 is responsible for over 60% of all BMP activity in serum, indicating BMP9's relative abundance [15]. Because synovial fluid partly consists of plasma filtrate [51], it is likely that chondrocytes are constantly exposed to BMP9 via synovial fluid, but this still has to be confirmed. Considering that BMP9 signaling can induce hypertrophy, inhibition of this signal would be of crucial importance for the maintenance of articular cartilage, and therefore an important role of TGFβ1. Additional in vivo experiments are needed to test this hypothesis. In a pilot study, we tried to investigate the ability of BMP9 to induce cartilage hypertrophy in vivo by intra-articular injection of BMP9, but severe osteophyte formation, resulting in disrupted joint homeostasis, in response to BMP9 made it very difficult to draw conclusions on BMP9's direct effect on cartilage.

BMP9 levels are not known to correlate with OA, but its serum level doesn’t decrease during aging in mice [18]. We think that this stable level of BMP9 becomes a problem for cartilage during aging, in view of age-related loss of TGFβ signaling [11,13]. From cartilage-specific TGFβ-receptor type 2 knockout mice it is known that mice lacking TGFβ-signaling develop increased cartilage degradation and expression of hypertrophy markers [52]. It would be very interesting to see if BMP9 is the driving force behind this process by crossing these mice with BMP9 knockout mice.

How BMP9 and TGFβ1 can synergize on pSmad2/3 phosphorylation and downstream gene expression is yet unknown. However, the synergy between both growth factors can be observed rapidly, which suggests a mechanism upstream in the signaling pathway. TGFβ-superfamily signaling requires formation of a heterotetrameric receptor complex containing two type II and two type I receptors, in which receptor-Smads are phosphorylated by the type I receptors [5]. TGFβ1 can bind two type I receptors, ALK1 and ALK5, and form complexes containing both. Furthermore, complex formation between ALK1 and ALK5 induces both pSmad1/5 and pSmad2/3 [39]. Putatively, a receptor-complex containing both ALK1 and ALK5 can phosphorylate less Smad2/3 compared to a complex containing ALK5 twice because ALK1 cannot phosphorylate pSmad2/3. Using radiolabeled ligands, it has been shown that BMP9 has a far greater affinity for ALK1 than TGFβ1 [15]. Possibly, BMP9 affects TGFβ1-induced receptor complex formation by sequestering ALK1, forcing TGFβ1 to signal via receptor complexes containing solely ALK5, thus resulting in more pSmad2. This idea is supported by the observation that two other BMPs, BMP2 and BMP7, do not synergize with TGFβ1 on pSmad2 even at a very high dosage. These BMPs share the induction of pSmad1/5 and
The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.

the use of type II receptors like BMPR2 with BMP9, but do not use ALK1, making it more likely that ALK1 is involved in the synergy between BMP9 and TGFβ1. Moreover, the synergy between TGFβ1 and BMP9 is most obvious at high levels of BMP9, which would sequester ALK1 more efficiently.

In summary, this study shows that in chondrocytes, the ALK1-ligand BMP9 induces pSmad1/5 and downstream gene expression, leading within a week to a hypertrophy-like state in chondrocytes characterized by \( b\text{Alpl}\) and \( b\text{ColX}\) expression. However, we also show that this can potently be inhibited by addition of a low dose of TGFβ1. Possibly, the observed synergy between BMP9 and TGFβ1 on Smad2 phosphorylation plays a role in this inhibitory effect. Although we only investigated this interaction \textit{in vitro}, we propose that the interaction of BMP9 with TGFβ1 underlines the importance of TGFβ1 in maintenance of chondrocyte phenotype and furthermore that our observations support the idea that pSmad1/5 and pSmad2/3 have opposing roles in regulation of chondrocyte differentiation.
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The high affinity ALK1-ligand BMP9 induces a hypertrophy-like state in chondrocytes that is antagonized by TGFβ1.
TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain?

Blaney Davidson EN, van Caam AP, Vitters EL, Bennink MB, Thijsen E, van den Berg WB, Koenders MI, van Lent PL, van de Loo FA, van der Kraan PM.

Osteoarthritis Cartilage. 2015 Mar;23(3):478-86
Abstract

Objective
Pain is the main problem for patients with osteoarthritis (OA). Pain is linked to inflammation, but in OA a subset of patients suffers from pain without inflammation, indicating an alternative source of pain. Nerve Growth Factor (NGF) inhibition is very efficient in blocking pain during OA, but the source of NGF is unclear. We hypothesize that damaged cartilage in OA releases Transforming Growth Factorβ (TGFβ), which in turn stimulates chondrocytes to produce NGF.

Design
Murine and human chondrocyte cell lines, primary bovine and human chondrocytes, and cartilage explants from bovine metacarpal joints and human OA joints were stimulated with TGFβ1 and/or Interleukin 1 (IL1)β. We analyzed NGF expression on mRNA level with QPCR and stained human OA cartilage for NGF immunohistochemically. Cultures were additionally pre-incubated with inhibitors for TAK1, Smad2/3 or Smad1/5/8 signaling to identify the TGFβ pathway inducing NGF.

Results
NGF expression was consistently induced in higher levels by TGFβ than IL1 in all of our experiments: murine, bovine and human origin, in cell lines, primary chondrocytes and explants cultures. TAK1 inhibition consistently reduced TGF-β-induced NGF whereas it fully blocked IL1β-induced NGF expression. In contrast, ALK5-Smad2/3 inhibition fully blocked TGFβ-induced NGF expression. Despite the large variation in basal NGF in human OA samples (mRNA and histology), TGFβ exposure led to a consistent high level of NGF induction.

Conclusion
We show for the first time that TGFβ induces NGF expression in chondrocytes, in a ALK5-Smad2/3 dependent manner. This reveals a potential alternative non-inflammatory source of pain in OA.
TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain?

Introduction

The main symptom of osteoarthritis (OA) is joint pain. However, what part of the joint is actually causing pain is somewhat obscure. Pain is usually linked to inflammation [1], but in early OA patients, 45% of patients report pain without any detectible inflammatory component like synovitis that could be driving their pain [2]. This suggests a non-inflammatory cause for joint pain in at least a part of OA patients.

In recent years, blocking Nerve Growth Factor (NGF) has surfaced as an efficient way to inhibit pain, also in OA [3–8].

NGF was elevated in an animal model of OA with cartilage damage without joint inflammation, indicating a non-inflammatory source of NGF in OA [9,10]. McNamee et al. investigated NGF expression in a DMM model in whole knee joints and found that tumour necrosis factor alpha (TNFα) induced NGF in the post-operative phase, but TNFα was not responsible for later phases of OA pain as anti-TNFα only abrogated pain in the post-operative phase, not in late OA [9]. In contrast, NGF was key in both OA phases as soluble NGF receptor TrkAD5 prevented pain in both phases [9]. Iannone et al. showed that chondrocytes produced NGF. NGF was low in normal chondrocytes, increased in minor damaged cartilage, and further enhanced in severely damaged human articular cartilage [10]. Pecchi et al. found that Interleukin 1 (IL1) dose-dependently increased NGF expression in OA articular chondrocytes up to 5.7 fold at 10 ng/ml, paralleled by NGF protein release [11]. However, IL1 still suggests an inflammatory component, but does not explain a non-inflammatory source.

We were intrigued by the fact that chondrocytes produced NGF and that levels were higher in damaged cartilage. In cartilage, Transforming Growth Factorβ (TGFβ1) is stored in high amounts [12]. Upon damage, TGFβ1 is released from the cartilage extracellular matrix [13]. During OA, TGFβ is upregulated in cartilage [14]. In addition, high levels of TGFβ1 were found in serum of OA patients with a strong correlation to a higher Kellgren–Lawrence grade and WOMAC scale [15]. Clearly, TGFβ1 levels are linked to cartilage damage and pain. By genome-wide gene expression analysis in murine chondrocytes, we showed that only six genes were enhanced by both IL1β and TGFβ1: one of these genes was NGF [16]. TGFβ1, like IL1β can signal through TAK1 [17,18]. This led to the hypothesis that TGFβ1 could be a driving factor in NGF expression in articular chondrocytes in a TAK1-dependent manner.

We showed, for the first time, that TGFβ1 induced NGF expression in articular chondrocytes of murine, bovine and human origin and that TGFβ1-induced NGF levels were higher than those induced by IL1β. We identified that TAK1 inhibition blocked NGF expression by IL1β, but only reduced TGFβ1-induced NGF expression, thereby suggesting another major driving pathway for NGF induction. Further investigation revealed that blocking ALK5-Smad2/3 signaling could fully prevent TGFβ1 induced NGF expression.
Materials and methods

Chondrocyte cell lines
The murine chondrocyte cell line H4 [19] and human G6 chondrocyte cell line were used in monolayer [20]. The G6 cell line proliferates at 32°C and stops proliferating at 37°C. H4 chondrocytes were cultured in DMEM/F12 (Gibco, UK) with 50 mg/L gentamycin (Centrafarm Services, the Netherlands) and 100 mg/L pyruvate (Gibco, UK) with 5% FCS until use. G6 chondrocytes were cultured in a 1:1 mixture of DMEM/F12 10% serum and Lonza Mesenchymal Stem Cell Growth Medium (Lonza).

Bovine primary chondrocytes and cartilage explants
Primary bovine chondrocytes were isolated from cartilage of the metacarpal joint, obtained from the local slaughterhouse within 3 h post-mortem. Cartilage was removed with a scalpel, obtaining the non-calcified cartilage layers. We used 3 mm punches to generate equal size explants, which were randomly distributed over the different conditions, four explants per condition per cow. Cartilage explants were left untreated in DMEM/F12 0% FCS for 24 h prior to performing experiments. Alternatively for chondrocyte isolation, cartilage was treated with 1.5 mg/ml Collagenase B (Roche Diagnostics, Germany) in DMEM/F12 with 50 mg/L gentamycin and 100 mg/L pyrovate (1 g cartilage per 10 ml medium) overnight. Chondrocyte suspension was filtered (70 μm cell strainer (Beckton Dickenson Biosciences), pelleted, washed in saline, and seeded in a density of 1 × 10^5 cells per cm^2. Cells were cultured for 1 week to allow adherence prior to experiments.

Human OA primary chondrocytes or cartilage explants
Human OA cartilage was obtained from anonimized patients undergoing joint-replacement surgery. Cartilage explants were used, or chondrocytes were isolated as described for the bovine material, with the exception of the 1 week adherence prior to use, as human chondrocytes were cultured in monolayer overnight prior to stimulation.

Experiments
Cells or explants were serum starved overnight prior to stimulation. Cultures were supplemented with recombinant human or murine IL1β (1 or 10 ng/ml) (R&D) for human or murine cells, respectively, or recombinant human TGFβ1 (0.1, 1 or 10 ng/ml) (Biolegend). In experiments with inhibitors, DMSO was used as vehicle control. To block TAK1 activity, we used (5Z)-7-oxozeaenol [21] (oxozeanol in text, or oxo in figures) (Tocris Bioscience) either 0.5 μM or 1 μM in the first experiment, but thereafter 0.5 μM. To inhibit TGFβ signaling via ALK5-Smad2/3, 5 μM of the ALK5 inhibitor SB-505124 [22] (SB in figures) (Sigma Aldrich) was used. For inhibition of the Smad1/5/8 pathway 0.05 μM LDN-193189 [23] was used (Axon Medchem). Cultures were pre-incubated with inhibitors for 1 h prior to addition of IL1β and/
or TGF-β1. Twenty-four hours after addition of IL1β and/or TGF-β1, medium was removed and TRI-reagent was added for RNA isolation. Unless otherwise indicated a standard dose of 10 ng/ml of TGFβ1 and/or 10 ng/ml IL1β was used for 24 h.

**RNA isolation and Q-PCR**

RNA isolation from explants was performed as previously described in Ref. [24] using the RNeasy Fibrous tissue Mini Kit (Qiagen) according to manufacturers protocol. RNA isolation from chondrocytes was performed using TRI-reagent (Sigma–Aldrich, Germany) as previously described in Ref. [25]. RNA concentration was measured using a Nanodrop photospectrometer (Thermo Scientific, USA), followed by cDNA preparation as previously described in Ref. [25]. The cDNA was diluted 10× in ultra pure water, and gene expression was measured using 1 μM of validated cDNA-specific primers (**Table I**) (Biologio, the Netherlands) in a quantitative real time polymerase chain reaction (qPCR) using SYBRgreen master mix (Applied Biosystems). The protocol was: after 10 min at 95°C, 40 cycles of 15 s 95°C and 1 min 60°C each were run. A melting curve was made to verify gene-specific amplification. Data was expressed as ΔΔCt values corrected for GAPDH and controls, or when multiple controls were used a –Δct value was depicted. Besides NGF, we used *MMP3* (for bovine) or *MMP13* (for mouse and human) as a control for IL1 responsiveness.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’→3’</th>
<th>Reverse 5’→3’</th>
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</thead>
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<td>GGGGAGCGCATCGAGTTT</td>
</tr>
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<td>TCCTGAAAGATTCCGCAA</td>
</tr>
<tr>
<td>hMMP13</td>
<td>ATTAAGGAGCATGCGACTTCT</td>
<td>CCCAGGAGAAAAGCATGAG</td>
</tr>
</tbody>
</table>

**Table I.** Primers used for QPCR

**Immunohistochemistry**

Cartilage explants were fixed for 24 h in 4% paraformaldehyde. Tissue was dehydrated with an automated tissue processing apparatus (Miles Scientific Tissue-Tek VIP tissue processor; Miles Scientific, now part of Bayer Corp., Emeryville, CA, USA) and embedded in paraffin. Seven μm sections were stained immunohistochemically as previously described in Ref. [26]. Primary antibody was anti-NGF (sc-33602, FL-241, Santa Cruz). A biotin–streptavidin detection system was used according to manufacturers protocol (Vector Laboratories, Inc.). Bound complexes were visualized via reaction with 3′,3′diaminobenzidine (Sigma Chemicals Co., Zwijndrecht, the Netherlands) and H₂O₂ resulting in a brown precipitate. Sections were counterstained with heamatoxylin and mounted with Permout.
Statistical analysis
There are experiments within this paper that were not suitable for statistical analysis because we considered repetition of important scientifically relevant groups in separate experiments and different species of higher importance than repeating the same experiments. For human OA chondrocytes we included many patients, but given the variation in basal NGF expression in human OA patients we considered it would only be fair to the readers to display their $-\Delta C_t$ values individually rather than pooling data and calculating $\Delta\Delta C_t$ values. For the remainder of the experiments statistical analysis was performed using Graphpad Prism software. Gaussian distribution was tested using D’Agostino & Pearson omnibus normality test followed by either a one-way ANOVA with post-hoc Bonferroni comparison in case of a normal distribution, or by a Kruskall–Wallis test followed by Dunn’s comparison (see figure legends).

Results

TGFβ1 induces NGF in chondrocytes in an ALK5-Smad2/3 and TAK-1 dependent manner
To investigate whether TGFβ1 induced NGF in chondrocytes, we exposed the murine articular chondrocyte cell line H4 to 10 ng/ml TGFβ1 for 24 h. This led to a 2.8 fold increase ($1.5 C_t$) in NGF mRNA levels compared to non-treated controls (Fig. 1A). This increase was fully blocked by inhibiting TGFβ1 signaling via ALK5-Smad2/3 with SB-505124 (Fig. 1A). To investigate potential TAK1 dependency we exposed H4 chondrocytes to 10 ng/ml TGFβ1 with or without oxozeaenol (0.5 or 1 μM). TGFβ1 again increased NGF mRNA expression by 2.6 fold ($1.4 C_t$) (Fig. 1B). When adding appropriate DMSO dose to match the DMSO content of the oxozeaenol 0.5 or 1 μM, TGFβ1-induced NGF expression increased by 3.2 and 4 fold ($1.7$ and $2.0 C_t$), respectively. Preventing TGFβ1 signaling via TAK1 with oxozeaenol prevented TGFβ1-induced NGF-expression in both 0.5 and 1.0 μM oxozeaenol (Fig. 1B). Our data clearly show for the first time that TGFβ1 is capable of elevating NGF mRNA expression in chondrocytes using ALK5-Smad2/3 and TAK1 dependent.

TGFβ1 induces higher NGF expression than IL1β in human chondrocytes
To investigate whether TGFβ1 induced NGF in human cells, we stimulated human G6 chondrocytes with 10 ng/ml of IL1β and compared that to a series of dosages of TGFβ1 (0.1, 1 and 10 ng/ml) in (Fig. 2A). As expected, 10 ng/ml of IL-1 led to an increase in NGF mRNA levels of 2.3 fold ($1.2 C_t$). Surprisingly, TGFβ1 exposure increased NGF mRNA even higher by 10.6 fold ($3.4 C_t$) and 6.1 fold ($2.6 C_t$) in dosages of 1 and 10 ng/ml, respectively (Fig. 2A). This shows that 1 ng/ml of TGFβ1 induced NGF expression that was 4.6 fold ($2.2 C_t$) higher compared to a high dose of IL1β (10 ng/ml) (Fig. 2A). Increasing the TGFβ1 dose from 1 to 10 ng/ml did not result in higher NGF levels, which might suggest a maximum level of NGF induction was already reached at 1 ng/ml of TGF-β1. These data show that TGFβ1 is more potent in inducing NGF than IL1β.
TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain?

**Fig. 1.** TGFβ1 induces NGF in murine articular chondrocytes in an ALK5-Smad2/3 and TAK1 dependent manner. Murine H4 chondrocytes were stimulated with 10 ng/ml TGFβ1 for 24 h in the presence or absence of ALK5-Smad2/3 signaling inhibitor SB-505124 and compared to controls. RNA was isolated and QPCR was performed for NGF mRNA expression. TGFβ1 stimulation upregulated NGF expression compared to controls, which could be prevented by inhibiting ALK5-Smad2/3 signaling. Statistical analysis was performed using Kruskall–Wallis and Dunn’s comparison (A). Murine H2 chondrocytes were stimulated with 10 ng/ml of TGFβ1 for 24 h in the presence or absence of 0.5 μM or 1 μM o xozeaenol to prevent TAK1 signaling. TGFβ1 exposure resulted in an upregulation of NGF, which could be prevented by blocking TAK1 (B).

**Fig. 2.** TGFβ1 induces NGF expression in human chondrocytes in higher levels than IL1β; TGFβ1 and IL1β do not have an additive effect on NGF expression. Human G6 chondrocytes were stimulated with TGFβ1 or IL1β for 24 h, followed by RNA isolation and QPCR for NGF expression. Clearly, TGFβ1 induced higher levels of NGF compared to IL1β already at a dose of 1 ng/ml TGFβ1 (A). When TGFβ1 and IL1β were combined, NGF levels were not enhanced, showing a lack of additive effect of TGFβ1 and IL1β (B).
**IL1β and TGFβ1 are not additive in inducing NGF expression**

To reveal whether or not TGFβ1 and IL1β could have an additive effect on NGF-expression in human G6 chondrocytes we combined IL1β and TGFβ1 in 1 and 10 ng/ml combinations. Again, TGFβ1 1 and 10 ng/ml had equal effects in upregulating NGF mRNA levels of 17.7 fold (4.1 C_t) and 24.3 fold (4.6 C_t), respectively. Again, IL1β exposure yielded lower NGF expression compared to TGFβ1 of 4.6 fold (2.2 C_t) and 6.1 fold (2.6 C_t) by 1 ng/ml and 10 ng/ml of IL1β, respectively. Combining TGFβ1 (either 1 ng/ml or 10 ng/ml) with IL1β (10 ng/ml) revealed no additive effect on NGF expression in any combination, but rather NGF levels were similar to the corresponding TGFβ1 levels without IL1β (Fig. 2B). Given the consistently lower IL1β responses compared to TGFβ1 and lack of additive effect, we wanted to confirm IL1β responsiveness and therefore additionally measured MMP13 mRNA expression. TGFβ1 stimulation alone upregulated MMP13 by 3.26 C_t and 1.99 C_t in 1 ng/ml and 10 ng/ml, respectively, whereas IL1β stimulation resulted in an increase of 6.87 C_t and 6.77 C_t in 1 ng/ml and 10 ng/ml, respectively. IL1β 1 ng with TGFβ1 1 ng/ml or 10 ng/ml resulted in a mean increase in MMP13 expression of 8.57 C_t and 7.81 C_t, respectively and using IL1β 10 ng/ml with TGFβ1 1 ng/ml or 10 ng/ml increased MMP13 expression with 8.63 C_t and 8.15 C_t. Thus IL1β increased MMP13 expression thereby confirming IL1β responsiveness.

**In primary bovine chondrocytes, TGFβ1 more potently induces NGF expression than IL1β**

So far, we used chondrocyte cell lines. To confirm our findings in primary chondrocytes, we freshly isolated chondrocytes from bovine metacarpal joints and stimulated those with TGFβ1 (1 and 10 ng/ml), IL1β (1 or 10 ng/ml) or a combination of TGFβ1 1 ng/ml with IL1β 10 ng/ml (Fig. 3A). This again showed that TGFβ1-induced NGF mRNA expression was comparable between 1 and 10 ng/ml leading to a 16 fold (4.0 C_t) and 19.7 fold (4.3 C_t) increase, respectively. This was again higher than NGF levels induced by IL1β, which were 2.6 fold (1.4 C_t) and 3.7 fold (1.9 C_t) for 1 and 10 ng/ml, respectively. Combining TGFβ1 with IL1β did not further enhance NGF expression beyond TGFβ1-induced levels. Pre-incubation with TAK1-inhibitor oxozeaenol reduced NGF expression in all groups (Fig. 3A). However, TGFβ1 stimulated groups with oxozeanol all still had elevated levels of NGF compared to baseline. Overall these data indicate that TGFβ1-stimulated NGF expression is only partially TAK1-dependent, whereas IL1β fully relies on TAK1 signaling.

**TGFβ1 increases NGF expression in bovine articular cartilage explants**

From experience we know that isolated chondrocytes can respond differently from intact cartilage. To more closely mimic in vivo articular cartilage, we freshly isolated cartilage explants from healthy bovine metacarpal joints, and exposed these to TGFβ1 and IL1β. Isolated explants were left untreated for 24 h to reach a state of equilibrium. When comparing values of control samples 30 h after isolation to snap frozen samples, we found that NGF levels were increased. The NGF levels had restored to baseline after 48 h (24 h equilibrium +
TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain?

24 h in experiment). Therefore, we used the 24 h stimulation after 24 h equilibrium for the remainder of our explants experiments. Upon TGFβ1 exposure, we again found a significant increase in NGF mRNA expression of 26 fold (4.7 Ct) on average compared to controls, whereas IL1β stimulation resulted in an average increase of 2.1 fold (1.1 Ct), which was not significantly different from controls (Fig. 3B). Combining TGFβ1 with IL1β again showed no additional effect on NGF expression levels. These data confirm that also in intact articular cartilage, TGFβ1 induces NGF mRNA expression levels that are higher than those induced by IL1β (Fig. 3B). We again wanted to confirm IL1β responsiveness in these samples and therefore measured MMP3 expression as MMP13 expression is below detection limit in bovine cartilage explants. This showed a significant increase in MMP3 expression in samples containing IL1β, thereby confirming IL1β responsiveness (Fig. 3C).

TGFβ1-induced NGF in bovine articular cartilage runs via ALK5-Smad2/3
As ozozeanol could partially reduce TGFβ1-induced NGF expression we wanted to investigate if TGFβ1 signaling induces NGF in cartilage in a similar way (Fig. 3C). Therefore, we stimulated cartilage explants of four different cows to TGFβ1 with or without inhibitors for TGFβ1 signaling via ALK5-Smad2/3 (SB-505124), TGFβ1 signaling via Smad1/5/8 (LDN) or TAK1 (ozozeanol). TGFβ1 induced a 58.9 fold increase (5.9 Ct) compared to control. When comparing TGFβ1 + DMSO to TGFβ1 with inhibitors, TAK1 inhibition reduced TGFβ1-induced NGF expression by 3.4 fold (1.8 Ct), but this was not significant. Blocking Smad1/5/8 signaling could not inhibit TGFβ1-induced NGF expression at all. In contrast, only blocking ALK5-Smad2/3 signaling was capable of significantly reducing TGFβ1-induced NGF expression (reduction of 55.6 fold (5.8 Ct). This shows that in intact articular cartilage, TGFβ1 signaling via ALK5-Smad2/3 induces NGF and that there might be a small role for TAK1 in TGFβ1-induced NGF expression, whereas Smad1/5/8 signaling is not involved.

TGFβ1 and IL1β induce NGF expression in human OA cartilage
Until now we investigated the effect of TGFβ1 on healthy articular chondrocytes and intact cartilage. However, people experience pain during OA. We wanted to investigate whether human OA chondrocytes were also capable of upregulating NGF in response to TGFβ1 and IL1β. We freshly isolated chondrocytes from human OA cartilage and treated those with TGFβ1 or IL1β. Similar to prior results, IL1β induced an increase in NGF mRNA expression of 6.1 fold (2.6 Ct) on average whereas TGFβ1 induced an increase of 18.4 fold (4.2 Ct) on average (Fig. 4A). We included MMP13 expression to confirm IL1β responsiveness in these samples (Fig. 4B). The most striking observation in these human OA samples was not so much the induced levels, but rather the massive variation in NGF mRNA expression in controls. We re-ordered the patients in the graph to clarify the large spread in basal NGF levels between OA patients, which clearly showed that upon induction with IL1β or TGFβ1 the levels of NGF mRNA seem to level out to a certain threshold, irrespective of the background levels. We isolated intact
Fig. 3. TGFβ1 induces higher levels of NGF than IL1β in bovine chondrocytes and bovine articular cartilage in a ALK5-Smad2/3 and partially TAK1 dependent manner. Primary bovine chondrocytes isolated from the bovine metacarpophalangeal joint were stimulated with TGFβ1 or IL1β or both with or without oxozeaenol. Both TGFβ1 and IL1β induced NGF, but TGFβ1 induced NGF levels were much higher. Co-incubation with oxozeaenol, thereby preventing TAK1 signaling, could reduce TGF-β1-induced NGF expression, and fully reverse IL1β induced NGF expression (A). Bovine articular cartilage explants were stimulated with TGFβ1 or IL1β or a combination of both for 24 h after which mRNA was isolated to measure NGF expression. This shows that TGFβ1 induced NGF expression is higher than IL1β induced NGF expression (B). Bovine articular cartilage was stimulated with TGFβ1 with or without inhibitors for ALK5-Smad2/3 signaling (SB-505124) 5 μM, Smad 1/5/8 signaling (LDN) 0.05 μM or TAK1 signaling (oxozeaenol) 0.5 μM for 24 h after which RNA was isolated to measure NGF expression. This showed that TGFβ1 induced NGF expression could only be fully blocked in the presence of SB-505124, could not be blocked by LDN and slightly reduced by oxozeaenol (C). Statistical analysis was performed using Kruskall–Wallis and Dunn’s comparison.
cartilage from three random OA patients and stained these for NGF with immunohistochemistry which illustrated that the staining patterns of NGF protein expression were very different for each patient (Fig. 4C). Whereas the first sample showed a light positive, but diffuse staining throughout the entire cell, sample 2 showed very intense nuclear staining, but not all cells were positive. The third showed a combination of both types of staining and some negative cells as well. Areas that had less damage seemed to contain less NGF positive cells, but since these were only three patients we cannot draw hard conclusions on this. However, our data underlines the diversity in NGF expression in human OA. Moreover, our stimulation data suggests that irrespective of the basal levels of NGF, upon stimulation with IL1β or TGFβ1 the chondrocytes will produce a relative maximum level of NGF mRNA compared to GAPDH, which again generally seems to be higher for TGFβ1 stimulation than IL1β stimulation.

**In human OA intact cartilage TGFβ1 induction of NGF is ALK5-Smad2/3 dependent**

We identified that TGFβ1-induced NGF was ALK5-Smad2/3 dependent in bovine and murine chondrocytes. We wanted to investigate whether this holds true for OA. Therefore, we exposed human OA cartilage explants to TGFβ1 and combined this with ALK5-Smad2/3 inhibition (SB-505124). We found that TGFβ1 induced a 6.5 fold increase of NGF (2.7 C_t) and that this was fully blocked when ALK5-Smad2/3 signaling was prevented in all but one patient (Fig. 4D). This shows that even in OA cartilage, TGFβ1 can induce NGF via ALK5-Smad2/3.
Fig. 4. TGFβ1 induces higher NGF expression than IL1β in human primary OA chondrocytes, but basal NGF levels show very high variation. Human primary OA chondrocytes were isolated from patients undergoing total joint replacement surgery after informed consent. After isolation, chondrocytes were stimulated with TGFβ1 or IL1β for 24 h followed by RNA isolation and Q-PCR for NGF. Clearly, TGFβ1-stimulated samples reached higher levels of NGF than IL1β-stimulated samples. However, a striking observation was that the basal levels of NGF in human OA chondrocytes showed a very large variation (A). In these samples MMP13 was measured to ensure IL1β responsiveness which showed that IL1β indeed induced MMP13 whereas TGFβ1 inhibited MMP13. Statistical analysis was performed using Kruskall–Wallis and Dunn’s comparison (B). Immunohistochemistry for NGF was performed on articular cartilage of three random OA patients showing diversity in NGF protein expression (C). Human OA cartilage explants were stimulated with TGFβ1 with or without an inhibitor for ALK5-Smad2/3 signaling (SB-505124) 5 μM for 24 h after which RNA was isolated to measure NGF expression. Co-incubation with SB-505124 resulted in a clear blockage of TGFβ1-induced NGF expression. After normality test, statistical analysis was performed with a one-way ANOVA and Bonferroni for comparison (D).
**Discussion**

NGF has been identified as a potential target for pain therapy in OA. Iannone *et al.* showed that chondrocytes produce NGF and that levels of NGF were linked to cartilage damage [10]. In articular cartilage, TGFβ1 is stored in high amounts and is released upon damage [12]. Since there are patients with pain without any inflammatory component [27], we hypothesized that the source of NGF might be TGFβ released from the cartilage. We showed that TGFβ1 induced NGF in articular cartilage via ALK5-Smad2/3 and these levels of NGF were much higher than those achieved by IL1β. This indicates that the non-inflammatory induction of NGF might have an even higher impact than presumed until now.

As NGF was originally considered driven by inflammatory mediators, IL1β and TNFα have been in focus as inducing factors. In synovial OA fibroblasts, IL1β and TNFα were capable of inducing NGF [27]. However, the same authors identified that in experimental arthritis, IL1β, but not TNFα increased NGF and that TNFα even lowered constitutive NGF amounts in murine knee joints [28]. TNFα, however, enhanced IL1β-induced NGF indicating an indirect effect of TNFα and IL1β on NGF. The tissue of origin was unknown as whole knee joints were used. We compared IL1β to TGFβ1 as a reference for the extent of NGF induction. In all our experiments IL1β induced NGF, but TGFβ1 was far more potent, even when using a low dose of 1 ng/ml TGFβ1 compared to IL1β 10 ng/ml. This dose of IL1β is the same as used by Pecchi *et al.* [11]. They showed a basal value of NGF protein of approximately 15 pg/ml. We show that inhibiting ALK5-Smad2/3 signaling with SB-505124 reduced values to lower than controls (not significant). This could indicate that endogenous basal NGF levels rely on TGFβ as well.

Pecchi *et al.* showed that mouse and human chondrocytes dose-dependently increased NGF expression, on mRNA level and protein, when exposed to IL1β [11]. Mechanical stimulation also induced NGF, but this could not be enhanced by IL1β. In fact, their finding could rely on TGFβ1 as a source of NGF induction, as we recently published that Smad2/3 signaling was enhanced by mechanical stimulation [29]. Moreover, we found that IL1β could not enhance TGFβ1-induced NGF expression, which could explain why IL1β could not enhance NGF when combined with mechanical stimulation in Pecchi’s experiments.

As both IL1β and TGFβ1 can signal via TAK1, we initially thought this could be the common pathway driving NGF. Moreover, our prior finding of NGF being one out of only six genes that were not counteracted, but rather enhanced by TGFβ1 and IL1β, strengthened our idea [16]. Indeed we found that TAK1 inhibition decreased TGFβ1-induced NGF expression, but not to the extent that we had originally expected. In contrast, IL1β-induced NGF expression was fully blocked by inhibition of TAK1, thereby confirming TAK1 dependency. This indicated involvement of another pathway in TGFβ1-induced NGF expression. By blocking different pathways activated by TGFβ1, we identified that TGFβ1-induced NGF in chondrocytes and cartilage explants was fully ALK5-Smad2/3 dependent.
In pancreatic cells it was shown that TGFβ1 induced NGF via ALK5 [30]. This fully matches our data in chondrocytes, where we show that TGFβ1 relies on ALK5-Smad2/3 signaling for induction of NGF. In line with that, Braunger et al. showed in retinal progenitor cells of TGFβ1 receptor II deficient mice, Smad3 phosphorylation was decreased, which led to lower levels of NGF mRNA. Similarly, mice deficient in Smad7, which is a TGFβ1 signaling inhibitor, had higher Smad3 phosphorylation and higher levels of NGF mRNA [31]. In rat hippocampus, TGFβ1 increased NGF mRNA expression in vivo [32]. Interestingly, Yu et al. showed four different transcripts of NGF that were differentially expressed comparing mouse and rat spinal cord and brain cells. This potentially relies on multiple TGFβ1 responsive elements in the NGF promoters located upstream of exons 1 and 3 [33]. TGFβ1 could induce NGF in astrocytes and glioma cells, but not in neuronal cells, whereas all cells expressed the same TGFβ1 receptors. It was concluded that differences were on a transcriptional level. They showed that in different cell types different combinations of NGF transcripts were induced by TGFβ1. We did not find species differences, but clearly IL1β and TGFβ1 showed different levels of NGF expression, which could correspond to different transcripts being expressed.

In human OA cartilage, we found that basal levels of NGF were highly different between patients, which was reflected by differences in immunohistochemical NGF staining. The finding of nuclear NGF staining could point towards a nuclear translocation as previously found by Yanker et al. [34]. We observed that intact cartilage areas showed less NGF staining compared to damaged areas, but given the limited number of patients we are reluctant to conclude on that. However, it does fit the data of Iannone et al. who also found that damaged cartilage expressed more NGF [10]. Strikingly, despite the vast differences in basal NGF levels, stimulating these highly variable samples with either TGFβ1 or IL1β, resulted in similar levels of NGF expression for which the height of expression was dependent on the stimulus. TGFβ1-induced NGF was overall higher than the IL1β-induced NGF. This again points towards a potential different regulatory mechanism: ALK5-Smad2/3 vs TAK1 for TGFβ1 and IL1β, respectively.

In this study we included multiple species and monolayer vs tissue explants culture to ensure NGF-induction was not limited by method or species selected. However, this introduced a limitation where for some experiments a low number of individual samples were used in which case statistical analysis was not possible. Therefore it is not possible to extrapolate beyond current experiments when sample number was low.

In conclusion, we show that TGFβ1 is a very potent inducer of NGF expression in chondrocytes and cartilage and its induction of NGF can be higher than IL1β. Moreover, we show that TGFβ1-induced NGF expression relies on ALK5-Smad2/3 signaling, even in human OA cartilage. In addition, we show that TGFβ1-induced NGF expression can be reduced by TAK1 inhibition. In contrast, IL1β induced NGF expression is fully TAK1 dependent. Overall our data strongly suggest that NGF-induced by TGFβ1 in chondrocytes could be a prominent alternative, non-inflammatory source of pain in OA.
TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALK5-Smad2/3 pathway. Potential role in OA related pain?

References

TGFβ is a potent inducer of Nerve Growth Factor in articular cartilage via the ALKS-Smad2/3 pathway. Potential role in OA related pain?
7.

TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity


Abstract

Objective
Dysregulated transforming growth factor β (TGFβ) signaling is implicated in osteoarthritis development, making normalizing TGFβ signaling a possible therapy. Theoretically, this can be achieved with small molecule inhibitors specifically targeting the various TGFβ receptors and downstream mediators. In this study we explore in primary chondrocytes the use of small molecule inhibitors to target TGFβ-induced pSmad1/5/9-, pSmad2/3- and TGFβ-activated kinase 1 (TAK1)-dependent signaling.

Design
Primary bovine chondrocytes and explants were isolated from metacarpophalangeal joints. To modulate TGFβ signaling the activin receptor-like kinase (ALK)1/2/3/6 inhibitor LDN-193189, the ALK4/5/7 inhibitor SB-505124 and the TAK1 inhibitor (5Z)-7-Oxozaenol were used. pSmad1/5 and pSmad2 were measured using western blot analysis and TGFβ1-induced gene expression was measured using quantitative real time PCR (qPCR).

Results
In chondrocytes, TGFβ1 strongly induced both pSmad1/5 and pSmad2. Remarkably, LDN-193189 did not inhibit TGFβ-induced pSmad1/5. In contrast, SB-505124 did inhibit both TGFβ-induced Smad2 and Smad1/5 phosphorylation. Furthermore, (5Z)-7-Oxozaenol also profoundly inhibited TGFβ-induced pSmad2 and pSmad1/5. Importantly, both SB-505124 and (5Z)-7-Oxozaenol did not significantly inhibit constitutively active ALK1, making an off-target effect unlikely. Additionally, LDN-193189 was able to potently inhibit BMP2/7/9-induced pSmad1/5, showing its functionality. On gene expression, LDN-193189 did not affect TGFβ1-induced regulation, whereas both SB-505124 and (5Z)-7-Oxozaenol did. Similar results were obtained in cartilage explants, although pSmad1/5 was not strongly induced by addition of TGFβ1.

Conclusion
Our data suggest that ALK5 kinase activity plays a central role in both TGFβ-induced Smad1/5 and Smad2/3 phosphorylation, making it difficult to separate both pathways with the use of currently available small molecule inhibitors. Furthermore, our data regarding (5Z)-7-Oxozaenol suggest that TAK1 facilitates Smad-dependent signaling.
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

**Introduction**

Transforming growth factor β (TGFβ) plays a crucial role in regulation of tissue homeostasis via its control over diverse cellular processes such as proliferation, differentiation and matrix formation. By binding to different receptors of the activin-receptor like kinase (ALK) family, TGFβ induces intracellular carboxy-terminal (C-terminal) phosphorylation of receptor-regulated Smad (R-Smad) proteins. Phosphorylated R-Smads form complexes with the common-mediator Smad; Smad4, and these complexes translocate to the nucleus where they bind DNA to regulate gene transcription via recruitment of transcription factors [1, 2]. Use of different ALKs can result in activation of different R-Smads; activation of ALK5 (TGFβR1) induces phosphorylation of Smad2 and Smad3, whereas activation of an ALK1 (or 2 or 3) complex mediates phosphorylation of Smad1, Smad5 and Smad9 [3, 4]. However, TGFβ signaling is not limited to R-Smads as Smad-independent signaling can occur by activation of e.g. TGFβ-activated kinase 1 (TAK1 or MAP3K7). Smad-independent signaling results in activation of various downstream signaling pathways, including the JUN N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways [5].

In many tissues, e.g. in cartilage, phosphorylated Smad2/3 and Smad1/5 have opposing functions [6, 7]. In cartilage, phosphorylated Smad3 guards chondrocyte phenotype against deleterious hypertrophy and production of catabolic enzymes like matrix metalloproteinases (MMPs) [8, 9, 10]. In contrast, phosphorylated Smad1/5 is essential for chondrocyte hypertrophy [11, 12], and is associated with expression of matrix metalloproteinase 13 (MMP13), the main cartilage-degrading enzyme [13]. Therefore, balancing TGFβ signaling via Smad2/3 or Smad1/5 is important for chondrocytes to maintain cellular homeostasis and deregulation of this balance has been proposed as a cause of disease, e.g. osteoarthritis (OA) [4, 13]. It would be of great benefit if cellular responses to TGFβ could be directed towards either the Smad1/5 or Smad2/3 pathway; this would enable blockade of deleterious pathways while maintaining beneficial signaling.

Inhibiting Smad phosphorylation can be achieved by blocking the kinase domain of ALK receptors. Small molecules have been developed that specifically inhibit the kinase activity of ALK4, ALK5 and ALK7, the receptors that phosphorylate Smad2 and Smad3, or ALK1, ALK2, ALK3 and ALK6, the receptors that phosphorylate Smad1, Smad5 and Smad9 [14, 15]. In this study we investigated in chondrocytes and cartilage explants the use of SB-505124 [15, 16], an inhibitor of ALK4/5/7 kinase activity and LDN-193189, an inhibitor of the ALK1/2/3/6 kinase activity [14, 16] to study their ability to direct R-Smad signaling and downstream effects. Furthermore, we used (5Z)-7-Oxozeaenol as a selective inhibitor of TAK1 kinase activity to evaluate the importance of TAK1 in TGFβ signaling in chondrocytes [17, 18], because of the ability of TAK1 to induce the JNK and p38 MAPK pathways and MMP production in cartilage [19, 20].
In this study we show that inhibition of ALK4/5/7 kinase activity prevented TGFβ1-induced C-terminal phosphorylation of both Smad2 and Smad1/5. Furthermore, treatment with (5Z)-7-Oxozeaenol, a selective TAK1 kinase inhibitor, attenuated phosphorylation of both Smad2/3 and Smad1/5 and expression of Smad target genes. Use of the ALK1/2/3 inhibitor LDN-193189 did not affect TGFβ1-induced R-Smad phosphorylation or gene expression. In conclusion, our data show that in cartilage the activity of the ALK5 kinase domain is essential for TGFβ1 signaling, whereas ALK1 kinase activity is not. Furthermore, our results obtained with (5Z)-7-Oxozeaenol underline that TAK1 kinase activity is facilitating R-Smad phosphorylation. Our study indicates that TGFβ1-induced Smad1/5 and Smad2/3 phosphorylation cannot be selectively targeted by using the small molecule inhibitors SB-505124 and LDN-193189.

Materials and methods

Chondrocyte culture
Primary bovine chondrocytes were isolated from the metacarpophalangeal joint of cows (2–5 years old) obtained from a slaughterhouse within 3 hours post mortem. Chondrocytes were obtained by incubating cartilage slices overnight in Collagenase B (for details see [21]). Chondrocytes were seeded in DMEM/F12 1:1 containing 10% fetal calf serum (Thermo Scientific UK) at a density of $0.5 \times 10^5$ cells per cm$^2$ in 6-well plates (Greiner Bio-one International, the Netherlands) for protein studies, or in 24-well plates (Bio-one International, the Netherlands) for mRNA experiments. Cells were cultured for 1 week at 37 °C and 5% CO$_2$ before the start of the experiments. For explant studies, four 7 mm$^2$ explants per condition were pooled in 1 ml of medium. Both monolayer and explant studies were repeated in different donors.

Adenoviral transfection of primary chondrocytes
Adenoviruses were used to induce expression of constitutively active (ca)ALK1 [4], constitutively active ALK5 [4] and the CAGA$\alpha_{12}$-luciferase reporter construct [22] in primary chondrocytes. Cells were rinsed twice with saline, and adenoviruses were added in a multiplicity of infection of 200 for 3 h at 37 °C in a minimal volume of DMEM/F12 1:1. Hereafter, cells were washed twice with saline, and DMEM/F12 1:1 was added. Cells were used 48 h after transfection.

Inhibition of TGFβ1 signaling
Before stimulation, cells were deprived of serum for 24 h and thereafter stimulated with 1 ng/ml recombinant human TGFβ1 (Biolegend, the Netherlands) for 2 or 24 h. In experiments where inhibitors were used, DMSO was used as vehicle control. To block TAK1 activity, we used (5Z)-7-Oxozeaenol [17] (Tocris Bioscience) in a concentration of 0.5 μM. To inhibit ALK5 kinase, we used SB-505124 [15] (Sigma Aldrich) in a concentration of 5 μM. For inhibition of ALK1 kinase, LDN-193189 [14] (Axon Medchem) was used in a concentration of 0.05
μM. This concentration of LDN-193189 is well above its reported half maximal inhibitory concentration (IC$_{50}$), 0.8 nM for ALK1, but far below its IC$_{50}$ for ALK5 of 350 nM [16, 23]. Cells were pre-incubated with the inhibitors for 1 h prior to addition of TGF-β1. Either 2 or 24 h after addition TGFβ1, medium was removed and TRI-reagent was added for RNA isolation.

**Protein isolation**

To isolate proteins from monolayer cultures, cells were lysed on ice using lysis buffer (Cell signaling, USA) containing a protease inhibitor cocktail (complete, Roche Diagnostics, Germany) and subsequently sonicated on ice using a Bioruptor (Diagenode, USA). With a BCA-assay (Thermo Scientific, USA) protein concentration was measured and after addition of Laemmli sample buffer, samples were boiled for 5 minutes at 99 °C.

To isolate proteins from explants, four 7-mm$^2$ explants were homogenized using a Mikro-dismembrator (B. Braun, Germany) and dissolved in 1 ml ice cold radioimmunoprecipitation assay (RIPA) buffer with added 1 mM Na$_3$VO$_4$ and protease inhibitor cocktail (Roche Diagnostics, Germany). After 1 h incubation on a roller bench at 4 °C, samples were spun down for 3 minutes at $10^4 \times g$ and the pellet was discarded. Cetylpyridinium chloride was added up to a concentration of 1% (m/v) and samples were incubated on a roller bench for 1 h at 4 °C. Hereafter, samples were spun down twice for 15 minutes at $10^4 \times g$ at 4 °C and the pellet was discarded. Using 10 kDa centrifugal filter units (Millipore, USA) the supernatant was concentrated to a volume of 50 μl. Subsequently, proteins were precipitated by addition of 950 μl of 20% m/v trichloroacetic acid and 0.1% m/v dithiothreitol in aceton at -20 °C and spinning samples down for 15 minutes at 6700 × $g$ at 4 °C. Next, the pellets were washed three times with 0.1% m/v dithiothreitol in aceton at -20 °C. Finally, the pellets were dried under vacuum for 20 minutes and dissolved in 100 μl 1% m/v sodium dodecyl sulfate in 100 mM tris(hydroxymethyl)aminomethane in H$_2$O pH 9.0.

**Detection of proteins using SDS-PAGE and western blot**

Proteins were separated on a 7.5% Bisacrylamide gel, and transferred to a nitrocellulose membrane using wet transfer (Towbin buffer, 2.5 h 275 mA at 4 °C). After overnight incubation at 4 °C with 1:1000 polyclonal Rabbit anti P-Smad1/5 (S463/465)/Smad8 (S426/428) (Cell signaling, USA) or anti P-Smad2 (S465/467) (Cell signaling, USA), membranes were incubated with 1:1500 polyclonal Goat anti Rabbit labeled with horseradish peroxidase (HRP) (DAKO, Belgium) for 1 h. Hereafter, enhanced chemiluminescence using ECL plus (GE Healthcare, UK) was used to visualize the proteins. To visualize overexpression of constitutively active ALKs, a rabbit polyclonal antibody directed against their internal HA tag was used: HA-probe Antibody (Y-11) (Santa Cruz, USA) (1:1000). As loading control either Gapdh was stained with an anti-Gapdh mouse mAb (1G5) (Sigma Aldrich, Germany) (1:10 000) in combination with IRDye 680RD Donkey anti mouse (1:10 000) (Licor, USA) using the Odyssey detection system (Licor, USA) or Vinculin with a rabbit pAb (H300) (Santa Cruz, USA) (1:1000) in combination
with HRP-labeled Goat-anti Rabbit (1:2000) (DAKO, Denmark) using ECL. Finally, blots were quantified using ImageJ.

**Detection of gene expression**

TRI-reagent (Sigma-Aldrich, Germany) was used for RNA isolation according to the manufacturer’s protocol (for details see [21]). RNA concentration was measured using a Nanodrop photospectrometer (Thermo Scientific, USA). Per sample, 1 μg of RNA was treated with DNAse (Life Technologies, USA), which was subsequently inactivated at 65 °C with 1 μl 25 mM EDTA (Life Technologies, USA). To perform reverse transcriptase reaction, 1.9 μl ultra pure water, 2.4 μl 10×DNAse buffer, 2.0 μl 0.1 M DTT, 0.8 μl 25 mM dNTP, 0.4 μg oligo dT primer, 1 μl 200 U/μl M-MLV Reverse transcriptase (all Life Technologies, USA) and 0.5 μl 40 U/μl RNAsin (Promega, the Netherlands) was added, and samples were incubated for 5 minutes at 25 °C, 60 minutes at 39 °C, and 5 minutes at 95 °C using a thermo cycler. The obtained cDNA was diluted 10 times in ultra pure water, and gene expression was measured using 1 μM of validated cDNA-specific primers (Biolegio, the Netherlands; see Table 1) in a quantitative real-time polymerase chain reaction (qPCR) using SYBR green master mix (Applied Biosystems). The following protocol was used: after 10 minutes at 95 °C, 40 cycles of 15 sec at 95 °C and 1 minute at 60 °C each were run. Hereafter a melting curve was made to verify gene-specific amplification. For calculations of the -ΔCt, two reference genes were used: glyceraldehyde 3-phosphate dehydrogenase and ribosomal protein S14.

**Statistics**

All quantitative data are expressed as a mean of multiple repeats ± SD. For every analysis data were checked for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) with Tukey multiple comparison post-hoc test was used to determine the significance. The statistical analyses were performed using Graphpad Prism 5.0 software.
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

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Table 1: Sequence and efficiency of the primers used in this study. P is product length in base pairs, E is efficiency in percentage. Alk1 is also known as Acrv1, Alk2 as Acrv1, Alk3 as Bmpr1a and Alk5 as Tgfr1

Results

SB-505124 and (5Z)-7-Oxozaenol both inhibit TGFβ1-induced Smad1/5 and Smad2/3 phosphorylation whereas LDN-193189 does not

To begin, we confirmed in primary bovine chondrocytes the expression of the receptors that TGFβ can use to induce Smad2/3 phosphorylation i.e. ALK5 or Smad1/5 phosphorylation, i.e. ALK1, ALK2 and ALK3 [3, 4]. All four receptors were readily detected using qPCR (Additional file 1: Figure S1), and indeed confirmed that stimulation of chondrocytes with TGFβ1 results in both Smad2 and Smad1/5 phosphorylation (Fig. 1A).

Subsequently, we analyzed the effects of the ALK4/5/7-kinase inhibitor SB-505124, the ALK1/2/3-kinase inhibitor LDN-193189 and the TAK1-kinase inhibitor (5Z)-7-Oxozaenol on TGFβ1-induced R-Smad phosphorylation (Fig. 1A, B and C). Strikingly, addition of 5 μM SB-505124 not only inhibited Smad2 phosphorylation but also pSmad1/5 phosphorylation. Remarkably, treatment with 0.5 μM (5Z)-7-Oxozaenol also resulted in inhibition of TGFβ1-induced Smad1/5 and Smad2 phosphorylation. Noteworthy, addition of 0.05 μM LDN-193189 did not affect TGFβ1-induced Smad1/5 phosphorylation but did significantly enhance TGFβ1-induced pSmad2.
Fig. 1. SB-505124 and (5Z)-7-Oxozeaenol inhibit both transforming growth factor β1 (TGFβ1)-induced Smad1/5 and Smad2/3 phosphorylation whereas LDN-193189 does not. (A) Primary bovine chondrocytes were pre-incubated with either 5 μM SB-505124, 0.5 μM (5Z)-7-Oxozeaenol or 0.05 μM LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGFβ1 for 1 h, after which phosphorylated Smads were visualized using western blot by specific antibodies. kDa, kilodalton. (B) Quantification of the pSmad1/5 signal (as shown in (A)) in four experiments. pSmad1/5 levels were normalized to vinculin levels and plotted as a relative amount in arbitrary units (AU) compared to the control group: #p ≤ 0.001 compared to unstimulated; ***p ≤ 0.001. (C) Quantification of the pSmad2 signal (as shown in (A)) in four experiments. pSmad2 levels were normalized to vinculin levels and plotted as a relative amount in AU compared to the control group: #p ≤ 0.001 compared to unstimulated; ***p ≤ 0.001. (D) Dose-response effect of SB-505124 on TGFβ1 signaling in primary chondrocytes. (E) Quantification of pSmad levels (shown in (D)); levels were normalized to vinculin levels and plotted as a relative amount in percentage compared to the control group.
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

Fig. 2. SB-505124 and (5Z)-7-Oxoeaenol do not affect constitutively active activin receptor-like kinase 1 (ALK1) whereas LDN-193189 does. (A) Primary bovine chondrocytes were transfected with either constitutively active ALK1 (caALK1) or caALK5 and 2 days later treated with inhibitors for 1 h. A virus overexpressing LacZ was used as control. Efficiency of overexpression was visualized on western blot by staining for the HA-tag attached to the caALKs. kDa kilodalton. (B) Quantification of the pSmad1/5 and pSmad2 signal (as shown in (A)) in three experiments; # p ≤ 0.001 compared to unstimulated. pSmad levels were normalized to vinculin levels and plotted as a relative amount in arbitrary units (AU) compared to the control group; § p ≤ 0.01 compared to unstimulated; ** p ≤ 0.01; *** p ≤ 0.001. C Dose-response effect of LDN-193189 on transforming growth factor β1 (TGFβ1) signaling in primary chondrocytes. pSmad levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) levels and indicated as a relative amount in AU compared to the control group. (D) Primary chondrocytes were incubated for 1 h with LDN-193189 and subsequently stimulated for 1 h with 1 ng/ml (78 pM) TGFβ1, 25 ng/ml (1.94 nM) recombinant human bone morphogenic protein 2 (rhBMP2), 25 ng/ml (3.06 nM) bone morphogenic protein 7 (BMP7) or 1 ng/ml (83 pM) bone morphogenic protein 9 (BMP9). pSmad levels were normalized to Gapdh levels and indicated as a relative amount in AU compared to the control group.
To investigate if the striking effect of SB-505124 on Smad1/5 phosphorylation was a dose-dependent effect, we performed a dose-response experiment (Fig. 1D and E). This experiment showed that at all tested doses (0.1, 0.5, 1, 5, 10 and 25 μM), SB-505124 inhibited TGFβ1-induced Smad1/5 phosphorylation, and that Smad1/5 phosphorylation was inhibited more potently than that of Smad2. Therefore, to address if ALK1 inhibition was a possible off-target effect of SB-505124, this compound was added to cells over-expressing constitutively active (ca)ALK1 or caALK5. This experiment showed that SB-505124 did not affect caALK1-induced Smad1/5 phosphorylation (Fig. 2A and B), but did inhibit caALK5-induced Smad2 phosphorylation (Fig. 2A and B). Therefore, SB-505124 is specific for ALK5 kinase and does not inhibit ALK1 kinase. The same experimental setup was used to address the specificity of (5Z)-7-Oxozeaenol, and no significant inhibitory effect of this compound on either caALK1 or caALK5 function was detected, making it unlikely that either the ALK1 or ALK5 kinase domain is an off-target of (5Z)-7-Oxozeaenol.

To investigate if the lack of effect of LDN-193189 on TGFβ1-induced Smad1/5 was maybe dose-dependent, we added a 10 times higher dose of LDN-193189 (0.5 μM) to the cells, but were still unable to inhibit TGFβ1-induced Smad1/5 phosphorylation (Fig. 2C). Therefore we sought to validate the function of LDN-193189. To do this, we added 0.05 μM LDN-193189 to chondrocytes over-expressing caALK1 and observed that this blocked caALK1-induced Smad1/5 phosphorylation (Fig. 2A and B). Furthermore, the effect of 0.05 μM LDN-193189 on bone morphogenic protein (BMP) signaling was also investigated (Fig. 2D) by stimulating primary chondrocytes for 1 h with either the ALK2/3 ligands BMP2 or BMP7 [24] or the ALK1/2 ligand BMP9 [25, 26]. We observed that BMP-induced Smad1/5 phosphorylation was clearly inhibited by 0.05 μM LDN-193189. Together, these experiments strongly confirm the function of 0.05 μM LDN-193189 in primary chondrocytes.

In conclusion, in primary chondrocytes, TGFβ1-induced Smad1/5 phosphorylation is unaffected by LDN-193189, whereas both Smad2/3 and Smad1/5 phosphorylation are inhibited by SB-505124 and (5Z)-7-Oxozeaenol.

Fig. 3. SB-505124 fully and (5Z)-7-Oxozeaenol partly inhibits transforming growth factor β1 (TGFβ1)-induced Smad-dependent gene expression whereas LDN-193189 does not. (A) Primary bovine chondrocytes were pre-incubated with 5 μM SB-505124, 0.5 μM (5Z)-7-Oxozeaenol or 0.05 μM LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGFβ1 for 2 h and 24 h. With the use of quantitative (q)PCR, expression of bSerpine1 was measured as response gene for pSmad3 signaling after 2 h, and bId1 for pSmad1/5 signaling after 2 h. (B) The pSmad3 responsive CAGA12-luciferase (CAGA12-luc) construct was placed in primary chondrocytes with the use of an adenovirus, and cells were stimulated with 1 ng/ml TGFβ1 for 8 h with or without inhibitors after 2 days. (C) Primary chondrocytes were stimulated with 1 ng/ml TGFβ1 for 24 h and gene expression was measured using validated cDNA-specific primers and qPCR. MMP matrix metalloproteinase, NGF nerve growth factor, ALK5 activin receptor-like kinase 5. (D) Gene expression was measured after pre-incubation of chondrocytes for 1 h with inhibitors followed by stimulation with 1 ng/ml TGFβ1 for 24 h. For qPCR data, average±sd (mean±sd) was plotted, with each dot representing the average of one donor (A) or four donors (C and D). Analysis was performed using one-way analysis of variance with Tukey’s post-hoc test: *p≤0.05; **p≤0.01; ***p≤0.001. A change of 1 ΔΔCt equals twofold upregulation.
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

Figure 3

A

B

C

D

Inhibition of TGFβ1-induced gene expression in monolayer chondrocytes (24 h)
SB-505124 fully and (5Z)-7-Oxozeaenol partly inhibit TGFβ1-induced Smad-dependent gene expression whereas LDN-193189 does not

Next, we analyzed the effects of TGFβ1 signaling inhibitors on TGFβ1-induced Smad-dependent gene expression. For this, \( \textit{bId1} \) and \( \textit{bSerpine1} \) were used as markers of pSmad1/5 and Smad3 signaling, respectively [4, 27] [22, 28]. Expression of \( \textit{bId1} \) was profoundly upregulated by stimulation with 1 ng/ml of TGFβ1 (Fig. 3A). In accordance with our western blot data on pSmad1/5, expression of \( \textit{bId1} \) was inhibited by both SB-505124 and (5Z)-7-Oxozeaenol, and was unaffected by LDN-193189. Expression of \( \textit{bSerpine1} \) was upregulated by 1 ng/ml of TGFβ1 and fully inhibited by SB-505124, but was only partly inhibited by (5Z)-7-Oxozeaenol (Fig. 3B). To confirm these effects on pSmad3-dependent gene expression, we used the CAGA\(_{12}\)-luc construct, which produces luciferase specifically in response to pSmad3.

After 8 h, TGFβ-induced CAGA\(_{12}\)-luc activity was significantly inhibited by (5Z)-7-Oxozeaenol and SB-505124, showing that pSmad3-signaling is indeed lowered in these conditions. When the effect of the inhibitors on multiple TGFβ1-induced (Fig. 3C), genes like \( \textit{bSmad7}, \textit{bMmp3}, \textit{bCol2a1}, \textit{bNgf}, \textit{bAlk5}, \textit{bJunb}, \textit{bFN1} \) and \( \textit{bTgfb1} \) was measured, none of these genes was significantly downregulated by LDN-193189, unlike SB-505124, which profoundly downregulated \( \textit{bSmad}, \textit{bNgf}, \textit{bAlk5}, \textit{bJunb}, \textit{bFN1} \) and \( \textit{bTgfb1} \), or unlike treatment with (5Z)-7-Oxozeaenol, which significantly lowered \( \textit{bNgf}, \textit{bTgfb1} \) and \( \textit{bMmp3} \) expression (Fig. 3D). Because a role for pSmad1/5 in the regulation of e.g. \( \textit{Smad7}, \textit{Junb} \) and \( \textit{Col2a1} \) has been established [29, 30, 31], these data indicate that the ALK5 kinase inhibitor SB-505124 inhibits gene expression downstream of both pSmad2/3 and pSmad1/5, whereas (5Z)-7-Oxozeaenol does so to a lesser extent and in a more limited amount of genes. No effect of LDN-193189 was observed on TGFβ1-induced gene expression.

Fig. 4. In cartilage, SB-505124 fully inhibits and (5Z)-7-Oxozeaenol partly inhibits transforming growth factor β1 (TGFβ1)-induced Smad2/3 phosphorylation and gene expression, whereas LDN-193189 does not. (A) Bovine cartilage explants were pre-incubated ex vivo with 5 μM SB-505124, 0.5 μM (5Z)-7-Oxozeaenol or 0.05 μM LDN-193189 for 1 h, and subsequently stimulated with 1 ng/ml TGFβ1 for 2 h, after which phosphorylated Smads were visualized by western blot using specific antibodies. kDa kilodalton. (B) With the use of quantitative (q)PCR, expression of \( \textit{bSerpine1} \) was measured as response gene for pSmad3 signaling after 24 h, and \( \textit{bId1} \) for pSmad1/5 signaling after 24 h. (C) Gene expression was measured after pre-incubation of explants for 1 h with inhibitors followed by stimulation with 1 ng/ml TGFβ1 for 24 h. For qPCR data, average ± sd (mean ± sd) was plotted, with each dot representing the average of one donor (B) or four donors (C). Analysis was performed using one-way analysis of variance with Tukey’s post-hoc test: *\( p \leq 0.05; **p \leq 0.01; ***p \leq 0.001 \). A change of 1 ΔΔC\(_t\) equals twofold upregulation or downregulation. TGF transforming growth factor, COL2A1 = collagen type II alpha 1 chain MMP matrix metalloproteinase, NGF nerve growth factor, ALK5 activin receptor-like kinase, \( \textit{JUNB} = \) JunB proto-oncogene, \( \textit{FN1} = \) fibronectin 1
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

**Figure 4**

**A**

+ 1 ng/ml TGFβ1

**B**

+ 1 ng/ml TGFβ1

**C**

Inhibition of TGFβ1-induced gene expression in cartilage explants (24 h)

Vehicle 5Z-7-Oxozeaenol SB-505124 LDN-193189

mean ± sd

+ 1 ng/ml TGFβ1

* p < 0.05
** p < 0.01
*** p < 0.001
In intact cartilage, SB-505124 and Oxozeaenol inhibit TGFβ1-induced pSmad1/5 and pSmad2
As TGFβ1 signaling is known to be affected by cellular context, we sought to validate our observations in monolayer chondrocytes in intact cartilage ex vivo. Therefore we stimulated cartilage explants with 1 ng/ml TGFβ1 with or without inhibitors, and investigated Smad phosphorylation. Explants were stimulated for 2 h to allow for diffusion of TGFβ1 through the cartilage matrix. In explants, 1 ng/ml TGFβ1 induced pSmad2, but Smad1/5 phosphorylation was more difficult to detect (Fig. 4A), in contrast to in monolayer cultures. Therefore BMP9 was used as positive control, which, in comparison to TGFβ, resulted in far stronger induction of pSmad1/5 (for quantification see Additional file 2: Figure S3). A smaller amount of pSmad1/5 was detected in explants treated with TGFβ1 and SB-505124, (5Z)-7-Oxozeaenol or LDN-193189 compared to TGFβ1-treated samples alone. TGFβ-induced phosphorylation of Smad2 was clearly inhibited by SB-505124 and partly by (5Z)-7-Oxozeaenol (Fig. 4A), but on gene expression bSerpine1 was only significantly inhibited by SB-505124 and not by (5Z)-7-Oxozeaenol (Fig. 4B). In explants, expression of bld1 could not be used as a read-out for pSmad1/5 signaling, because TGFβ1 downregulated its expression, in contrast to its induction in monolayer experiments. This is a well-known Smad3-dependent cytostatic effect of TGFβ1 [32], and further indicates that TGFβ1 signaling differs between chondrocytes in monolayer and chondrocytes in intact cartilage. Notably, LDN-193189 did downregulate bld1 expression, even in absence of TGFβ1 (Additional file 3: Figure S2), demonstrating the bioactivity of this compound in cartilage explants. Again, when multiple, TGFβ1-induced, Smad-dependent, genes were measured (Fig. 4C) SB-505124 inhibited TGFβ1-induced expression of bSmad7, bNgf, bFn1 and bTgfb1, and (5Z)-7-Oxozeaenol inhibited TGFβ1-induced expression of bFn1, bTgfb1, whereas no effect of LDN-193189 was observed. Therefore, SB-505124 also inhibits both TGFβ1-induced Smad1/5 and Smad2 phosphorylation and gene expression in intact cartilage, whereas LDN-193189 does not. Furthermore, (5Z)-7-Oxozeaenol partly inhibited both TGFβ1-induced Smad1/5 and Smad2 phosphorylation.

Discussion

TGFβ1 signaling is a crucial regulator of chondrocyte homeostasis. We investigated the impact of small molecule inhibitors on TGFβ1 signaling in primary chondrocytes both in vitro and ex vivo. We report that inhibition of ALK4/5/7 kinase with SB-505124 lowered both TGFβ1-induced Smad1/5 and Smad2/3 phosphorylation and downstream gene expression, whereas inhibition of ALK1/2/3/6 kinase with LDN-193189 did not affect TGFβ1-induced Smad1/5 phosphorylation or transcriptional activity. In addition, we showed that treatment with (5Z)-7-Oxozeaenol, a selective TAK1 kinase inhibitor, attenuated TGFβ1-induced R-Smad phosphorylation and downstream gene expression.

In many tissues, including cartilage, TGFβ1 induces pSmad1/5 and pSmad2/3 [3, 4, 7]. ALK5 is essential for TGFβ1-induced Smad2/3 phosphorylation, whereas a role for ALK1 [4], ALK2 and ALK3 [3] has been demonstrated in TGFβ-induced Smad1/5 phosphorylation (see Fig. 5).
Remarkably, we found that specific inhibition of ALK4/5/7 kinase with SB-505124 was sufficient to inhibit both TGFβ1-induced Smad2/3 and Smad1/5 phosphorylation and transcriptional activity. In contrast, we were unable to block TGFβ1-induced Smad1/5 phosphorylation with LDN-193189, even though we used a dose (0.05 μM) well above the reported IC₅₀ of this inhibitor for ALK1 (0.8 nM), ALK2 (0.8 nM) and ALK3 (5.3 nM) [23]. Additionally, even a 10 times higher dose of LDN-193189 (0.5 μM) was unable to block TGFβ1-induced Smad1/5 phosphorylation. LDN-193189 has been established as an efficient inhibitor of BMP type I receptors, i.e. ALK1/2/3 and ALK6, both in vitro and in vivo [14, 23, 33]. We were able to confirm this inhibitory effect of LDN-193189 on BMP-induced Smad1/5 phosphorylation in primary chondrocytes by using BMPs that signal via ALK1, ALK2 and/or ALK3, i.e. BMP2, BMP7 and BMP9 [24, 25]. Furthermore, we were also able to block caALK1 activity with LDN-193189 in our primary cell cultures. Together, these experiments indicate that LDN-193189 is functional in primary bovine chondrocytes and blocks the ALK1, ALK2 and ALK3 kinase activity at a dose of 0.05 μM. Therefore, our data indicate that ALK1, ALK2 or ALK3 kinase activity is not required for TGFβ1-induced Smad1/5 in primary chondrocytes and cartilage. Notably, various studies using the LDN-193189 precursor dorsomorphin also show that this BMP type I receptor blocker is unable to block TGFβ1-induced Smad1/5 phosphorylation, supporting our observations [34, 35].

Based on structural analyses of R-Smad-ALK interactions, the ability to phosphorylate Smad1/5 is thought to be limited to BMP type I receptors [36, 37]. Our data indicate that ALK5 kinase activity is crucial, and possibly sufficient, for TGFβ1-induced Smad1/5 phosphorylation. In line with our data, it has previously been demonstrated that inhibition of ALK5-kinase with SB-431542 in endothelial cells [6] or with SB-505124 in synovial fibroblasts [34] attenuates TGFβ1-induced phosphorylation of Smad1/5. Furthermore, by knocking down ALK5, it has been shown by others that this receptor is necessary for TGFβ-induced Smad1/5 phosphorylation [3, 6]. Possibly, ALK5 can directly phosphorylate Smad1/5 independently of BMP type I receptors in cartilage. Direct phosphorylation of Smad1 by caALK5 has been demonstrated by in vitro kinase assays [35, 38], and it has been shown that TGFβ1-induced Smad1 phosphorylation is inhibited in cells expressing a kinase defective ALK5 variant: ALK5KR [38]. Together, these studies indicate a direct induction of pSmad1/5 by ALK5, which would be blocked by SB-505124, but challenge the dogma that ALK5 cannot directly interact with Smad1 [36, 37]. In contrast, multiple studies show that in the absence of BMP type I receptors, TGFβ1-induced pSmad1/5 is reduced [3, 6] indicating at least a role for these receptors.

Apart from R-Smad phosphorylation, TGFβ1 also induces TAK1 activation, a key component of Smad-independent TGFβ signaling [5]. Noteworthy, with the use of the specific TAK1 kinase inhibitor (5Z)-7-Oxoozaenol [17] at 0.5 μM, we showed that TAK1 kinase activity facilitates canonical C-terminal R-Smad phosphorylation in chondrocytes and cartilage. Previous studies support a role for TAK1 in R-Smad-dependent signaling; with the use of cartilage-specific TAK1 knockout animals, it has been shown that loss of TAK1 greatly
Fig. 5. Schematic overview of transforming growth factor β (TGFβ)-induced Smad signaling versus bone morphogenic protein (BMP)-induced Smad signaling in cartilage and the effects of small molecule inhibitors. To signal, a TGFβ dimer binds and induces dimerization of its type II receptor: TGFBR2. This complex subsequently recruits a dimer of the type I receptors TGFβ can bind: activin receptor-like kinase (ALK)1, ALK2, ALK3 and ALK5. Signaling complexes containing ALK5 homodimers will induce Smad2/3 phosphorylation, whereas complexes containing ALK5 combined with ALK1, ALK2 or ALK3 will induce both Smad1/5 and Smad2/3 phosphorylation. However, our current study shows that the kinase domain of ALK1, ALK2 or ALK3 is not involved in TGFβ-induced pSmad1/5 because TGFβ-induced pSmad1/5 is inhibited by SB-505124 but not by LDN-193189. Possibly, ALK1, ALK2 and ALK3 function to recruit Smad1/5 to TGFβ receptor complexes but are not activated by TGFBR2 themselves and are therefore not active. For BMP signaling a heterotetrameric complex is also formed from its different type II and type I receptors; BMPR2, ACVR2a and ACVR2b can be used as type II receptors, whereas ALK1, ALK2, ALK3 and ALK6 function as type I receptors. These complexes will induce pSmad1/5 but this is potently blocked by LDN-193189 (as shown in this article) or partially blocked by (5Z)-7-Oxozeaenol (evidence from the literature).

diminishes BMP-induced C-terminal Smad1/5 phosphorylation while not affecting total R-Smad levels [39, 40]. Furthermore, tissue specific knockout of TAK1 in mesenchymal cells of neural crest origin shows that also C-terminal Smad2/3 phosphorylation is inhibited in the absence of TAK1 [41]. The pronounced effects of TAK1 on canonical C-terminal Smad phosphorylation have been ascribed to TAK1-induced Smad-linker phosphorylation, a post-translational Smad modification [41, 42].

Linker modification of R-Smads is an essential aspect of TGFβ1-signaling (reviewed in [43, 44]). The linker region of R-Smads contains threonine and serine residues that can be phosphorylated by various intracellular kinases like extracellular signal-regulated kinases (ERK), cyclin-depdendent kinases (CDKs) and JNKs [43, 45]. Degradation, nuclear transport and interaction of R-Smads with other proteins are regulated by phosphorylation of this region, enabling context-dependent TGFβ signaling [43, 44]. TAK1 can directly interact with R-Smads via the R-Smad MH2 domain [42], and phosphorylate, e.g. Smad2 at threonine 220 (Thr-
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

Phosphorylation of this threonine greatly affects cellular localization and transcriptional activity of this Smad [41, 42]. TAK1 kinase activity is essential for this interaction of TAK1 with R-Smads [42]. As a consequence of this kinase dependency, it has been reported that the use of (5Z)-7-Oxozeaenol results in similar effects on C-terminal Smad1/5 and Smad2/3 phosphorylation as knockout of TAK1 [41]. We did not have access to cartilage-specific TAK1 knockout animals, but with the use of (5Z)-7-Oxozeaenol, we show for the first time that this selective TAK1 kinase inhibitor greatly affects TGFβ1-induced C-terminal R-Smad phosphorylation both in vitro and ex vivo in articular cartilage.

A limitation of our study is the possibly imperfect specificity of the used compounds, making it possible that off-target effects can explain our observations and interfere with our conclusions. However, in the experiments with the caALKs, we did not observe a significant effect of SB-505124 or (5Z)-7-Oxozeaenol on caALK1-induced pSmad1/5, or an effect of (5Z)-7-Oxozeaenol on caALK5-induced pSmad2. Therefore we do not think that these compounds have off-target effects on ALK1. Furthermore, a dose-response curve showed that SB-505124 inhibited TGFβ-induced Smad1/5 phosphorylation more potently than Smad2/3 phosphorylation, demonstrating that this effect of SB-505124 on TGFβ signaling is not an off-target effect obtained at high dosage, and this compound therefore cannot be used to specifically inhibit TGFβ-induced Smad2 in chondrocytes.

Another limitation of our study is that we did not include in vivo data. Unfortunately, the in vivo use of both SB-505124 and LDN-193189 is difficult because of their unfavorable pharmacokinetic properties, resulting in the need for daily re-administration via injection [33]. Up to now, only one study has used SB-505124 as a TGFβ blocker in vivo in a model of OA; i.e. in anterior cruciate ligament transection in mice [46]. In this study, inhibition of TGFβ signaling with 1 mg/kg/day of SB-505124 attenuated OA development, confirming that inhibition of TGFβ signaling with a small molecule inhibitor is a feasible and promising approach to treat OA. However, the use of a higher dose of SB-505124 (2.5 mg/kg/day) was detrimental for cartilage because this resulted in proteoglycan depletion, indicating that total TGFβ inhibition comes with a risk. Possibly, a small molecule inhibitor that prevents TGFβ-induced pSmad1/5 but leaves pSmad2 unaffected will not have this risk. To our knowledge, both LDN-193189 and (5Z)-7-Oxozeaenol have not been used in vivo to study their effects on OA development. They have been used in other applications, e.g. LDN-193189 in atherosclerosis research and (5Z)-7-Oxozeaenol in cancer studies; however, this was not to specifically modulate TGFβ signaling but to inhibit BMP and TAK1 signaling, respectively. Therefore it is as yet unknown how these inhibitors modulate TGFβ signaling in vivo or in OA.

In conclusion, with the use of the small molecule inhibitors SB-505124 and LDN-193189 we were unable to direct TGFβ1-signaling towards either Smad1/5 or Smad2/3 phosphorylation in cartilage. Our data suggest that in cartilage, ALK5 plays a central role in both TGFβ-induced Smad1/5 and Smad2/3 phosphorylation, making it difficult to separate the Smad pathways with the use of currently available intracellular small molecule inhibitors.
of the ALK receptors. Furthermore, we showed that treatment with the TAK1 inhibitor (5Z)-7-Oxoozaenol inhibits TGFβ-induced C-terminal phosphorylation of both Smad1/5 and Smad2/3, suggesting a link between the non-canonical and the canonical TGFβ pathway in cartilage.
Supplementary data

**Figure S1.** Expression of ALK1, ALK2, ALK3 and ALK5 mRNA in primary bovine cartilage and chondrocytes. With the use of qPCR, expression of ALK1, ALK2, ALK3 and ALK5 was measured in both freshly isolated cartilage explants and in primary chondrocytes after 1 week of cell culture in DMEM/F12 supplemented with 10% non-heat-inactivated FCS without passage. All four ALKs were readily detected in both groups, but expression of all the receptors was higher in freshly isolated tissue. For calculations of the -ΔCt, two reference genes were used: βGapdh and βRps14.

**Figure S2.** LDN-193189 in a concentration of 0.05 μM inhibits basal ID1 expression in cartilage explants. Primary chondrocytes were incubated with LDN-193189 for 2 h in a dose of 0.05 μM and bId1 expression was measured using qPCR. LDN-193189 significantly inhibited bId1 expression showing the bioactivity of this compound in cartilage explants.
Figure S3. Quantification of TGFβ-induced pSmad2 and pSmad1/5 in cartilage explants. Quantification of the western blot as shown in Fig. 4a. The experiment was repeated three times. Significance was not obtained due to variation between experiments. pSmad levels were normalized to vinculin levels and plotted as a relative amount in arbitrary units (AU) compared to the control group.
TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.

References


TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity.


8. Summary and concluding remarks
Aim of this thesis

TGFβ-family signaling regulates the homeostasis of many tissues, including cartilage [1]. Via induction of SMAD-dependent and SMAD-independent signaling, members of the TGFβ-family regulate gene expression, protein expression, cell survival, cell growth and cell division [2, 3]. In view of these diverse functions, it is not surprising that disturbed TGFβ-family signaling is linked to the development of many pathologies, including osteoarthritis (OA), the world’s most common joint disease [1]. Currently, there is no disease modifying treatment available for OA yet, fueling the search for such a treatment. To aid in the development of such a treatment, a better understanding of the disease process is of great importance. In part, such comprehension comes from a good knowledge of how tissue homeostasis is regulated. Another part of this understanding can be gained by investigating how risk factors affect tissue homeostasis and contribute to disease development. Therefore, the aims of this thesis were to further characterize (1) the importance of TGFβ-family signaling in cartilage homeostasis and (2) how ageing, the main risk factor for OA, impacts this signaling. To achieve these aims we studied TGFβ-family signaling and its activation in ageing (healthy) cartilage and characterized its effects.

Role of loading-induced TGFβ signaling in maintenance of articular chondrocyte phenotype.

Loading has long been identified as an important contributor to cartilage health and homeostasis [4]. In this thesis we demonstrated in chapter 2 that a crucial role for loading lies in the activation of pSMAD2/3 signaling. We identified this crucial role by first observing that unloading of (bovine) articular cartilage led to a rapid decrease in pSMAD2 levels and pSMAD2/3-mediated gene expression. This decrease in pSMAD2/3-mediated gene expression was even so severe that addition of a functional pSMAD2/3 inhibitor (which works by inhibiting its activating kinases, i.e. the ALK4/5/7 inhibitor SB-505124), had no additional effect, indicating that there was no residual pSMAD2/3 signaling left. Hereafter, we observed that loading was able to rapidly and repeatedly induce both pSMAD2 levels and pSMAD2/3-mediated gene expression to great extent in cartilage explants, thus confirming the importance of loading in SMAD2/3 signaling in cartilage.

After having identified that loading is essential for pSMAD2/3 levels in cartilage, we sought to find its physiological role in cartilage homeostasis. We first investigated the importance of loading-mediated SMAD2/3 signaling in maintenance of cartilage proteoglycan content. We did this by comparing the sulfated glycosaminoglycan (GAG) content of cartilage which was regularly loaded for two weeks to the content of cartilage which was not loaded at all. During the course of these two weeks, the GAG content of unloaded cartilage greatly diminished by 80%, but regular loading did not counteract this decrease in the slightest. In contrast, the addition of fetal calf serum or IGF1 both resulted in maintenance of cartilage...
GAG content. These observations show that loading-induced signaling is unable to maintain GAG content in cartilage, and GAG maintenance is thus not likely a physiological role for loading-mediated pSMAD2/3 signaling.

Subsequently we investigated if loading-mediated pSMAD2/3 signaling has a role in the maintenance of chondrocyte phenotype. Again, we compared cartilage which was regularly loaded to cartilage which was not loaded at all. We observed that in the absence of loading chondrocyte hypertrophy, as measured by collagen type X expression, was greatly induced. Strikingly, loading, even for only a relatively short time interval, was able to potently inhibit this induction of chondrocyte hypertrophy. In contrast, addition of serum (or IGF1) was unable to inhibit or prevent chondrocyte hypertrophy. This led us to the conclusion that prevention of chondrocyte hypertrophy is an important physiological role of loading-mediated SMAD2/3 signaling.

As the explanation behind loading-induced pSMAD2/3 signaling in cartilage, we would like to argue that this is activation of matrix-bound inactive TGFβ. To begin with, we observed that loading-induced pSMAD2/3 signaling can be blocked with the ALK4/5/7 kinase inhibitor SB-505124. This indicates that activation of (one of) these receptors is responsible for loading-mediated pSMAD2/3 signaling. Next, we measured that both ALK4 and ALK5 are well expressed in cartilage, but ALK7 is not, making it unlikely that ALK7 activation is responsible. Therefore we next tested if loading-induced effects can be mimicked by addition of the ALK4 ligand activin A, and/or by the ALK5 ligand TGFβ1 [5]. This showed us that the addition of activin A had no effect but that the addition of TGFβ resulted in similar effects as loading, suggesting a role for TGFβ in loading-induced pSMAD2/3 signaling. Such a role for TGFβ is further supported by the observation that the cartilage matrix contains high levels of inactive TGFβ [6], and in various tissues mechanical force is a potent activator of TGFβ due to mechanical unwinding of the LAP inactivation domain associated with TGFβ [7]. Therefore, we think that loading activates pSMAD2/3 signaling via activation of inactive TGFβ.

Finally, we explored how loading further affects chondrocyte phenotype. We did this by measuring gene expression of chondrocytes shortly and one day after they were exposed to loading. In these experiments we observed that loading (rapidly) induced the expression of TGFβ1 and ALK5, while it inhibited the expression of ALK1. These observations indicate that upon compression a positive feedback loop is activated in chondrocytes which induces the production of TGFβ1 and helps direct TGFβ1 signaling to pSMAD2/3 by changing the TGFβ receptor ratio in favour of more ALK5 expression and less ALK1 expression. This positive feedback loop further strengthens the importance of loading in pSMAD2/3 signaling in cartilage.

**Ageing negatively affects TGFβ signaling in cartilage**

After we established the importance of loading in activation of pSMAD2/3 signaling in cartilage via activation of TGFβ, we realized that ageing might negatively affect this process.
In a previous study of our lab we had identified age-related changes in expression of the TGFβ receptors ALK1 and ALK5 that resulted in relatively higher ALK1 expression levels in old mice compared to young mice [8, 9]. These changes positively correlated with OA development in both the DMM model of OA and STR/ort mice. However, in mice, ageing and OA development go hand in hand and therefore it was unclear if this receptor change preceded or reflected OA development. Furthermore, it was also still unclear if and how such a change in receptor expression affected their function. Therefore we re-investigated age-related changes in TGFβ-family signaling components, but this time in a model organism in which ageing can be separated from OA development; the cow. Bovine cartilage can be obtained from a wide age-range of individuals, and OA affected cartilage can be visually detected and excluded from analysis.

In chapter 3 we used this approach to study cartilage ageing on histology and gene and protein expression. The cartilage samples we studied were obtained from metacarpophalangeal joints of 42 animals ranging from 1 to 10 years old. In this age range, many aspects of ageing cartilage could be observed; cartilage thinning, loss of cellularity, profound (1000-fold) loss of collagen type 2 expression and tidemark duplication(s) [10, 11]. Importantly, we also observed age-related loss of the ALK5 receptor but saw only minor changes in ALK1 expression levels. This observation supports the idea that changing receptor levels precede the development of OA, and can therefore be a cause for OA. To further support this idea, we also investigated if a change in ALK1 and ALK5 receptor levels affects TGFβ signaling. We did this by exposing bovine cartilage of different ages to TGFβ ex vivo and measuring the induction of Serpine1, a gene whose TGFβ-induced expression is SMAD3 dependent [12]. This experiment showed us that with advancing age less Serpine1 expression is induced by TGFβ, indicating a loss of TGFβ potency in inducing pSMAD3 in old cartilage. To evaluate if ALK1 signaling was affected, we exposed cartilage explants in a similar setting to BMP9, a high affinity ALK1 ligand [13], and measured ID1, a SMAD1/5-dependent gene. Strikingly, ageing did not negatively affect BMP9 signaling, and BMP9 signaling was possibly even increased with advancing age. Together these observations indicate that ageing differentially affects the ALK1 and ALK5 receptors and their ability to signal. This implicates that ageing can alter the balance between TGFβ-induced SMAD2/3 and SMAD1/5 signaling and thus change the effects that TGFβ has on chondrocyte phenotype from protective to deleterious.

Now that we confirmed that ageing indeed negatively impacts TGFβ signaling in old (bovine) cartilage, we investigated if ageing also negatively affects compression mediated pSMAD2/3 (TGFβ) signaling in chapter 4. We did this by exposing bovine explants of different ages ex vivo to loading and measured phosphorylated SMAD2 levels. The induction of pSMAD2 by compression was markedly decreased by up to 90% in old cartilage. Also Serpine1 gene expression was significantly less increased in old cartilage compared to young cartilage upon compression. Together, these observations show that loading-induced pSMAD2/3
signaling is impaired in old cartilage. Because the loading we applied to the cartilage was force controlled, we noticed that older cartilage was more difficult to compress at a physiological force of 3 MPa. However, the reduced activation of pSMAD2/3 is not due to this increased stiffness of old cartilage. We tested this by increasing the force from 3 MPa to 12 MPa and repeating the experiment. This increased force resulted in a similar amount of cartilage deformation between young and old, but did not affect the difference in pSMAD2/3 activation between these two age groups. In conclusion, age-related changes have a significant impact on the response of chondrocytes to mechanical stimuli, including their pSMAD2/3 levels. These observations indicate that the essential role of TGFβ in maintenance of cartilage we postulated in chapter 2 is negatively impacted by ageing.

New insights into the role of TGFβ signaling in cartilage

Now that it was clear that loading-induced TGFβ signaling is essential for cartilage homeostasis, and that ageing negatively affects this protective mechanism, we wanted to further explore the importance of TGFβ signaling in cartilage.

In view of the observations we did in ageing cartilage, we began by further exploring the impact of ALK1 signaling on chondrocyte phenotype. We did this by stimulating chondrocytes with BMP9, a high affinity ALK1 ligand [13], and measured the effects thereof on SMAD activation, gene expression and chondrocyte phenotype. We chose to use BMP9 instead of TGFβ to separate (putative) deleterious ALK1 signaling from (putative) protective ALK5 signaling which would both be activated simultaneously in case of TGFβ [14]. In chapter 5 we show that BMP9 potently induced SMAD1/5/8 phosphorylation and SMAD1/5-dependent gene expression. In chondrocytes that were exposed to BMP9 for 1 week, BMP9 signaling led to increased expression of collagen type X and alkaline phosphatase, two markers of chondrocyte hypertrophy [15]. This indicates that long term ALK1 signaling is detrimental to chondrocyte phenotype.

However, remarkably, BMP9 also induced some SMAD2 phosphorylation, and more importantly, synergized with TGFβ on induction of SMAD2 phosphorylation and SMAD3-dependent [16] luciferase production in an CAGA₃-luciferase reporter assay. Furthermore, TGFβ was able to potently inhibit BMP9-induced collagen type X and alkaline phosphatase expression, possibly facilitated by the synergy between these ligands. These results again demonstrate the importance of TGFβ signaling for maintenance of chondrocyte phenotype. Notably, the synergy between BMP9 and TGFβ was an unique aspect of BMP9 signaling compared to the signaling of two other BMPs, BMP2 and BMP7, showing that this synergy is not a generalized effect of BMPs. A possible explanation for the synergy between TGFβ and BMP9 is that BMP9 outcompetes TGFβ for ALK1 binding due to its higher affinity, forcing TGFβ to make use of ALK5-ALK5 signaling complexes which are assumed to produce twice as much pSMAD2/3 as ALK1-ALK5 complexes because ALK1 cannot phosphorylate SMAD2/3 [17].
We would like to argue that the inhibition of BMP9 induced hypertrophy is an important function of TGFβ in cartilage. To begin, BMP9 is circulating in large amount [18], making it likely that chondrocytes are (continuously) exposed to this growth factor. Because we observed that prolonged BMP9 signaling induces chondrocyte hypertrophy, we therefore think that chondrocytes are continuously exposed to a drive towards hypertrophy. An inhibitor of this would thus be required to maintain articular cartilage phenotype and in view of our results we propose that this is TGFβ. Importantly, in \textit{ex vivo} cartilage explants, BMP9 strongly induces cartilage matrix production (i.e. collagen type 2 and proteoglycans) (literature [19] and own observations) but TGFβ does not negatively affect this positive aspect of BMP9 signaling (own observation). Therefore, TGFβ signaling can separate the good from the bad in BMP9 signaling in cartilage.

After having established that TGFβ inhibits BMP9-mediated chondrocyte hypertrophy, we wanted to further examine TGFβ-induced gene expression in chondrocytes and cartilage to gain more insights into its role in cartilage biology. One approach we took was by stimulating (primary) human, murine and bovine chondrocytes with TGFβ for both short and long time intervals and measuring changes in gene expression.

In \textit{chapter 6} the results of these experiments are shown that led us to conclude that TGFβ potently induces nerve growth factor (NGF), an important pain stimulus [20], via an ALK5-dependent mechanism. In human, murine and bovine chondrocytes, TGFβ induced \textit{NGF} mRNA expression after 24 hours. Strikingly, TGFβ was very potent in inducing this \textit{NGF} mRNA expression, it was even more potent than IL1β which we had used as positive control. Next we used the ALK1 inhibitor LDN-193189, the ALK5 inhibitor SB-505124 and the TAK1 kinase inhibitor 5Z-(7)-oxozeaenol to test which receptor was crucial for this TGFβ-induced \textit{NGF} mRNA expression and if SMAD-independent signaling was involved. In this experiment, we observed that SB-505124 fully inhibited TGFβ-induced \textit{NGF} expression, whereas LDN-193189 had no effect. Therefore we concluded that this effect of TGFβ is ALK5 dependent. Furthermore, The 5Z-(7)-oxozeaenol compound inhibited TGFβ-induced \textit{NGF} expression by approximately 50%, indicating involvement of SMAD-independent signaling. These observations make it clear that TGFβ can potently induce \textit{NGF} mRNA expression. This links TGFβ to pain in OA, because NGF is a key driver of OA pain, because clinical studies have shown that inhibiting it (fully) blocks OA related pain [20]. However, we did notice that \textit{in vitro} it was very difficult to detect NGF protein production in either human, murine or bovine chondrocytes after stimulation with TGFβ, even though mRNA expression was abundantly present. Only in human OA cartilage we were able to detect NGF protein using immunohistochemistry. To us, this indicates that a second stimulus (which would be present in OA cartilage) is required to mediate the translation of \textit{NGF} mRNA into protein. Such a mechanism would prevent the (unwanted) continuous production of NGF in healthy cartilage exposed to TGFβ signaling \textit{e.g.} by loading, but would allow for rapid NGF production in case of tissue damage or inflammation.
Modulation of TGFβ signaling using small molecule inhibitors

Based on our previous results we think it is clear that TGFβ-induced pSMAD2/3 via ALK5 is chondroprotective and an especially potent inhibitor of chondrocyte hypertrophy. In contrast, ALK1 signaling via pSMAD1/5 was associated with chondrocyte hypertrophy, and many SMAD-independent pathways like TAK1 signaling are associated with cartilage damage in literature. Therefore TGFβ has both good and bad sides to its signaling with respect to cartilage homeostasis [1]. For this reason, we wanted to explore if we could separate the for cartilage deleterious signaling from the chondroprotective pSMAD2/3 signaling with the use of small molecule inhibitors. Our approach was to add either LDN-193189; i.e. an ALK1/2/3/6 kinase inhibitor, or SB-505124, i.e an ALK4/5/7 inhibitor, or (5Z)-7-oxozeaenol, i.e a TAK1 inhibitor, to primary bovine chondrocytes in combination with TGFβ, and investigate SMAD phosphorylation and TGFβ-induced gene expression. In chapter 7 the results hereof are shown. To our surprise, we observed that the SB-505124 compound fully inhibited not only TGFβ-induced SMAD2/3 phosphorylation and downstream signaling, but also SMAD1/5 phosphorylation and signaling. In contrast, we observed no effect of the LDN-193189 compound on TGFβ signaling. These results would indicate that ALK5 kinase activity is phosphorylating both SMAD2/3 and SMAD1/5, and that ALK1 kinase activity is not involved in TGFβ signaling. Because this challenges the dogma that ALK5 kinase is not able to phosphorylate SMAD1/5 due to steric hindrance between SMAD1/5 and the ALK5 kinase pocket [17], we sought to validate the specificity of SB-505124. We did this by overexpressing a constitutively active form of ALK1, a form of the receptor which continuously phosphorylates SMAD1/5 [14]. Importantly, the function of this receptor was not inhibited by SB-505124, showing that this compound does not affect ALK1 kinase activity. In contrast, constitutively active ALK1 was inhibited by the LDN-193189 compound. These results support the previous results that the ALK5 kinase activity is crucial for both TGFβ-induced SMAD2/3 and SMAD1/5 phosphorylation. Furthermore, these show that with these compounds it is not possible to separate TGFβ-induced pSMAD1/5 from pSMAD2/3. However, we did observe that the IC50 of SB-505124 for pSMAD1/5 is much lower than that for pSMAD2/3. This indicates that the phosphorylation of pSMAD1/5 by ALK5 is much more sensitive for ALK5 kinase inhibition, and that therefore a dose of this inhibitor might be found that already fully inhibits TGFβ-induced pSMAD1/5 but only marginally affects SMAD2/3 phosphorylation.

When we studied the effects of (5Z)-7-oxozeaenol on TGFβ signaling, we noticed that this compound inhibited both TGFβ-induced SMAD2/3 and SMAD1/5 phosphorylation, but the latter more extensively. Because (5Z)-7-oxozeaenol is not known to inhibit ALK1 or ALK5 kinase activity, this indicates that SMAD-independent signaling at least partly regulates SMAD-dependent signaling. This inhibition of SMAD phosphorylation was also reflected in TGFβ-induced gene expression, further illustrating the importance of SMAD-independent signaling in transduction of TGFβ signaling. Possibly the SMAD-independent signaling interacts directly with SMAD proteins in order to facilitate their C-terminal phosphorylation. This
would also explain why SMAD activation by TGFβ-family members is relatively slow compared to the induction of SMAD-independent signaling, e.g. in primary chondrocytes it takes approximately 1 hour for SMAD dependent signaling to reach its maximum, whereas SMAD-independent signaling takes only 15 minutes to reach its peak (own observation). Possibly, the SMAD linker role plays an important role in this aspect of TGFβ signaling [21]. This SMAD-linker is a domain connecting the MH1 DNA binding domain of SMADs to their MH2 protein binding domain. The linker region can be extensively post-translationally modified, e.g. via ubiquitination, phosphorylation, acetylation etc, regulating the function of both the MH1 and MH2 domain and SMAD half-life [21]. These modifications are done by intracellular kinases, including those that are activated by SMAD-independent signaling like TAK1. This makes the SMAD-linker a bridge between SMAD-dependent and SMAD-independent signaling.

SMAD linker modifications are dependent on cellular context [21]; this context regulates the kinases making the modifications. We think this can explain the results we also obtained in chapter 7 when studying TGFβ signaling in cartilage explants and compared the results hereof to those we obtained in chondrocytes in monolayer. Remarkably, we had problems detecting TGFβ-induced SMAD1/5 phosphorylation in healthy bovine cartilage explants; whereas in chondrocytes in monolayer this pathway is abundantly activated upon addition of TGFβ, in explants this was not the case. Furthermore, also regulation of ID1 expression by TGFβ was totally opposite in explants compared to monolayer; from induction in monolayer to inhibition in explants. Both these observation nicely illustrate the importance of cellular context in TGFβ signaling; the cellular environment regulates TGFβ signaling and its outcome.

Final considerations
The goals of this thesis were to further characterize (1) the importance of TGFβ-family signaling in cartilage homeostasis and (2) how ageing, the main risk factor for OA, impacts this signaling. We succeeded in characterizing the importance of pSMAD2/3 signaling in loading-mediated regulation of cartilage homeostasis. Moreover, we identified that ageing negatively impacts this pSMAD2/3 signaling by lowering ALK5 expression. Furthermore, we established the importance of TGFβ signaling especially to protection of chondrocyte phenotype against hypertrophy inducing processes like BMP9 signaling. The observations we did on TGFβ-induced NGF expression help us link TGFβ signaling to OA pain, and we learned that separating TGFβ-induced pSMAD1/5 from pSMAD2/3 is more difficult than expected, due to the reliance of both processes on ALK5.

In conclusion, based upon these results we suggest a model for cartilage homeostasis in which TGFβ is the factor that keeps articular cartilage healthy and protects it from hypertrophy (figure 1). The cartilage ECM contains a large amount of (inactive) TGFβ which is activated upon loading of the cartilage. This subsequently activates a protective pathway that results in TGFβ and ALK5 expression, and inhibition of ALK1 and chondrocyte

Summary and concluding remarks
hypertrophy. This inhibition of chondrocyte hypertrophy is important because articular cartilage is continuously exposed to hypertrophy inducing factors like BMP9 via the synovial fluid. Unfortunately, ageing negatively affects this protective mechanism by reducing ALK5 expression, the receptor TGFβ uses to establish these protective effects. Because we observed that the reduction in ALK5 expression precedes OA development, we think this is a cause for OA; the protective, anti-hypertrophic, loading-mediated mechanism is becoming less and less effective with advancing age, eventually leading to failure of this protective system. This model underlines that osteoarthritis is a disease and not just “wear and tear”, and indicates that reverting or inhibiting this age-related decrease in ALK5 expression will be a challenge for the future to combat age-related development of OA.
Fig. 1. Proposed model for cartilage homeostasis. TGFβ keeps young articular cartilage healthy by counteracting hypertrophy-promoting signaling like that of BMP9. Mechanical stimulation of cartilage activates latent TGFβ, which induces pSMAD2/3 via ALK5. This pSMAD2/3 induces TGFβ and ALK5 expression, establishing a positive feedback loop, and lowers ALK1 expression and downstream effects of BMP9 like hypertrophy. However, upon ageing, ALK5 expression is gradually lowered, resulting in less activation of this cartilage protective mechanism, slowly leading to failure and the onset of cartilage damage.
References


Nederlandse samenvatting

De transforming growth factor β (TGFβ)–familie is een verzameling van groeifactoren die allen gebruik maken van activin-receptor like kinases (ALK1 t/m ALK7) om signalen door te geven aan cellen. ALKs activeren intracellulaire SMAD eiwitten door deze te fosforyleren, waarna de SMADs naar de kern van de cel migreren. In de kern binden de SMADs aan het DNA en sturen ze genexpressie. Op deze manier reguleren leden van de TGFβ-familie de homeostase van vele weefsels in het lichaam, waaronder die van artificial kraakbeen. Articulair kraakbeen is het weefsel dat de botuiteinden in synoviale gewrichten bedekt, en dit weefsel is zeer belangrijk voor de absorptie van krachten die vrijkomen bij bewegen en maakt wrijvingsarme beweging van de gewrichtsvlakken mogelijk. Een verstoorde werking van leden van de TGFβ-familie is gekoppeld aan het ontstaan van artrose, de meest voorkomende gewrichtsziekte ter wereld. Artrose wordt gekenmerkt door afbraak van het kraakbeen, wat leidt tot functieverlies van het gewricht en pijn. Er is helaas nog geen afdoende behandeling voor artrose afgezien van vervanging van het aangedane gewricht door een kunstgewricht. Hierdoor is er een grote behoefte aan een therapie die het ziekteproces aanpakt. De ontwikkeling van zo'n behandeling heeft baat bij een goed begrip van hoe de homeostase van kraakbeen gereguleerd wordt en bij een goed begrip van hoe risicofactoren deze homeostase beïnvloeden. Daarom waren de doelen van dit proefschrift om in kaart te brengen (1) het belang van de TGFβ-familie, en dan met name van TGFβ, voor de homeostase van kraakbeen en (2) hoe veroudering, de belangrijkste risicofactor voor artrose hierop ingrijpt.

Het belang van regelmatige belasting voor het behoud van het fenotype van de kraakbeencel

Het is al langere tijd bekend dat regelmatig belasten goed is voor articulair kraakbeen; zonder belasting gaat articulair kraakbeen snel kapot. In hoofdstuk 2 van dit proefschrift wordt aangetoond dat belasting SMAD2/3 signalering activeert (pSMAD2/3) in chondrocyten, de enige cellen in het kraakbeen en daarom verantwoordelijk zijn voor het onderhoud van dit weefsel. Het belang van belasting voor SMAD2/3 signalering in chondrocyten werd voor het eerst geobserveerd in onbelast kraakbeen waaraan een remmer van SMAD2/3 signalering was toegevoegd; deze remmer bleek, tegen de verwachting in, geen effect te hebben. Uit nadere analyse bleek dat dit onbelaste kraakbeen amper tot geen pSMAD2/3 bevatte waardoor deze remmer onverwacht geen effect kon hebben. Vervolgens bleek dat experimentele mechanische belasting van kraakbeen een sterke pSMAD2/3 activiteit tot gevolg had, die weer verdween na het weghalen van de belasting. Samen bevestigden deze experimenten dus het belang van belasting voor pSMAD2/3 activiteit in chondrocyten.

Nadat zo het belang van belasting voor pSMAD2/3 activiteit in kraakbeen was aangetoond, is onderzocht wat het belang hiervan is voor kraakbeenhomeostase. Als eerste werd een mogelijke rol in het onderhoud van de hoeveelheid glycosaminoglycanen
in kraakbeen onderzocht. Deze glycosaminoglycanen zijn essentiële bouwstenen van het kraakbeen en belangrijk voor de schokabsorberende functie van kraakbeen. Van dwarslaesiepatiënten is bekend dat het niet gebruiken van kraakbeen leidt tot een verlies van deze bouwstenen. Ook in de gedane experimenten bleek onbelast kraakbeen over een tijdsbestek van 2 weken een zeer aanzienlijk deel (80%) van deze bouwstenen te verliezen. Dit verlies was echter tegen te gaan door toevoeging van serum of insulin-like growth factor 1 (IGF1), een andere belangrijke signaalstof voor kraakbeen. Echter belasting noch TGFβ, een sterke pSMAD2/3 activator, hadden enig effect op de hoeveelheid glycosaminoglycanen in kraakbeen. Hierdoor is het onwaarschijnlijk dat belasting of TGFβ signalering de hoeveelheid glycosaminoglycanen in kraakbeen reguleert. Een ander aspect van de kraakbeenbiologie dat vervolgens onderzocht werd, is de handhaving van het chondrocytfenotype. Een belangrijk aspect van artrose is namelijk het verlies van dit fenotype en het ontstaan van zogenaamde hypertrofe chondrocyten, cellen die hun omgeving, en dus het kraakbeen, actief afbreken. Uit experimenten bleek dat ook het aantal hypertrofe chondrocyten aanzienlijk toeneemt als kraakbeen twee weken lang niet belast wordt. Zowel belasting als TGFβ bleken echter de vorming van deze cellen sterk te remmen, terwijl serum of IGF1 dit niet deden. Hieruit kan geconcludeerd worden dat het remmen van de vorming van hypertrofe chondrocyten een belangrijke rol is van belasting en TGFβ.

Een mogelijke verklaring voor de pSMAD2/3 activiteit in kraakbeen na belasting is dan ook de activering van TGFβ, en wel op basis van de volgende argumenten: (1) de activering van pSMAD2/3 in kraakbeen is geheel te remmen met SB-505124, een remmer van ALK4/5/7. Dit laat zien dat (een van) deze receptoren essentieel zijn (is) voor dit proces. (2) Van deze receptoren zijn ALK4 en ALK5 te detecteren in kraakbeen maar ALK7 niet of nauwelijks. Dit maakt een rol van ALK7 of een ligand hiervan onwaarschijnlijk. (3) De effecten van belasting op chondrocythypertrofie kunnen geheel nagebootst worden met behulp van de ALK5 ligand TGFβ maar niet met de ALK4 ligand Activin A. (4) De extracellulaire matrix van kraakbeen bevat zeer veel TGFβ gebonden in inactieve vorm, en uit studies met fibroblasten is gebleken dat belasting deze inactieve TGFβ kan activeren. Samen ondersteunen deze argumenten dus de hypothese dat activering van TGFβ de verklaring is voor belasting-gerelateerde pSMAD2/3 activering.

Opvallend genoeg had belasting niet alleen een remmend effect op chondrocythypertrofie, maar verhoogde het ook de expressie van TGFβ1 en diens receptor ALK5, terwijl het de expressie van ALK1 verlaagde. Omdat ALK5 voor activering van pSMAD2/3 zorgt, en ALK1 voor pSMAD1/5, duiden deze observaties erop dat belasting in kraakbeen een positieve terugkoppeling beweert: belasting activeert TGFβ, en de pSMAD2/3 signalering hiervan zorgt voor nieuwe TGFβ en verhoogt de SMAD2/3 activerende receptor. Ook verlaagt belasting de SMAD1/5/9 activerende receptor waardoor TGFβ signalering eerder via pSMAD2/3 zal verlopen. Door dit terugkoppelingsmechanisme wordt dus pSMAD2/3 signalering door zichzelf in stand gehouden.
De effecten van veroudering op TGFβ signalering in chondrocyten

Nadat het belang van belasting voor pSMAD2/3 activering en chondrocyt-fenotype was aangetoond, is vervolgens onderzocht hoe risicofactoren hierop ingrijpen. De belangrijkste risicofactor voor artrose is veroudering, en daarom is voornamelijk hieraan aandacht besteed. Om veroudering in kraakbeen te kunnen bestuderen is gebruik gemaakt van de koe als model. Koeienkraakbeen lijkt namelijk sterk op humaan kraakbeen en is verkrijgbaar in een leeftijdsreeks waarin alle veranderingen waargenomen kunnen worden die ook in verouderend menselijk kraakbeen gezien worden; zoals dunner worden, het toenemen van de gecalcificeerde zone, het ontstaan van meerdere tidemarks, het verlies van chondrocyten en het verlies van collageen type 2 expressie. Gezond menselijk kraakbeen zelf was niet te gebruiken vanwege schaarste. Het gebruik van koeienkraakbeen had verder als voordeel dat artrotisch kraakbeen visueel uitgesloten kon worden van verdere analyse, zodat artrose en veroudering goed van elkaar gescheiden konden worden. Veroudering en artrose kunnen namelijk heel tegengestelde effecten in kraakbeen teweeg brengen, zoals bijvoorbeeld chondrocyt-senescence versus proliferatie, waardoor de interpretatie van resultaten bemoeilijkd word indien beide processen niet gescheiden worden.

In *hoofdstuk 3* wordt beschreven dat in koeienchondrocyten veroudering de hoeveelheid ALK5 verlaagt, terwijl het weinig effect heeft op ALK1 expressie. Deze verandering in receptorenniveaus heeft gevolgen voor de cellulaire gevoeligheid voor TGFβ; jong kraakbeen reageert potenter op TGFβ dan oud kraakbeen, met name als een pSMAD3-gevoelig gen (PAI1 of Serpine1) gemeten wordt. Voor ALK1 signalering via pSMAD1/5 werd geen verlaging geobserveerd tussen jong en oud: toevoeging van BMP9, een ligand met hoge affiniteit voor ALK1 resulteerde in oud kraakbeen in een expressie van het pSMAD1/5-gevoelige gen ID1 die zeker zo hoog, zo niet hoger, was als in jong kraakbeen. Deze observaties laten zien dat veroudering de ALK1 en ALK5 receptoren en hun signalering verschillend beïnvloedt, en impliceren dat de signalering van een ligand die van beide receptoren gebruik kan maken, zoals TGFβ, dus kan verschuiven bij veroudering.

Na zo te hebben vastgesteld dat veroudering ALK5 expressie en functie negatief beïnvloedt, is vervolgens in *hoofdstuk 4* onderzocht of veroudering ook door belasting geïnduceerde pSMAD2/3 signalering negatief beïnvloedt. Dit is gedaan door koeienkraakbeen van verschillende leeftijden te belasten en de uitkomsten te vergelijken. Deze experimenten lieten zien dat leeftijd ook op door belasting geïnduceerde pSMAD2/3 grote nadelige effecten had: in oud kraakbeen kon slechts 10% van de pSMAD2/3 en bijbehorende genexpressie van jong kraakbeen gemeten worden. Om uit te sluiten dat dit kwam doordat ouder kraakbeen moeilijker samen te drukken is dan jong kraakbeen zijn de experimenten ook herhaald met een hogere druk; 12 MPa in plaats van 3 MPa. Bij deze grotere druk wordt zowel jong als oud kraakbeen vergelijkbaar samengedrukt, maar dit had geen effect op het verschil in pSMAD2/3 activering dat gemeten werd tussen jong en oud. Veroudering verlaagt dus inderdaad de belasting-geïnduceerde pSMAD2/3 niveaus in chondrocyten.
Nieuwe inzichten in de rol van TGFβ signalering in kraakbeen.

Nu het belang van TGFβ signalering voor kraakbeen en de nadelige effecten van veroudering hierop duidelijk waren geworden is vervolgens gekeken welke rollen TGFβ nog meer vervult in kraakbeen naast bescherming van het chondrocytenfototype.

Vanwege de resultaten aangaande ALK1 expressie bij veroudering is in hoofdstuk 5 eerst gekeken naar de effecten van BMP9, een ligand met hoge affiniteit voor deze receptor, op chondrocyten en de effecten van TGFβ hierop. BMP9 induceerde een krachtig pSMAD1/5 signaal in kraakbeen met chondrocythypertrofie tot gevolg zoals bleek uit het meten van collageen type X en alkalische fosfatase. Deze observatie impliceert dat over langere duur ALK1 signalering inderdaad schadelijk is voor kraakbeen. Echter, BMP9 induceerde ook pSMAD2 en, zeer belangrijk, vertoonde ook synergie met TGFβ op de activering van pSMAD2/3. Mogelijk geholpen door deze synergie was TGFβ in staat om de door BMP9 geïnduceerde hypertrofie geheel te remmen. Deze resultaten onderstrepen nogmaals het belang van TGFβ voor het behoud van chondrocytenfototype. Opmerkelijk was ook dat deze synergie van BMP9 met TGFβ uniek was in vergelijking met andere BMPs zoals BMP2 en BMP7. Een mogelijke verklaring voor de synergie kan competitie zijn tussen BMP9 en TGFβ zijn voor de ALK1 receptor; vanwege diens hogere affiniteit zal BMP9 deze competitie winnen waardoor TGFβ gedwongen wordt via ALK5-ALK5 complexen te signaleren. Theoretisch geven deze ALK5-ALK5 complexen twee keer zoveel pSMAD2/3 als ALK5-ALK1 complexen omdat ALK1 geen pSMAD2/3 kan activeren.

De rol van TGFβ in het tegengaan van BMP9 geïnduceerde hypertrofie is waarschijnlijk een belangrijk mechanisme voor de handhaving van kraakbeenhomeostase in vivo. BMP9 is namelijk een groeifactor die in hoge concentratie gemeten kan worden in bloed, waardoor het waarschijnlijk is dat chondrocyten continu aan deze factor worden blootgesteld via de synoviale vloeistof in de gewrichtsholte. Omdat BMP9 signalering voor hypertrofie kan zorgen, is er dus continu een rem nodig om dit te voorkomen. TGFβ is bij uitstek geschikt om als deze rem te functioneren gezien de observaties uit hoofdstukken 1 en 5. Opmerkelijk is ook dat BMP9 in kraakbeen kan zorgen voor de aanmaak van glycosaminoglycanen en collagenen, maar dat TGFβ op deze positieve effecten van BMP9 geen remmende werking heeft. TGFβ kan dus het goede van het kwade scheiden in BMP9 signalering.

In hoofdstuk 6 wordt een geheel nieuwe functie van TGFβ besproken; diens rol in de expressie van nerve growth factor (NGF), een zeer belangrijke pijnprickel in artrose. Na stimulatie van chondrocyten en kraakbeen met verschillende concentraties TGFβ bleek dat TGFβ een potente stimulus is voor de transcriptie van NGF. Zowel in humaan-, als muizen- en koeienkraakbeen induceerde TGFβ hoge genexpressie van NGF (mRNA). De door TGFβ geïnduceerde expressie was zelfs hoger dan de NGF expressie die door IL1β, een krachtige ontstekingsprickel, bereikt werd. Deze studie verbindt dus TGFβ aan pijn in artrose, omdat uit studies is gebleken dat NGF bestrijding de pijn in artrose volledig laat verdwijnen. Om te onderzoeken via welke intracellulaire signaleringsroute TGFβ dit effect teweeg bracht, zijn de
het redigeren van TGFβ signalering met small molecule inhibitors

Uit de voorgaande resultaten is gebleken dat ALK5 signalering via pSMAD2/3 beschermend is voor het fenotype van de chondrocyte, terwijl ALK1 signalering via pSMAD1/5 gekoppeld kon worden aan chondrocysthypertrofie. Omdat TGFβ beide receptoren en signaleringsroutes kan activeren, kan TGFβ dus zowel goede als slechte effecten hebben op chondrocyten. Vanuit therapeutisch oogpunt is het interessant om te proberen beide routes te scheiden en wel zodanig dat alleen de positieve effecten behouden blijven. Daarom is in hoofdstuk 7 geprobeerd om met behulp van zogenaamde “small molecule inhibitors” de TGFβ signaleringsroutes van elkaar te scheiden. Hiervoor hebben we wederom gebruik gemaakt van de ALK1 remmer LDN-193189, de ALK5 remmer SB-505124 en de TAK1 remmer (5Z)-7-Oxozeaenol. Onverwacht bleek dat SB-505124 niet alleen de TGFβ-geïnduceerde pSMAD2/3 remde, maar ook de TGFβ-geïnduceerde pSMAD1/5, en dat LDN-193189 geen enkel effect had. Deze resultaten impliceren dat ALK5 zowel pSMAD2/3 als pSMAD1/5 kan activeren. Omdat deze gevolgtrekking in gaat tegen het dogma dat dit onmogelijk zou zijn vanwege sterische hindering van SMAD1/5 met het kinasedomein van ALK5, is de functie van SB-505124 extra gevalideerd. SB-505124 bleek echter naar behoren te werken en had geen enkel effect op ALK1, waardoor het niet uit te sluiten is dat ALK5 inderdaad ook pSMAD1/5 activeert. Deze onverwachte mogelijke dubbele rol van ALK5 bemoeielt dus het scheiden van beide signaleringsroutes. Een aanknopingspunt voor het scheiden van beide SMAD signaleringsroutes is echter wel dat er veel minder SB-505124 nodig was om TGFβ-geïnduceerde pSMAD1/5 volledig te remmen dan dat er nodig was om TGFβ-geïnduceerde pSMAD2/3 te remmen. Door slimme dosiskeuze zou de gewenste scheiding van signaleringsroutes dan alsnog bereikt kunnen worden.

Naast SB-505124 had ook (5Z)-7-Oxozeaenol een sterk effect op TGFβ-geïnduceerde pSMADs; zowel pSMAD2/3 als pSMAD1/5 werden geremd. Een rechtstreeks effect van (5Z)-7-Oxozeaenol op de ALK receptoren was moeilijk aan te tonen waardoor een verklaring voor deze remming ook in een ander mechanisme gezocht kan worden. Mogelijk speelt een direct effect van TAK1 op de SMAD eiwitten een belangrijke rol. TAK1 kan zowel
SMAD2/3 als SMAD1/5 eiwitten modifieren door ze te fosforyleren in hun linker domein. Uit literatuur is bekend dat deze linker modificaties verstrekende gevolgen kunnen hebben voor het functioneren van de SMADs. Mogelijk speelt zo’n door (5Z)-7-Oxozeaenol geremde modificatie een belangrijke rol bij activering van de SMADs, bijvoorbeeld als deze modificatie een vereiste is voor transport van de SMAD naar de receptor.

**Conclusie**

De doelen van dit proefschrift waren om in kaart te brengen (1) het belang van de TGFβ-familie, en dan met name van TGFβ, voor de homeostase van kraakbeen en (2) hoe veroudering, de belangrijkste risicofactor voor artrose hierop ingrijpt. De experimenten die gedaan zijn om deze doelen te bereiken hebben laten zien dat belasting pSMAD2/3 activeert in kraakbeen, en dat dit een belangrijk mechanisme is voor het tegengaan van chondrocythypertrofie. Veroudering ontregelt dit systeem door expressie van de ALK5 receptor te verlagen, waardoor chondrocyten niet langer in staat zijn pSMAD2/3 te fosforyleren na blootstelling aan TGFβ. Hierdoor valt het beschermende mechanisme tegen hypertrofie weg bij veroudering. Het belang van TGFβ en SMAD2/3 signalering in het tegengaan van hypertrofie werd ook gevonden in chondrocyten die blootgesteld waren aan BMP9. In dit proefschrift is ook voor het eerst TGFβ gekoppeld aan pijn in artrose via NGF productie. Het scheiden van de intracellulaire signaleringsroutes van TGFβ om zo de goede effecten van de kwade los te kunnen koppelen bleek echter moeilijker dan gedacht door de centrale rol van ALK5 in alle SMAD-afhankelijke signaleringsroutes van TGFβ.

Op basis van alle resultaten in dit proefschrift kan een hypothetisch model opgesteld worden over kraakbeenhomeostase waarin TGFβ de factor is die kraakbeen gezond houdt door het te beschermen tegen chondrocyt hypertrofie. In jong kraakbeen activeert belasting TGFβ waardoor via een positieve terugkoppeling nieuwe TGFβ gemaakt wordt en chondrocyt hypertrofie onderdrukt wordt. Dit bescherm het kraakbeen tegen de schadelijke effecten van BMP9 waaraan het continu is blootgesteld via de synoviale vloeistof in de gewrichtsholte, maar laat wel de positieve effecten van BMP9 op glycosaminoglycan- en collageenproductie toe als onderhoud van de extracellulaire matrix. Veroudering verstoort echter dit beschermende mechanisme door verlaging van ALK5 waardoor de positieve terugkoppeling verstoord raakt met alle gevolgen van dien. Dit model onderstreept dus dat artrose een ziekte is en geen slijtage, en dat het voorkomen of bestrijden van leeftijdsgere lateerde ALK5 verlaging een uitdaging voor de toekomst is om leeftijdsgere lateerde artrose te voorkomen.
List of publications


Portfolio

Awards
Pauline van Wachem award for best presentation, NVMB 2012

A.van den Hooff award for best debater, NVMB 2016

Young investigator award, OARSI 2016 (shared with W.Madej)

Attended (Inter)national congresses


ORS 2013, San Antonio USA, Annual meeting of the European Orthopaedic Research society. Podium presentation.


MBE 2014, Rotterdam, Annual meeting of the Matrix Biology Europe society. Podium presentation.


ORS 2016, Orlando USA, Annual meeting of the European Orthopaedic Research society. Podium presentation.


Research data management

Findable:
All (raw) data described in this thesis can be found at the department of Experimental Rheumatology of the Radboud university medical center.

Accessible:
All (raw) data described in this thesis can be obtained on request from the department of Experimental Rheumatology of the Radboud university medical center. The same holds true for the protocols of the experiments described in this thesis.

Interoperable:
The (raw) data in this thesis is documented in a formal, accessible, shared, and broadly applicable language for knowledge representation.

Reusable:
The (raw) data shown in this thesis are sufficiently documented to be reusable.
Curriculum Vitae

Dankwoord

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