A Novel Approach to Stimulate Cartilage Repair:
Targeting Collagen Turnover

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Targeting Collagen Turnover

Een nieuwe aanpak om kraakbeenherstel te stimuleren
gericht op collageen turnover

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List of abbreviations

ADAMTS	A Desintegrin And Metalloproteinase with Trombospondin motif
BAPN	Beta-Aminopropionitril
COL9	Collagen type 9
COMP	Cartilage Oligomeric Matrix Protein
CS	Chondroitin Sulphate
DKO	Double Knock-out
DMOAD	Disease Modifying Osteoarthritic Drug
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
HP	Hydroxylysylpyridinoline
Hyl	Hydroxylysine
Hyp	Hydroxyproline
IGF	Insulin-like Growth Factor
IL	Interleukin
KS	Keratan Sulphate
LOX	Lysyl Oxidase
LH	Lysyl Hydroxylase
LP	Lysylpyridinoline
Lys	Lysine
MMP	Matrix Metalloproteinases
NSAID	Non-Steroidal Anti-Inflammatory Drug
OA	Osteoarthritis
PDGF	Platelet Derived Growth Factor
PNPX	Para-Nitrophenyl-β-D-Xyloside
PG	Proteoglycan
Pro	Proline
QPCR	Quantitative Polymerase Chain Reaction
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitors of Matrix Metalloproteinase
TLH	Telopeptide Lysyl Hydroxylase
TNF	Tumor Necrosis Factor

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General Introduction

Articular Cartilage

Cartilage present in articulating joints is referred to as articular cartilage. Articular cartilage is highly specialized connective tissue. It is resilient, distributes forces onto subchondral bone, and provides smooth joint movement. Articular cartilage is avascular, aneural and alymphatic [1]. The extracellular matrix (ECM) of cartilage is produced by chondrocytes which represent only a small percentage (1-5%) of the wet weight [2]. 70-75% of the cartilage is water. The most abundant protein is collagen type II, which is present at levels up to 60-80% by dry weight of the tissue. Proteoglycans form 20-30% of the proteins found in cartilage. The collagen molecules form a highly organized fibrillar network, defining form and tensile strength of the articular cartilage within which the highly hydrophilic proteoglycans are immobilized due to their large size. Many other structural proteins (collagens and non-collagenous proteins) enmeshed within the collagen network, are present at varying but low amounts. Some of these minor proteins might play important roles in the assembly of a complex three-dimensional collagen network and cell-matrix interaction. Besides its mechanical importance, the extracellular matrix represents the microenvironment for the chondrocytes.

Chondrocytes

Since articular cartilage is non-vascular, nutrition of the chondrocytes is dependent on synovial fluid and its diffusion through the cartilage facilitated by intermittent loading of the cartilage [3]. More recently it was discovered that the subchondral bone also plays a role in cartilage nutrition [4, 5]. Consequently, chondrocytes exist under oxygen tensions as low as 1% and thus rely mainly on anaerobic metabolism. The primary function of chondrocytes is to produce the cartilage ECM during growth and to maintain the integrity of the tissue. To this purpose chondrocytes adjust their behavior in response to external signals such as growth factors [6], cytokines [7, 8], and ECM degradation products [9, 10].

Collagen

Collagen is the main protein in articular cartilage; approximately 60-80% of cartilage dry weight is collagen. On the basis of their mode of assembly and structural features, the collagen superfamily is subdivided into three groups: the fibril forming collagens, the fibril associated collagens and the non-fibrilforming collagens [11]. Of the collagens present in articular cartilage, 95% is collagen type II, which belongs to the fibril forming collagens. Collagen type XI accounts for 3% of the total collagen amount, which is also fibril forming. Two percent is collagen type IX, which belongs to the fibril associated collagens.

   Each collagen molecule is composed of three alpha chains and depending on the collagen type, the collagen molecule is either composed of three identical alpha chains or of two or three different alpha chains. In its mature form, each fibrillar collagen

molecule consists of three domains: a short amino-terminal non-triple helix domain (N-telopeptide), a long central triple helical domain and a short carboxy-terminal non-triple helix domain (C-telopeptide). Intracellular, it also contains propeptides on the N- and C-terminal sides of the molecule. In order for the three alpha chains to form a triple helix, the small amino acid glycine must be present in every third position of the polypeptide chain. This results in the characteristic sequence of glycine-X-Y in which proline most often occupies the X position and hydroxyproline the Y position [12]. Collagen type II is a trimer composed of three α1(II) chains [13]. After translation of the procollagen α-chain, multiple post-translational modifications take place (Figure 1.1). Hydroxylation of proline residues is catalyzed by prolyl-hydroxylases [14]. Hydroxyproline stabilizes the triple helix by providing hydrogen bonds and water bridges. The presence of hydroxyproline is also required to stabilize the collagen triple helix at body temperature. In addition, lysine residues are hydroxylated by lysyl hydroxylases (LH) [15]. Hydroxylysine is a requisite for covalent intermolecular cross-linking and acts as acceptor site for carbohydrate units. Galactose is transferred to some of the hydroxylysine residues and glucose to some of the galactosylhydroxylysine residues. The function of the hydroxylysine-linked carbohydrate units is unknown but regulating the organization of the collagen molecules into fibrils is suggested [16]. Hydroxylation and glycosylation stop when the individual α-chains entwine to form the procollagen triple helix in the rough endoplasmatic reticulum. The procollagen is packaged into secretory vesicles and transported outside the cell.

In the extracellular space, the procollagen is converted to collagen by removing the C- and N-propeptides, steps catalysed by C- en N-proteinase [17]. The removal of the propeptides results in a decrease in water solubility, forcing the triple helical collagen molecules to aggregate into fibrils. These fibrils are finally stabilized by the formation of covalent cross-links. In cartilage, the hydroxyallysine route is the most important route for the formation of cross-links. This process is initiated by oxidation of two hydroxylysyl residues in the telopeptide by lysyl oxidase (LOX) to form aldehyde precursors that spontaneously condense into difunctional cross-links [18]. The formed hydroxyallysine reacts with a triple helical lysine residue into lysylpyridinoline (LP) or with a hydroxylysine residue into hydroxylysylpyridinoline (HP) to form a trifunctional crosslink that is responsible for the structural integrity of the collagen network. In cartilage, HP cross-links are found 30-50 fold more than LP cross-links [19].

Collagen type IX molecules are attached the surface of type II collagen fibrils, particularly the thin fibrils around chondrocytes [20]. Seven cross-linking sites have been defined in the collagen type IX molecule. These interact with type II collagen and with other collagen type IX molecules, functioning as an interfibrillar connector facilitating the formation and organization of the network [21-24]. Collagen type XI molecules are primarily cross-linked to each other in a head-to-tail manner, and are believed to form a
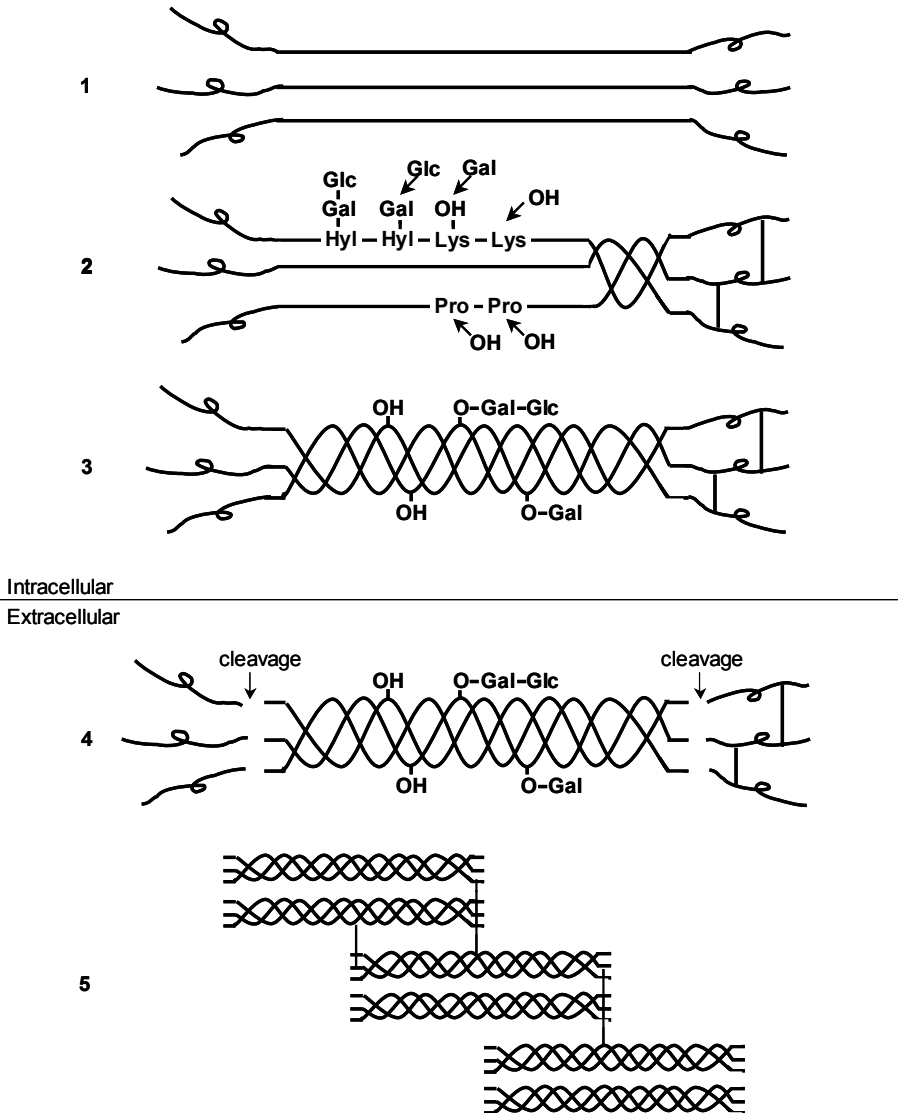
Figure 1.1. Collagen biosynthesis. Collagen is synthesised as pro-peptide-containing α-chains (1) that are post-translationally modified by hydroxylation of lysine (Lys) and proline (Pro) residues and by glycosylation of hydroxylysyl (Hyl) residues (2). These modifications stop when the individual α-chains associate to form the procollagen triple helix (3). The procollagen is transported outside the cell and the procollagen is converted to collagen by removing the propeptides (4). The triple helical collagen molecules aggregate into fibrils, which are stabilised by the formation of covalent cross-links (5).

template that constrains the lateral growth of the type II collagen hetero-fibril [25]. Other types of collagen found in articular cartilage include types III [26, 27], IV [28], VI [29], X (only in hypertrophic cartilage) [30, 31], XII [32, 33], XIII [34], XIV [33], XXVII [35]

Proteoglycans

Proteoglycans (PGs) consist of a protein core with carbohydrate side chains called glycosaminoglycans (GAGs). Aggrecan is the most common PG in articular cartilage and consists of a central core protein with several domains providing the molecule with specific characteristics. Highly negatively charged chondroitin sulphate (CS) and keratan sulphate (KS) side chains are attached to the core protein resulting in a characteristic brush-like structure [36]. The N-terminal region of the aggrecan core protein contains two globular domains (G1 and G2) of which the G1 domain interacts with hyaluronan [37]. The G3-domain at the C-terminal side of the core protein interacts with other cartilage matrix constituents [38] (Figure 1.2). The GAGs are covalently joined to the protein core by a glycosidic bond between xylose and the hydroxyl group of a serine residue [39]. After xylosylation of serine, linkage region synthesis occurs by the addition of two galactosyl moieties by galactosyltransferase I [40] and glucuronic acid by glucuronosyltransferase I [41]. Elongation of chondroitin sulphate chains proceeds by the alternate transfer of N-acetylgalactosamine by N-acetylgalactosaminyltransferase and glucuronic acid by glucuronosyltransferase II [42]. The sulphation of hexosamine moieties

Figure 1.2. Schematic structure of aggrecan. Aggrecan consists of a core protein with mainly chondroitin sulphate side chains. In addition, the core protein also contains keratan sulphate and linked oligosaccharides. Two globular domains (G1 and G2) are present at the N-terminal side of the aggrecan core protein, one (G3) at the C-terminal side of the core protein.
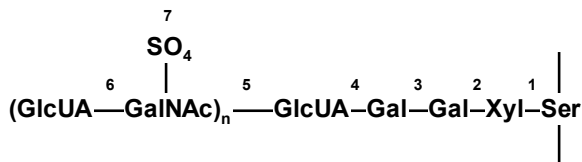
Figure 1.3. The linkage of chondroitin sulphate side chains to the protein core and chain elongation. After xylosylation of serine (l), linkage region synthesis occurs by the addition of two galactosyl moieties (2 and 3) and glucuronic acid (4). Elaboration of chondroitin sulfate chains proceeds by the alternate transfer of N-acetylgalactosamine (5) and glucuronic acid (6). The sulfation of hexosamine moieties occurs (7) by the transfer of sulfate from phosphoadenylylsulfate.

occurs by the transfer of sulphate from phosphoadenylylsulfate [43] (Figure 1.3[44]). Each core protein contains approximately 100 CS chains and 50 KS chains. Aggrecan associates with hyaluronan and a small glycoprotein, link protein, to form aggregates. These multimolecular structures can contain over 100 molecules of both aggrecan and link protein associated with a single hyaluronan chain. The aggregates are hydrated due to their high fixed negative charge resulting from the very large numbers of polyanionic glycosaminoglycan chains on aggrecan, providing cartilage with a high water content (about 70% by wet weight of the tissue) [45, 46].

A small fraction of the total mass of proteoglycans within the cartilage ECM comprises small leucine-rich proteoglycans. These small proteoglycans include decorin, fibromodulin, perlecan, and biglycan. Decorin and fibromodulin interact with collagen type II and are suggested to play a role in the formation and maintenance of the collagen fibrils [20, 47]. Biglycan and perlecan are found mainly in the pericellular matrix of the chondrocyte [48, 49].

Other matrix constituents

Finally, a small fraction of the total weight of cartilage ECM is formed by other matrix constituents. One of them is cartilage oligomeric matrix protein (COMP), which is a homo-pentameric protein originally found in cartilage [50] but is also present in a large variety of other tissues such as tendon [51], synovium and skin [52]. Subunits are joined close to their N-terminus, creating a five-stranded coiled coil that is stabilized by two disulphide bonds [53]. COMP is suggested to play an important role in matrix assembly and its response to external stimuli because it interacts with several components of the extracellular matrix such as collagen type I, II [54] and IX [55] and aggrecan [56]. In addition, COMP was found to influence the fibril formation of collagen type I and II leading to more and organized collagen fibrils in vitro. However, COMP was not associated with mature collagen fibrils suggesting a role as catalyst in fibrillogenesis for COMP [57].

The matrilins form a four-member family of modular, multi-subunit matrix proteins, which are expressed in cartilage but also in many other forms of extracellular matrix. They participate in the formation of fibrillar or filamentous structures and are often associated with collagens. They mediate interactions between collagen-containing fibrils and other matrix constituents, such as aggrecan. Matrilin-1 was first discovered from bovine cartilage [58]. Matrilin-3 is the smallest family member and is mostly co-expressed with matrilin-1 [59, 60]. Recent studies have revealed that matrilin-1, -3, and -4 form complexes with biglycan and decorin [61], bind to COMP [62], and that matrilin-3 interacts with collagen IX [63]. These matrilin-containing molecular complexes are in turn connected to collagen fibrils via COMP and collagen IX [61, 64] (Figure 1.4).

Other non-collagenous proteins found in articular cartilage include fibronectin [65, 66] and cartilage intermediate layer protein (CILP) [67].
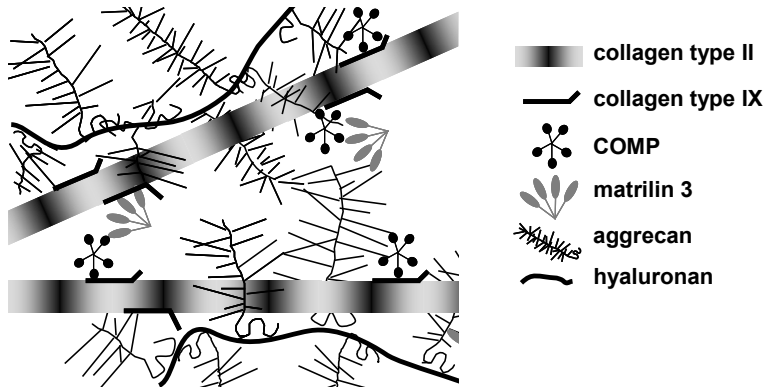
Figure 1.4. Interaction of a few of the cartilage matrix constituents. Collagen type IX is bound to the collagen type II fibrils. The aggrecan-hyaluronan complexes reside within the collagen network. COMP, matrilin 3 and collagen type IX interact with each other and with collagen type II and aggrecan forming the typical cartilage ECM network

Matrix turnover

Even though the half-life of cartilage collagen is more than 100 years [68], the cartilage matrix (including collagen) slowly remodels. Cartilage matrix turnover is a process of synthesis and degradation, which is balanced in healthy individuals. The Ca^{2+}- and Zn^{2+}-dependent matrix metalloproteinases (MMPs), members of a larger metalloprotease superfamily, are involved in the degradation of the cartilage ECM. Due to their wide substrate specificity, the MMP family is able to degrade virtually all components of the ECM [69]. MMP activity is controlled by the endogenous inhibitor α_2-Macroglobulin (α_2M) [70] and by Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) [71]. Growth factors, hormones and cytokines regulate the expression of the MMPs and TIMPs [72].

The ADAMs family (A Desintegrin and Metalloproteinase) includes proteinases of the subfamily 'A Desintegrin and Metalloproteinase with Trombospondin motifs' (ADAMTS) and is also involved in cartilage matrix turnover. ADAMTSs are involved in the cleavage of the propeptide from procollagen. ADAMTS -1, -4, -5, -8, -9, and -15 are able to cleave aggrecan at four different sites. Aggrecanase 1 and Aggrecanase 2 (ADAMTS-4 and ADAMTS-5 respectively) are the best studied aggrecanases and are able to cleave aggrecan at an additional site [73].

In addition, serine proteinases such as elastase and cathepsin G from polymorpho-nuclear granulocytes play a critical role in articular cartilage degradation. Not only as proteolytic enzymes able to degrade the extracellular matrix [74] but also by additionally modulating the level of active MMPs [75].

Functioning of articular cartilage

Articular cartilage normally functions as a low friction, wear-resistant, load-bearing material that facilitates joint motion. Because of this function, cartilage experiences

intermittent loading and deformation. Loading and deformation of cartilage will gener-
ate a combination of tensile, compressive and shear stresses in the material. The response
of cartilage to these stresses is determined by the tissue's unique composition and
structural organization. The proteoglycans in articular cartilage are negatively charged
due to the presence of carboxyl and sulphate groups on the glycosaminoglycans, result-
ing in a net negative charge of the extracellular matrix. To balance this negative charge,
cations are drawn into the tissue, generating a large osmotic potential. As a result,
cartilage is highly hydrophilic with the tendency to retain fluid in order to maintain
chemical equilibrium. The resulting large swelling force is balanced by the confining
influence of the collagen network. This property contributes to the mechanical function
of articular cartilage by generating a large swelling pressure which facilitates load sup-
port and tissue recovery from deformations [76]. Resistance to tensile and compressive
deformations and loads is generated principally by the intrinsic stiffness of the collagen
fibers and depends on the density and orientation of the collagen fibers, and the type or
amount of collagen cross-links [77-82]. The tensile modulus reflects the flow-independent
properties of the cartilage solid matrix when determined from stress-strain data at
equilibrium [83]. Conversely, the hydraulic permeability of cartilage is more associated
with the proteoglycan content [79, 84]. Nevertheless, the collagen network has also been
suggested to play a role in determining the hydraulic permeability [85]. In addition, the
swelling and deformation of cartilage is highly associated with the amount of degraded
collagen molecules [86].

Osteoarthritis

Osteoarthritis (OA) is the most prevalent disorder that affects diarthrodal joints [87]. The
prevalence of OA based on radiographic evidence has been recorded as high as 70%
in people aged >65 [88]. Joint degeneration seen in OA is characterized by cartilage and
bony changes, which lead to impaired joint motion, pain and disability. Macroscopically,
cartilage degeneration has been described such as fibrillation of the articular surface,
the presence of cracks or fissures, and partial or complete loss of the tissue [89]. Additional
signs of OA include an increase in cartilage hydration, joint space narrowing, subchon-
dral bone changes, and osteophyte formation. Moreover, OA is associated with an
inflamed synovial membrane with evident vascularisation, infiltration of T lymphocytes
and mononuclear cells, and proliferation of synovial cells [90, 91]. Although the etiology
of OA is unknown, many risk factors for OA are identified. These include age, gender,
obesity, genetic predisposition, and trauma [92, 93].

   In early OA, synthetic activity is increased, which is considered as a possible attempt
to regenerate the matrix [94-96]. In addition, collagen type III (which is present at low
levels in normal cartilage), the chondroprogenitor splice variant of the collagen type II
gene (type IIA) and collagen type X, have been detected in OA cartilage. This indicates,

next to an increase in cell proliferation, a change in phenotype of the chondrocyte during the OA process.

Increased production of proteinases, including the metalloproteinases (MMPs), MMP-1, MMP-3, MMP-13, and the aggrecanases, mainly ADAMTS-5, is associated with cartilage damage [97, 98]. Local loss of proteoglycans and cleavage of type II collagen occurs initially at the cartilage surface resulting in an increase in water content and loss of tensile strength in the cartilage matrix as the lesion progresses. The limited intrinsic repair capacity of cartilage is most probably due to the difficulties to repair the cartilage collagen network whereas proteoglycan depletion is often reversible. Collagen turnover is low in normal adult cartilage and increased in OA [68, 99], which is consistent with the presence of type II collagen propeptides in both healthy and osteoarthritic cartilage [96]. In OA, increased collagen turnover is accompanied by increased collagen denaturation, suggesting ineffective repair [100]. These data strongly support the idea that collagen turnover can be increased in healthy adult cartilage which can result in a normal collagen network.

It is believed that cytokines and growth factors also play a role in the pathophysiology of OA. Cytokines with destructive properties for articular cartilage are Interleukin-1 (IL-1) [101, 102], Tumor Necrosis Factor α (TNFα) [103, 104], and the more recently discovered IL-17 [105]. These cytokines have a pronounced suppressive effect on chondrocyte proteoglycan synthesis, and also stimulate the chondrocyte to release destructive proteases, which then mediate cartilage breakdown. IL-1 and TNFα are mainly produced by chondrocytes [106] or activated macrophages in the synovium [107], whereas IL- 17 is a T-cell-derived interleukin [108].

Anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 inhibit the destructive properties of these pro-inflammatory cytokines via the upregulation of inhibitors of IL-1 (e.g. IL-1 receptor antagonist) and TNFα (soluble TNFα receptor), or TIMPs [109, 110].

Growth factors are able to counteract the catabolic effect of IL-1. IGF-1 (Insulin-like Growth Factor-1) is the main anabolic growth factor for adult articular cartilage under physiological conditions but signaling is altered in OA. Enhanced levels of IGF-1 binding proteins (IGFBPs) [111] or inadequate signaling by the IGF-1 receptor on the chondrocyte [112] results in suppressed chondrocyte proteoglycan synthesis and enhanced breakdown of proteoglycans. In contrast, blockade of IGFBPs restores IGF-1-dependent proteoglycan synthesis in OA cartilage [113].

Transforming Growth Factor-β (TGFβ) is expressed in high levels in normal cartilage, but low in OA cartilage [114]. Blocking TGFβ makes cartilage more susceptible to damage [115]. TGFβ is a potent inducer of cartilage ECM synthesis [116] and a very potent counteracting agent of IL-1 actions [117-119]. Therefore, lack of TGFβ causes a reduction in ECM deposition and suppression of catabolic stimuli is drastically reduced. Next to IGF-1 and TGFβ, a range of other growth factors can stimulate chondrocyte activity,

including Fibroblast Growth Factor (FGF) and Platelet Derived Growth Factor (PDGF). Since their important role in OA, their effect on cartilage matrix production and chondrocyte behavior, the above mentioned growth factors are often used in cartilage repair strategies [106, 120-123].

Aims and outline of this thesis

The recommended management for OA consist of non-pharmacological treatments such as weight loss [124-127], then pharmacological intervention, and then, if necessary, surgery [128-130]. Pharmacological intervention includes non-steroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics for pain management [131]. However, all these interventions aim at pain management but no therapy is yet available to repair cartilage damage in OA.

Even though cartilage has a low turnover and is often thought to have no repair capacities, matrix production including collagen type II is upregulated in OA suggesting a potential for repair. This indicates that regeneration is possible and lead to the following goal:

Identification of biochemical factors that modulate cartilage metabolism focusing on a functional collagen type II network

Cartilage repair strategies often include the use of growth factors. IGF-1, FGF-2 (also known as basic FGF) and TGFβ are frequently used growth factors that also play a significant role in joint development and matrix homeostasis [132-134]. In **chapter 2**, we examined the effect of these three growth factors on cartilage matrix deposition, collagen cross-linking and functionality of the newly formed matrix using bovine chondrocytes cultured in alginate beads as a model.

One of these growth factors, TGFβ, is known to be a potent inducer of fibrosis. Increased amounts of TGFβ are found in fibrotic lesions [135, 136] and administration of TGFβ to laboratory animals leads to the development of fibrosis [137, 138]. Fibrosis is a complex process finally resulting in excessive deposition of highly cross-linked collagen [139, 140]. In our growth factor study with chondrocytes we observed contrasting effects of TGFβ, mainly in collagen cross-linking. Since TGFβ is a widely used growth factor in cartilage repair strategies on a large variety of culture substrates, we decided to investigate the effect of TGFβ on chondrocytes and their matrix production in more detail in **chapter 3**. Here, we used four different cell populations in two very different physical environments; primary and expanded chondrocytes and fibroblasts (as a control) embedded in alginate gel and attached to tissue culture plastic.

Since collagen cross-links are essential for cartilage matrix production and functionality, we inhibited an enzyme involved in collagen cross-linking in our third study. How this temporary cross-linking affected collagen turnover (synthesis and MMP-mediated degradation) and functionality of the matrix was examined in **chapter 4**.

Collagen fibrils interact with all other cartilage matrix molecules, directly or indirectly. Modifying matrix components next to collagen might influence collagen deposition and function. In addition, previous disruption of the proteoglycan network resulted in enhanced integrity of the collagen network [141]. In **chapter 5**, proteoglycan synthesis was inhibited by prevention of the linkage of GAG side chains to the protein core of the proteoglycans. How this influenced the collagen deposition, distribution and functionality was described there.

Next to proteoglycans, other matrix components such as COMP and collagen type IX are likely required for the formation of the collagen fibrils and the structure and integrity of the network. To get more insight into these requirements and their contribution in matrix regeneration, we investigated COMP and collagen type IX in more detail in chapter 6 and 7. The effect of the previously used growth factors (chapter 2) on COMP deposition was examined next to the effect of COMP overexpression on collagen biochemistry in **chapter 6**.

In **chapter 7**, COMP and collagen type IX were absent in chondrocyte cultures from double knock-out mice. How the absence of these to matrix molecules influenced matrix production and functionality *in vitro*, was investigated here.

In **chapter 8**, some of our previously used approaches are combined to examine their effect on cartilage matrix production as investigated earlier. In parallel, cartilage explants were also used to examine the effect of the combinations on cartilage integration.

Finally, **chapter 9** presents the general discussion; summarizing and discussing the results of the work performed and elaborating on future applications for cartilage repair in osteoarthritis.

2

Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFβ2 and FGF2

Yvonne M. Jenniskens, Wendy Koevoet, Anton C.W. de Bart, Harrie Weinans, Holger Jahr, Jan A.N. Verhaar, Jeroen DeGroot, Gerjo J.V.M. van Osch

Abstract

Background

Examine effects of IGF1, TGFβ2 and FGF2 on proteoglycan and collagen network and biomechanical properties of the newly formed cartilage matrix.

Methods

Bovine articular chondrocytes were cultured in alginate beads for three weeks with or without FGF2, TGFβ2 or IGF1 in the presence of 10% FCS. Proteoglycan content, collagen content, hydroxylysylpyridinoline cross-links (HP) and overall MMP activity in the culture medium were measured. Alginate disks cultured for five weeks were used to evaluate the effect of growth factors on mechanical properties of the construct by determining the equilibrium aggregate modulus and secant modulus.

Results

IGF1 increased collagen and proteoglycan deposition. FGF2 mainly decreased collagen deposition and TGFβ2 proteoglycan deposition. A decrease in cross-links was observed in matrix produced by chondrocytes cultured in the presence of TGFβ2. IGF1 and FGF2 had no influence on the number of cross-links per collagen molecule. Overall MMP-activity was significantly higher in culture medium of cells cultured with FGF2. TGFβ2 and IGF1 had no effect on MMP-activity. After 35 days of culture, the matrix produced under influence of IGF1 had a lower permeability and a trend to increase stiffness. FGF2 showed a trend to lower both properties. TGFβ2 had no effect on these parameters.

Conclusion

IGF1, TGFβ2 and FGF2 had differential effects on collagen network formation. Of the three growth factors tested, IGF1 seems to be best in promoting the formation of a functional collagen network since it increased proteoglycan and collagen deposition and improved the mechanical properties.

Introduction

Once damaged, adult articular cartilage has a poor repair capacity, which is probably due to the ineffective repair of the collagen network, since proteoglycan depletion is often reversible [142]. Although collagen turnover is increased in osteoarthritis, this does not lead to the formation of a functional network [26, 68, 96, 143-145]. A similar phenomenon is seen in cartilage tissue engineering both in vitro and in vivo, where proteoglycans are abundantly produced but the amount of collagen produced is limited, probably impairing functional properties of the newly formed matrix [146].

Type II collagen belongs to the fibril-forming collagens and accounts for more than 90% of the collagenous protein in adult articular cartilage, where it defines the basic architecture and provides tensile strength [25, 147]. Together with type IX and XI, collagen type II forms fibrils [20, 25], which are stabilized by the formation of intermolecular pyridinoline cross-links. In cartilage, collagen molecules are cross-linked via the hydroxyallysine route, which is mediated by enzymes such as lysyl hydroxylases (LH) and lysyl oxidase (LOX) [148], resulting in lysylpyridinoline (LP) or hydroxylysylpyridinoline (HP) [19, 149]. HP and LP are chemical variants of a mature trifunctional collagen cross-link [150], both giving strength to the cartilage. In cartilage, HP is found at 30-50 fold higher levels than LP.

In healthy cartilage, the extracellular matrix is continuously remodeled although slowly: this is a balance between enzymatic degradation of matrix components and de novo synthesis by chondrocytes. One group of matrix degrading enzymes is the matrix metalloproteinases (MMP) family. Members of the MMP family are capable of degrading practically all components of the extracellular matrix including collagen and aggrecan in their substrates [69]. A variety of cells including chondrocytes secrete MMPs and their production can be influenced by several factors including growth factors [151].

Generation of new cartilage repair tissue can also be influenced by growth factors. In previous studies, growth factors have demonstrated to influence proteoglycan production and deposition. In culture, Insulin-like Growth Factor 1 (IGF1) stimulates proteoglycan production in a dose-dependent manner [152]. Fibroblast Growth Factor 2 (FGF2) has pleiotropic effects: both stimulation and inhibition of proteoglycan deposition have been described, depending on the FGF-2 concentration [153], but also on the presence or absence of fetal calf serum [154] or the employed cell culture system (monolayer versus alginate) [154, 155]. Transforming Growth Factor β2(TGFβ2) also has variable effects when used in in vitro studies; while a dose-dependent inhibition of proteoglycan production is observed; a stimulation has also been described, depending on the culture conditions [152, 156].

While most studies focus on proteoglycan metabolism, we are interested in the effects of growth factors on the functional quality of the matrix and therefore choose collagen as our main focus in this study. Specifically, we investigated the amount of

collagen and proteoglycan synthesized, the number of collagen cross-links formed and the mechanical properties of the formed matrix as a consequence of IGF1, TGFβ2 and FGF2 addition to bovine chondrocytes.

Materials and Methods

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged 6–12 months. To isolate chondrocytes, full-thickness slices of non-calcified articular cartilage were subjected to pronase (2 mg/ml, Sigma, St. Louis, MO, USA) digestion for two hours followed by overnight collagenase B (1.5 mg/ml, Roche Diagnostics) digestion. The chondrocytes were resuspended in 1.2% (w/v) low viscosity alginate (Keltone) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/ml and beads were made as describes previously [157, 158]. Briefly, the cell-alginate suspension was pressed through a 22-gauge needle in 105 mM $CaCl_2$. Beads were washed with 0.9% NaCl and Dulbecco's Modified Eagle Medium (DMEM)/F12 (GibcoBRL, Gaithersburg, MD, USA) and inspected visually. Beads that appeared smaller or larger were not included in the experiment. After transfer to a six-well plate (BD Falcon, Bedford, MA, USA) they were cultured in 75 µl/bead DMEM/F12 supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). Cells were cultured with and without 2.5 ng/ml or 25 ng/ml FGF2 (Serotex, Oxford, UK), TGFβ2 (recombinant human, R&D systems, Abington, UK) or IGF1 (Sigma) and harvested after 11, 20 and 35 days of culture. Growth factors and their concentrations were chosen based on previous results [123, 152, 159]. Part of the cell containing beads was used immediately for RNA extraction; the rest was stored at –20 °C until further biochemical analyses.

Biochemical assays

Alginate beads were digested overnight at 56°C in papain buffer (200 µg/ml papain in 50 mM Ethylene Diamintetraacetate (EDTA) and 5 mM L-cystein). Glycosaminoglycan (GAG) amount in de digest was quantified using dimethylmethylene blue (DMB) assay [160]. The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio $A_{540}:A_{595}$ was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. The amount of DNA in each papain-digested sample was determined using Hoechst 33258 dye [161] with calf thymus DNA (Sigma) as a standard.

For high-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP and lysylpyridinoline, LP), the

papain digest was hydrolyzed (108°C, 18-20 h) with 6 M HCl. The hydrolyzed samples were dried and redissolved in 200 µl water containing 2.4 mM homoarginine (internal standard for amino acids) (Sigma). Samples were diluted 25-fold with 50% (v/v) acetic acid for cross-link analysis and diluted 250-fold with 0.1 M sodium borate buffer pH 8.0 for amino acid analysis. Additionally, to determine hydroxyproline, amino acids were labeled with 9-fluorenylmethyl chloroformate. Reversed-phase high-performance liquid chromatography of amino acids and cross-links were performed as described previously [162, 163]. The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

Immunohistochemical staining for collagen type I and II

To prepare histochemical cytospins, three alginate beads cultured for 35 days were dissolved in 55 mM sodium citrate acid. They were fixed in cold acetone and pre-treated with 1% hyaluronidase (Sigma). The cytospins were incubated with monoclonal antibodies against either type I collagen (1:100, M28; Developmental Studies Hybridoma Bank), or type II collagen (1:100, II-II6B3; Developmental Studies Hybridoma Bank) at room temperature for 2 h, followed by incubation with anti-mouse F_{ab}-fragments conjugated with alkaline phosphatase (GAMAP; 1:100; immunotech, Marseille, France) for 30 minutes and mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; 1:100; Dakopatts, Copenhagen, Denmark; 1:100) for 30 minutes. Freshly prepared neo-fuchsin substrate was used to achieve staining.

After staining, cytospins were scored for the number of cells positive for collagen types I and II per 100 cells in four fields of sight per specimen, resulting in a percentage of positive cells. An arbitrary scoring system from 1 to 5 was used, with 1 representing less than 10% of the cells positive, 2 representing 10-40% positive, 3 representing 40-60% positive, 4 representing 60-90% positive and 5 representing more than 90% of the cells positive. A cell was considered positive, when the matrix around the cell or the cytoplasm was stained red.

Gene expression analysis

For total RNA isolation, alginate beads were dissolved in 150 µl/bead 55 mM sodium citrate acid. Cell pellet was suspended in 1000µl RNA-Bee™ (TEL-TEST, Inc.; Friendswood, TX, USA) per million cells according to manufacturer's guidelines, and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using Ribogreen™ reagent (R-11490, Molecular Probes Europe BV, Leiden, the Netherlands) according to manufacturer's instructions, and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany).

Table 2.1: sequences of primers and probes for real-time PCR.

	Primers	Probe
GAPDH	Forward: GTCAACGGATTTGGTCGTATTGGG Reverse: TGCCATGGGTGGAATCATATTGG	Fam-TGGCGCCCCAACCAGCC-Tamra
Aggrecan	Forward: GGACACTCCTTGCAATTTGAGAA Reverse: CAGGGCATTGATCTCGTATCG	Fam-CCATCACCATCTTCCAGGAGCGAGA-Tamra

Table 2.2: sequences of primes and beacons real-time PCR.

	Primers	Beacon
PLOD2	Forward: TTAAAGGAAAGACACTCCGATCAGAGATGA Reverse: AATGTTTCCGGAGTAGGGGAGTCTTTTT	Fam-cgtgcgCGTGATAAACTGGATCCTGATATG GCTCTTcgcacg-Dabcyl
LOX	Forward: GTGGCCGACCCCTACTACATCC Reverse: AGCAGCACCCTGTGATCATAATCTC	Fam - cgtgccATGCGCGGCGGAGGAAAACTG ggcacg- Dabcyl
MMP-1	Forward: ACCCCAGACCTGTCAAGAGCA Reverse: TCCCCTGACAAAGGATATCATTAT	Fam-cgtgccAAAGCCTTTCAACTCTGGAGCAAT GTCACggcacg-Dabcyl

For Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aggrecan, each cDNA sample was amplified using specific primers and specific probe (Table 2.1, Eurogentec, Seraing, Belgium). For Collagen type 2 (COL2), Matrix Metalloproteinase-1 (MMP-1), Lysyl Oxidase (LOX) and Lysyl Hydroxylase 2b (LH2b), each cDNA sample was amplified using specific primers and specific molecular beacons (Table 2.2, Biolegio, Nijmegen, The Netherlands). All amplifications were done in a total reaction volume of 25 µl containing 1×PCR buffer, 0.4 mM of each dNTP, Mg^{2+} (3.5 mM for the molecular beacons and 5 mM for the probes), 0.5 unit of Taq polymerase (all from Eurogentec) and each primer and probe or beacon. PCR was performed in an ABI PRISM® 7000 (Applied Biosystems, Foster City, CA, USA) sequence detection system and consisted of a 5-min interval at 95 °C followed by 40 cycles of 95 °C for 30 s and 60°C for 1 min for hydrolysis probe assays and 40 cycles of 95 °C for 30 s, 56 °C for 40 s, and 72 °C for 30 s for beacon assays. Data were analyzed using Sequence Detector version 1.7 (Applied Biosystems) software and normalized for GAPDH expression and expression in the control condition without growth factors [164].

Total MMP assay

The culture medium was used to determine general MMP activity as described earlier [165]. General MMP activity was measured using 5 µM (all concentrations are final) fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH_2) in the presence or absence of 12.5 µM BB94 (a general MMP inhibitor). Medium samples were diluted (final dilution 1/2) in MMP buffer (50 mM Tris [pH 7.5], 5 mM CaCl2, 150 mM NaCl, 1 µM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3) containing a general proteinase inhibitor (Complete, EDTA-free, one tablet in 12.5 ml, Roche). The MMP activity in

each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence in time, expressed as relative fluorescence units (RFU) per second) between samples with and without BB94 addition. Fluorescence was measured for six hours at 30°C using a Cytofluor 4000 (Applied Biosystems, Foster City, CA, USA). This assay is considered to represent overall metalloproteinase activity.

Mechanical testing

For mechanical characterization, we used 4×10^6 cells/ml in 1.2% (w/v) alginate constructs of 3 mm thick and 6 mm in diameter. The constructs were prepared as previously described by Wong et al.[166]. Directly after making the cell containing alginate disks, samples were taken for mechanical testing at day 0, which is indicative for the mechanical strength of the alginate. Constructs were mounted on a materials testing machine (type LRX, Lloyd Instrument, Farnham, UK) in a radially unconfined stress relaxation test with the construct between impermeable platens, and hydrated in 0.9% saline with protease inhibitor Complete (Sigma). A 20% uniaxial compressive strain was then applied within 10 seconds at a ramped displacement strain rate of 2% s^{-1}, based on the measured thickness. The strain was maintained constant at this plateau for 30 min with the load recorded at a sampling frequency of 20 Hz using a 10 N load cell. The applied load recorded by the load cell was divided by the cross-sectional area of the construct to calculate the applied stress. The secant modulus was calculated as stress/strain at 10 seconds, where the stress had its peak response and the strain reached its maximum plateau. At the end of the test, the equilibrium aggregate modulus was determined again as stress/strain at 30 minutes creep.

Data analysis

The experiments at day 11 were repeated twice with 3 samples of 7 beads per experimental condition for biochemical analysis and one sample of 10 beads per experimental condition for gene expression analysis. The experiments at day 20 were repeated four times with 3 samples of 7 beads per experimental condition for biochemical analysis and one sample of 10 beads per experimental condition for gene expression analysis. The experiments at day 35 were repeated three times with a total of 10 samples for the control, IGF1 and TGFβ2 condition and 8 samples for the FGF2 condition. Overall MMP activity in the culture medium and collagen type I and II deposition with cytospins was determined in 1 experiment in triplicate.

Statistical analysis was performed using SPSS 11.5 (SPSS Inc. Chicago, IL) software. All data are presented as mean ± standard deviation, except for the gene expression data, which are presented as boxplots with the median and the 25th and 75th quartile. Control groups without growth factors and groups supplemented with growth factors were compared using a Kruskall-Wallis H test with a post hoc Mann–Whitney U test.

Results

Effects of growth factors on matrix deposition and cross-links

To investigate the effect of different growth factors on matrix deposition, we cultured chondrocytes in 3D alginate beads in the presence of growth factors. The amount of DNA was determined in the alginate beads, to give an indication of the number of chondrocytes present in the beads. Between the experiments, the amount of DNA at day 0 was 0.18 µg DNA/bead ± 0.025 µg/bead. After 20 days, the amount of DNA in the control condition was 0.27 µg/bead ± 0.04. In time, the used growth factors had no significant effect on the amount of DNA per bead compared to the control condition without growth factors. Addition of IGF1 resulted in 97%, TGFβ2 in 95% and FGF2 in 112% of the amount of DNA compared to the control condition at day 20. Because the used growth factors had no differential effects on the cell proliferation and we were interested in the overall matrix production, not in the matrix production per cell, results of matrix deposition is expressed per alginate bead.

Chondrocytes in alginate produced considerable amounts of proteoglycans over the three weeks culture period, resulting in a proteoglycan concentration in the control condition of 8.1 µg/bead at day 11 and 29.9 µg/bead at day 20. Cultured in the presence of 25 ng/ml IGF1, the chondrocytes deposited more proteoglycans than in the control condition resulting in 1.4-fold increase of proteoglycans at day 11 and 1.8-fold increase of proteoglycans at day 20 (Figure 2.1). 2.5 ng/ml IGF1 resulted in more proteoglycans at day 20 but not at an earlier time point. Addition of TGFβ2 to the culture medium of chondrocytes resulted in less proteoglycans at both time points, although the effect was not significant with 25 ng/ml TGFβ2 at day 20. Cells stimulated with 2.5 ng/ml

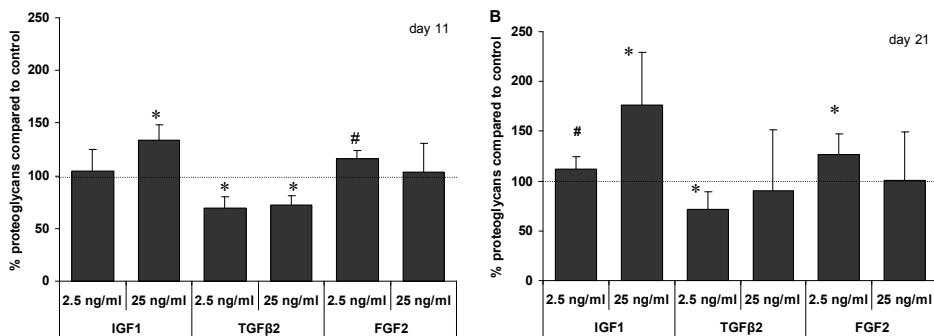**Figure 2.1.** Effect of growth factors on proteoglycan deposition in 3D chondrocyte cultures. Proteoglycan deposition is shown after A) 11 days of culture (n=6) and B) 21 days of culture (n=12). * indicates $p<0.01$ and # indicates $p<0.05$ compared to the control condition without growth factors, which is set at 100% (sd = 8.2% at day 11 and sd = 6.8% at day 21). Data are presented as mean % ± SD.
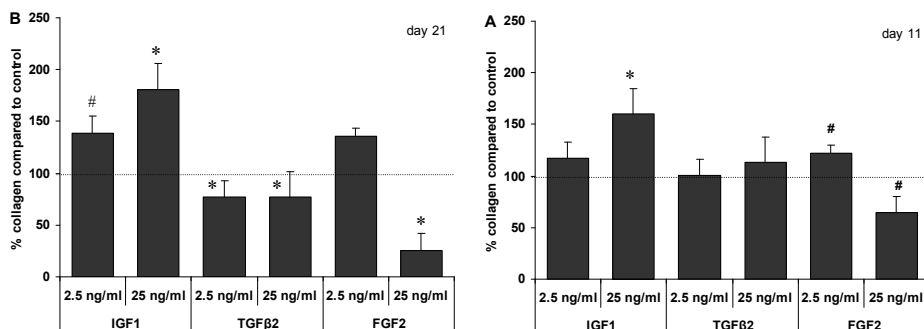
Figure 2.2. Effect of growth factors on collagen deposition in 3D chondrocyte cultures. Collagen deposition is shown after A) 11 days of culture (n=6) and B) 21 days of culture (n=12). * indicates p<0.01 and # indicates p<0.05 compared to the control condition without growth factors, which is set at 100% (sd = 2.5% at day 11 and sd = 16.1% at day 21). Data are presented as mean % ± SD.

FGF2 deposited more proteoglycans at both time points whereas 25 ng/ml FGF2 had no significant effect on the deposition (Figure 2.1).

The effect of growth factors on collagen, the other main constituent of cartilage, was also investigated in our study. Chondrocytes in alginate beads produced 4.8 μg collagen /bead at day 11 and 8.3 μg collagen/bead at day 20. Addition of IGF1 resulted in approximately the same effect as that seen on proteoglycan deposition; 1.6-times more collagen at day 11 and 1.8-times more collagen at day 20 with 25 ng/ml IGF1. With 2.5 ng/ml IGF1 the upregulation was only significant at day 20. At day 11, TGFβ2 had no effect on collagen deposition but by day 20, both concentrations resulted in 25% less collagen. FGF2 had a bi-directional effect on collagen deposition; 2.5 ng/ml FGF2 resulted in more collagen deposition, although this was not significant at day 20; whereas 25 ng/ml resulted in less collagen deposition, up to a 1.75-fold downregulation at day 20 (Figure 2.2).
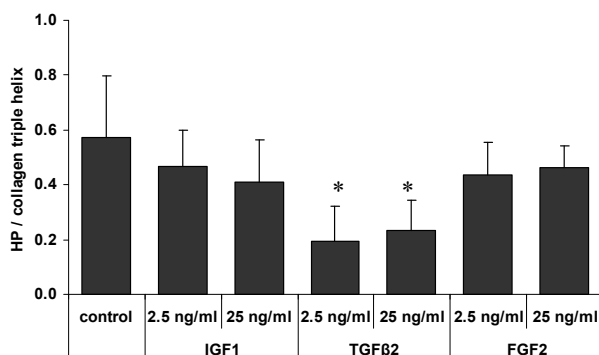

Figure 2.3. Effect of growth factors on collagen cross-linking. Hydroxylysylpyridinoline (HP) crosslinks in newly formed collagen are shown after 20 days of culture (n=12). * indicates p<0.01 Data are presented as mean ± SD.

After 20 days of culture, HP cross-links were present in the collagen network in contrast with LP cross-links, which were almost undetectable in the extracellular matrix.
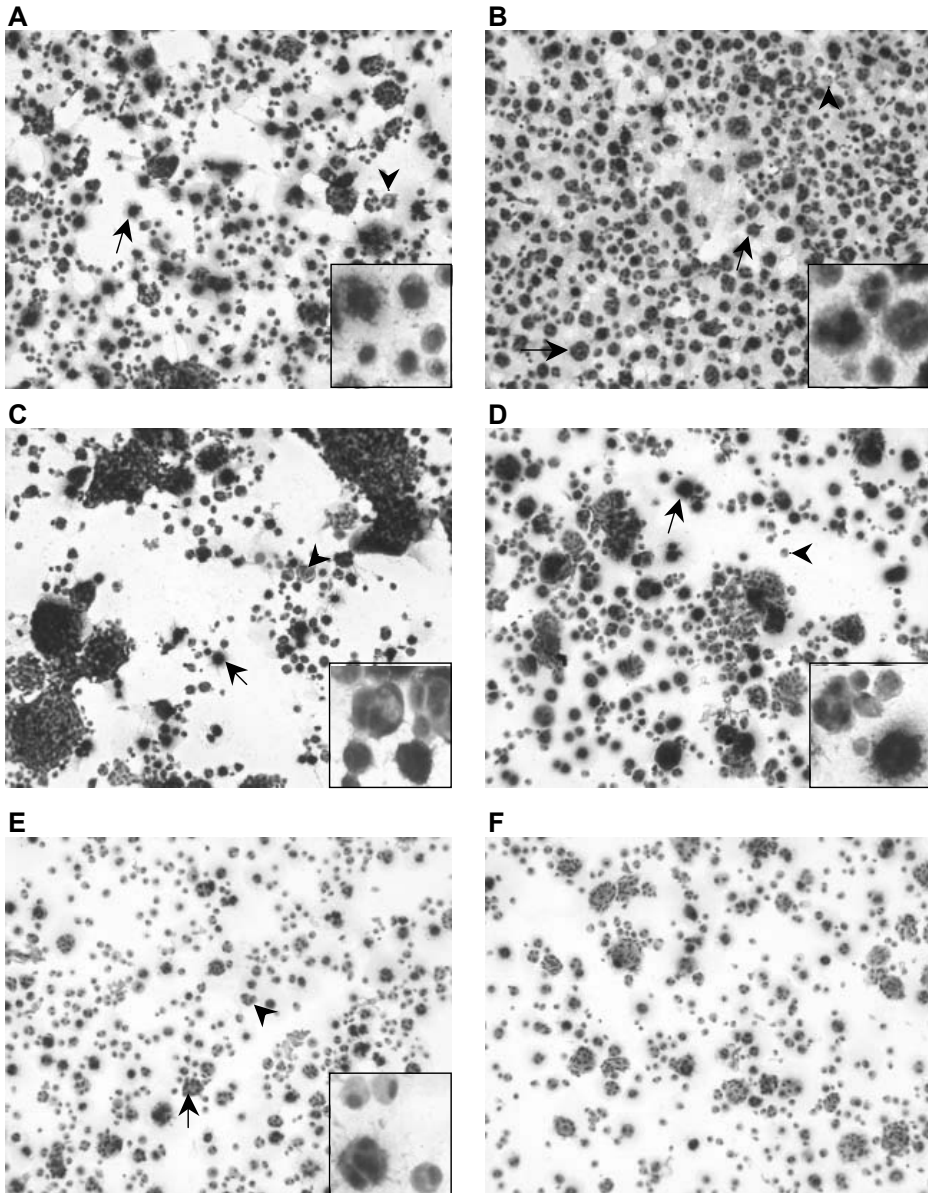
Figure 2.4. Immunohistochemical analysis of produced matrix. Collagen type II deposition in a) cells cultured in the presence of IGF1 b) TGFβ2 and c) FGF2 and d)cells cultured without growth factors; e) collagen type I is mainly only present in large cell clusters, not in matrix of single cells, f) is the isotype control. Magnification is 100 times, with a 400 times inset. Positive cells are indicated arrows, arrowheads indicate negative cells.

During the 20 days of culture, IGF1 and FGF2 had no significant effect on the HP-cross-links. On the other hand, the use of 2.5 or 25 ng/ml TGFβ2, unexpectedly downregulated HP cross-links per collagen molecule 2.5 times and 3.1 times, respectively (Figure 2.3).

Type of collagen production

To confirm whether the appropriate type of collagen (collagen type II) is made in our alginate system, a immunostaining for collagen type I and type II was performed on cytospins at the end of the experiment after 35 days of culture. 60-90% of the cells cultured in the presence of 25 ng/ml IGF1 (Figure 2.4A) and TGFβ2 (Figure 2.4B) were positive for collagen type II , in contrast to the control and the FGF2 condition where
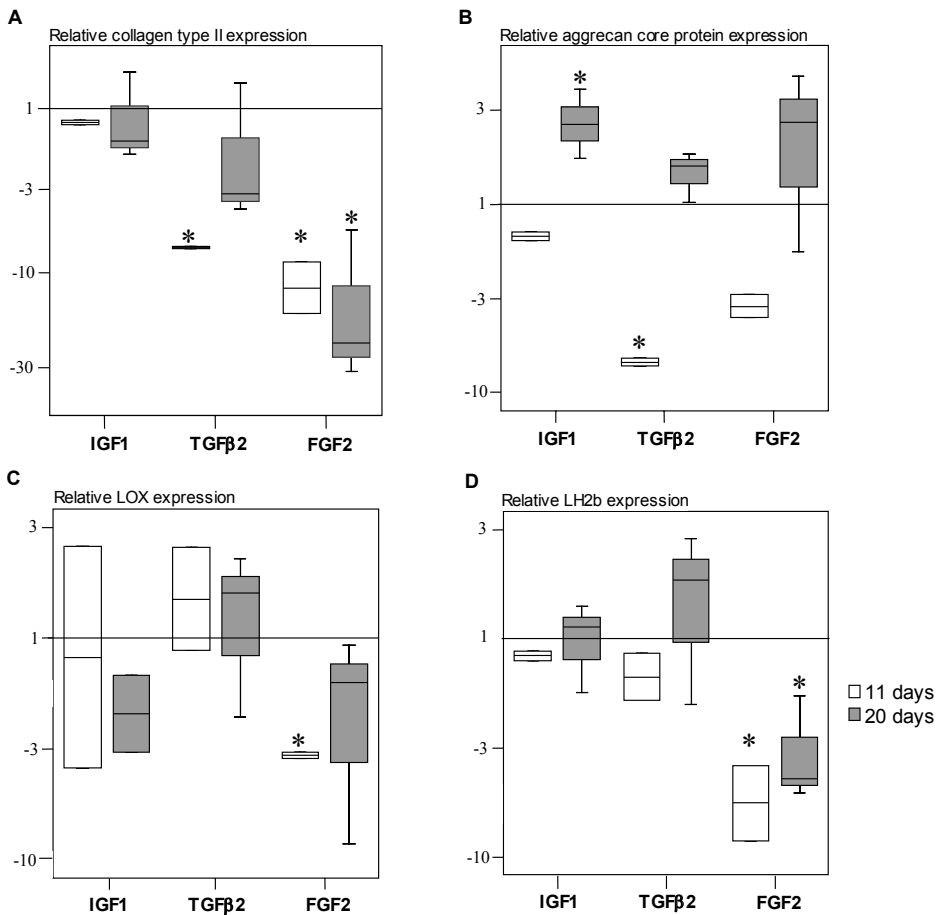

Figure 2.5. Gene expression analysis after 11 and 20 days of culture as n-fold change compared to the untreated control. A) collagen type II, B) aggrecan, C) LOX, and E)LH2b expression. * indicates p<0.01 compared to control. Data are presented as boxplots with median, 25th and 75th quartile for the gene expression with the control condition without growth factors set at 1.

approximately 40-60% of the cells were positive for collagen type II (Figure 2.4C and 4D). In every condition, approximately 10-40% of the cells stained positive for collagen type I of which a representative example is shown in Figure 2.4E.

Gene expression of matrix constituents and collagen modifying enzymes

To examine early regulation in matrix deposition and collagen cross-linking, gene expression of collagen type II (COL2), aggrecan, LOX and LH2b was determined after 11 days and 20 days of culture. COL2 expression was not influenced by 25 ng/ml IGF1 at both time points (Figure 2.5A). Furthermore, there was almost no effect of IGF1 on aggrecan, LOX and LH2b expression at day 11 (Figure 2.5B, C and D). At day 20, on the other hand, aggrecan expression is 2.7 times upregulated compared to the control (Figure 2.5B).

Expression of the matrix constituents collagen type II and aggrecan was considerably downregulated when cells were cultured in the presence of TGFβ2 for 11 days (Figure 2.5A and B), in contrast to the culture after 20 days where TGFβ hardly had an effect on COL2 and aggrecan expression. In contrast to the expectations, 25 ng/ml TGFβ2 had no apparent effect on the enzymes LOX and LH2b involved in collagen cross-linking during the culture period (Figure 2.5C and D).

25 ng/ml FGF2 downregulated COL2 expression up to 23 times at day 20 (Figure 2.5A) and had little or no effect on aggrecan expression. The expression of the collagen cross-linking enzymes LH2b and LOX was also downregulated in the presence of FGF2.

MMP activity and gene expression

Because MMPs influence matrix quality and content, we determined overall MMP activity in the culture media and gene expression for the specific collagenase MMP-1 at day 20. Because we were interested in possible breakdown of collagen type II, we did not analyze the MMP-2 expression since this is a gelatinase, which can only cleave unwound collagen, following initial cleavage by a collagenase. MMP-8 and MMP-13 were not examined because of their minor role in normal chondrocytes. 25 ng/ml IGF1 and TGFβ had no significant effect on the MMP activity in the culture medium at day 20 compared to the control. On the other hand, 25 ng/ml FGF2 increased the overall MMP activity (Figure 2.6A). Gene expression at day 11 and day 20 of MMP-1, the most important collagenase in healthy cartilage, was downregulated when chondrocytes were cultured with IGF1 and even more with TGFβ2, up to 20 times less at day 11. FGF2 had no effect on MMP-1 expression at both day 11 and day 20 (Figure 2.6B).
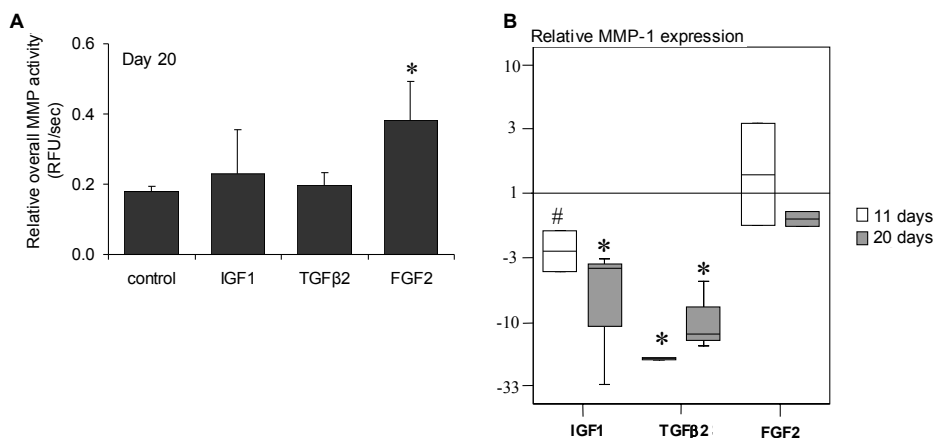
Figure 2.6. Overall MMP activity and MMP-1 expression. a)Overall MMP activity was measured in the culture medium after 20 days of culture together with b) the gene expression of the specific collagenase MMP-1 after 11 and 20 days of culture. * indicates p<0.01 and # indicates p<0.05 compared to the control condition. Data are presented as mean ± SD for the overall MMP activity and as boxplots with median, 25th and 75th quartile for the gene expression with the control condition without growth factors set at 1.

Association between biochemical and biomechanical properties

To test whether the growth factors could influence mechanical properties via their effect on amount of extracellular matrix, its composition and cross-linking, we examined the effect of the three growth factors on the equilibrium aggregate modulus and the secant modulus. Direct after making the cell containing alginate disks, the mechanical properties indicated by the equilibrium aggregate modulus and the secant modulus were 0.36 kPa and 2.1 kPa respectively, in concordance to previous results[167]. After five weeks of culture, matrix production contributed to the mechanical properties, indicated by the increase of the equilibrium aggregate modulus to 1.5 kPa and the secant modulus to 7.0 kPa in the control condition.

Cultures with IGF1 resulted in an upregulation of the secant modulus and a trend to upregulation of the equilibrium aggregate modulus. The proteoglycan and collagen content (Figure 2.7C and D) in these mechanically tested constructs was also higher than in the control condition without growth factors. The number of cross-links per collagen molecule (Figure 2.7E) was equal compared to the control. When chondrocytes were cultured in the presence of TGFβ2 for 5 weeks, both mechanical properties were unaffected compared to the control (Figure 2.7A and B), while the proteoglycan deposition and the number of cross-links per collagen molecule were greatly reduced in the same alginate disks (Figure 2.7C and 2.7E). After five weeks of culture with TGFβ2, collagen deposition was unaffected (Figure 2.7D). FGF2, on the other hand, resulted in decreased mechanical properties, although not significant compared to the control (Figure 2.7A
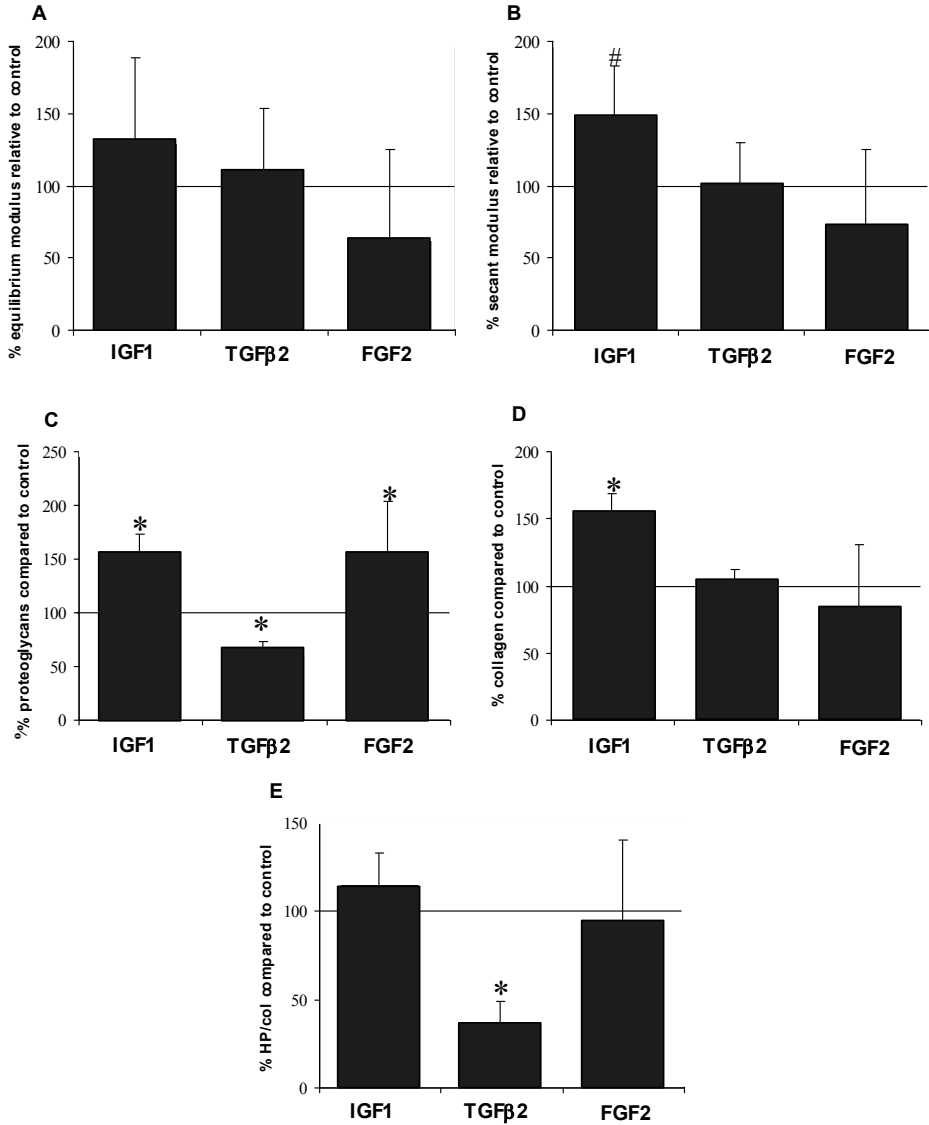
Figure 2.7. Relation between mechanical properties and matrix constituents after 35 days. Biochemical and biomechanical properties of tested alginate disks after 35 days of culture as percentage compared to the control with a)the equilibrium modulus of the newly formed matrix (control is 100 ± 26%) b)the secant modulus of the newly formed matrix (control is 100 ± 23%), c)proteoglycan deposition (control is 100 ± 11%) d)collagen deposition (control is 100 ± 12%) and e)HP-crosslinks per collagen molecule (control is 100 ± 10%). * indicates p<0.01 and # indicates p<0.05 compared to control. Data are presented as mean ± sd.

and B). These poor properties can partially be explained by the downregulation of collagen deposition, which is significant after 11 and 20 days of culture (Figure 2.2), and also appears to be downregulated but not significant at day 35. This is apparently not compensated by more proteoglycans (Figure 2.7C) or an equal number of cross-links per collagen molecule (Figure 2.7E).

Discussion

The formation of cartilage matrix in vitro or after damage in vivo is characterized by low collagen production and high proteoglycan production [142, 146]. The present study shows differential effects of IGF1, FGF2 and TGFβ2 on proteoglycan and collagen synthesis, collagen cross-links and the mechanical properties of the newly formed matrix. Of these three growth factors, IGF1 is most promising with regard to promoting a functional extracellular matrix with high proteoglycan and collagen deposition without altering the relative number of cross-links per collagen. Moreover, the mechanical properties of constructs cultured with IGF1 were improved compared to control constructs.

Using IGF1 in the alginate culture system resulted in approximately a 1.5-fold increase in both proteoglycan and collagen deposition after 35 days of culture. Upregulation of proteoglycan deposition with IGF1 is consistent with previous data [152, 153, 159, 168, 169]. The effect of partially blocking IGF1 previously described, which led to a reduction in proteoglycan production [170], further supports the importance of IGF1 in proteoglycan synthesis. On the other hand, the effect on collagen deposition in the presence of IGF1 was less intensively investigated and less consistent. IGF1 did not affect collagen deposition in one study [168] but caused an upregulation of collagen synthesis in other studies [153, 171]. In contrast to the increase in collagen protein that we observed, gene expression of collagen type II was not influenced whereas aggrecan expression was slightly upregulated in the presence of IGF1. This suggests that many of the synthesized collagen might be incorporated in the matrix. Also, the significant downregulation of MMP-1 expression might have contributed to more matrix deposition. MMP activity is regulated on several levels; transcription, translation, activation and inhibition. Therefore, the amount of MMP-1 mRNA is only indicative for the MMP-1 activity. The substrate used in our activity assay is mainly degraded by other MMPs than MMP-1, possibly explaining the discrepancy between the overall activity assay and the MMP-1 gene expression data.

The quality of the cartilage matrix is determined not only by the amount of matrix produced, but also by the number of cross-links between collagen molecules. We therefore determined the number of HP and LP cross-links, the two chemical variants of the mature collagen cross-links found in cartilage. In adult cartilage, the average is 1.5 HP

cross-links per collagen molecule [172], in immature cartilage the number is 0.8 HP. After 35 days of culture, a small although not significant increase in number of HP-cross-links per collagen molecule was detectable in the presence of IGF1 compared in the control condition, approaching the physiological number of cross-links in immature cartilage. The expression of the collagen cross-linking enzymes LH2b and LOX was not influenced by the presence of IGF1 even though more collagen was present in this culture condition. To our knowledge, there are no other publications about the effect of growth factors on collagen cross-linking in cartilage.

For the adequate repair of articular cartilage defects, it is especially important that the deposited matrix constituents are able to resist load in an articular joint. Different types of collagen have different properties concerning cross-linking and mechanical behavior. Therefore, we determined whether the appropriate type of collagen (collagen type II and not collagen type I) was produced in our culture system. Collagen type II was the most abundant type in the newly formed matrix in the IGF1 and TGFβ2 conditions. 20% of the newly formed matrix was positive for collagen type I, which was most probably deposited by cells in the outer layer of the alginate bead as shown previously [157].

Increased matrix deposition and increased cross-links per collagen molecule are expected to result in improved mechanical properties. In this study, these mechanical properties are measured as the equilibrium aggregate modulus and the secant modulus. The secant modulus is an indication for the friction between the solid and the liquid phase (e.g. the ability to hold water), while the equilibrium aggregate modulus is an indication for the stiffness of the formed matrix. The increase in extracellular matrix production in reaction to IGF1 might have resulted in a significantly higher secant modulus. This increased secant modulus indicates more friction between the water and the matrix resulting from a more condense or low permeable matrix. The equilibrium aggregate modulus also appears to be improved, although not significantly, indicating a stiffer solid phase. This property is defined mainly by the amount of collagen and relative number of collagen cross-links in the matrix [173], which also explains our result: more collagen deposition and the appropriate number of cross-links per collagen molecule. In addition, downregulation of MMP-1 gene expression might have resulted in less collagen breakdown, contributing to better mechanical properties, although this does not have to be in direct relation to each other since one MMP can digest several matrix molecules. Findings on the equilibrium modulus are consistent with the study by Mauck et al.[169], although this group found no result of IGF1 on the secant modulus.

With up to 25% less collagen and proteoglycan deposition, TGFβ2 had less prominent, but significant effects on matrix deposition. Decreased proteoglycan deposition is consistent with previous data [152, 174]. However, upregulation of proteoglycan and collagen deposition using TGFβ is also shown by some authors [156, 168, 169, 173, 174]. Factors that might explain these varying results in literature include different isoforms of

TGFβ (TGFβ1 vs. TGFβ2) the difference in cartilage donor (human, rabbit or bovine), the amount of matrix surrounding the cell, the presence of serum in the culture or the scaffold used for culture (polyglycolic acid, agarose or alginate). In concordance to the inhibition of collagen and proteoglycan deposition, gene expression at day 11 of collagen type II and aggrecan was decreased compared to the control condition but recovered at day 20. Although the immunostaining for collagen type II is used to determine the type of collagen produced, it is striking to see more positive cells in the TGFβ2 condition than in the control condition, which is in contrast to less collagen deposition in the TGFβ2 condition. Possibly, TGFβ2 had a positive effect on the type of collagen produced but not on the amount of collagen produced. The downregulation of MMP-1 gene expression had no effect on collagen deposition. Surprisingly, we found that TGFβ2 resulted in more than a two-fold downregulation of HP-cross-links per collagen molecule. This is in contrast to what we previously found after addition of either isoform of TGFβ to fibroblasts [175], this addition resulted in an almost four-fold increase of the cross-linking enzyme lysyl hydroxylase (LH) 2b expression and more HP and LP cross-links. In the present study, no effect was seen on LH2b gene expression. Whether this discrepancy may be explained by different cell types or a different type of collagen produced (collagen type II by chondrocytes vs. collagen type I by fibroblasts) is currently under investigation. The mechanical properties in cultures with TGFβ2 did not differ from the control condition, which is unexpected regarding the low number of collagen cross-links, the downregulation of MMP-1 gene expression and in contrast to the previous study by Mauck et al [169]. It appears that, in our system, collagen and proteoglycan amount have more effect on mechanical properties than collagen cross-links. Moreover, in a previous study we have found that addition of TGFβ2 changed the distribution of proteoglycan over "cell-associated" (pericellular and territorial) and further-removed (interterritorial) matrix in the alginate bead; relatively more of the proteoglycan was laid down in the further-removed matrix compared to the cell-associated matrix [156]. Since the interterritorial matrix mainly contributes to the mechanical properties of the construct, this might be a mechanism to retain functional properties, independently of the relative number of cross-links and the absolute amount of matrix components.

FGF2 had an interesting effect on matrix deposition. Proteoglycan deposition could be upregulated by FGF2, collagen deposition on the other hand, was slightly increased with the low concentration of FGF2 and reduced with the high concentrations FGF2. This so called bi-directional effect is described by others for proteoglycan deposition, but not for collagen deposition [153], and it can not be completely excluded that this results from the use of 25 ng/ml FGF2, a concentration which could be beyond the optimal concentration for stimulating matrix deposition in this culture system. In concordance with our results, the group of Shida et al. [176] found a decrease in mRNA levels for α1(II) procollagen when FGF2 was injected in rat knee joints. When chondrocytes are cultured

in alginate disks for 35 days with the addition of 25 ng/ml FGF2, the proteoglycan deposition is increased and collagen deposition is decreased but not significantly different from the control, indicating a difference in FGF2 effect depending on the culture environment (bead vs. disc) or time of culture (three vs. five weeks). Immunohistological analysis of the produced matrix in presence of FGF2, confirms the low collagen deposition during the culture while only 40% of the cells were positive for collagen type II and even less cells for collagen type I. Less collagen deposition can be partially explained by the high overall MMP activity. Collagen cross-linking levels were not influenced by neither the different concentrations of FGF2 nor in time, although LH2b and LOX mRNA levels were downregulated. This is not surprising, as less collagen needs to be cross-linked in this situation. Looking at the mechanical properties, both the equilibrium aggregate modulus and the secant modulus appear to be lower than in the control condition without growth factors. The decrease in collagen deposition might have resulted in a softer and also more permeable matrix indicated by both the lower equilibrium aggregate and secant modulus.

In summary, this study demonstrates differential effects of IGF1, TGFβ2 and FGF2 on generation of new cartilage. It indicates that the use of IGF1 in culturing chondrocytes is useful in promoting collagen synthesis and improving mechanical properties of newly formed cartilage whereas TGFβ2 and FGF2 do not clearly enhanced these parameters. Future studies will further investigate the quality of the produced matrix by examining the degradability of the matrix and the organization on a nanohistological (electron microscopic) level. Eventually, this may contribute to the optimalization of culture conditions in cartilage tissue engineering and might be applied in in vivo studies for the repair of articular cartilage defects.

3

TGFβ affects collagen cross-linking independent of chondrocyte phenotype but strongly depending on physical environment

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Abstract

Background

Transforming Growth Factor β (TGFβ) is often used in cartilage tissue engineering to increase matrix formation by cells with various phenotypes. However, adverse effects of TGFβ such as extensive cross-linking in cultured fibroblasts have also been reported. Our goal was to study effects of TGFβ on collagen cross-linking, evaluating the role of cellular phenotype and physical environment.

Method

We therefore used four different cell populations in two very different physical environments; primary and expanded chondrocytes and fibroblasts embedded in alginate gel and attached to tissue culture plastic. Matrix production, collagen cross-linking and α-smooth muscle actin (αSMA) were analyzed during 4 weeks with(out) 2.5 ng/ml TGFβ2.

Results

TGFβ2 did not affect collagen deposition by primary cells. In expanded cells, TGFβ2 increased collagen deposition. Chondrocytes and fibroblasts in monolayer produced more collagen cross-links with TGFβ2. In alginate, primary and expanded cells displayed an unexpected decrease in collagen cross-linking with TGFβ2. αSMA was not present in alginate cultures and barely upregulated by TGFβ2. Organized αSMA fibers were present in all monolayer cultures and became more pronounced with TGFβ2.

Discussion

This study demonstrates that the physical environment determined by the substrate used, co-determines the response of cells to TGF-beta. The presence of mechanical stress, determined with αSMA-staining, is probably responsible for the increase in collagen cross-linking upon addition of TGFβ.

Introduction

TGFβ plays an important role in cartilage repair. In tissue engineering studies, TGFβ is often used to redifferentiate expanded chondrocytes. Expansion, needed to obtain sufficient cell number for tissue-engineering purposes, results in dedifferentiation and a more fibroblast-like phenotype of the cells. In combination with other growth factors and in a serum-free culture medium, TGFβ induces proteoglycan production and collagen type II gene expression by expanded chondrocytes [177-179]. In addition, adenoviral overexpression of TGFβ results in constitutive collagen type II expression by expanded chondrocytes [180].

Next to redifferentiation of expanded chondrocytes, TGFβ is also used to induce a chondrogenic phenotype in bone marrow derived mesenchymal stem cells resulting in the deposition of typical cartilage proteins such as aggrecan and gene expression of collagen type II [181, 182]. Besides effects on cell phenotype, TGFβ induces production of cartilage matrix proteins. TGFβ leads to an increase in proteoglycan synthesis and content in healthy murine articular cartilage and protects against proteoglycan depletion in murine experimental osteoarthritis (OA) [183, 184]. Addition of TGFβ to chondrocytes in culture leads to various results: stimulation of proteoglycan and collagen production [156, 185-187] but also inhibition of proteoglycan deposition [152, 188]. These results indicate differential effects of TGFβ1 and TGFβ2, that most likely depend on cell type, cell source and culture substrate but not necessarily on the isoform [189].We recently demonstrated that TGFβ downregulates the formation of collagen pyridinoline cross-link formation by articular chondrocytes in alginate culture [188].

In contrast to the positive effects of TGFβ in cartilage repair, TGFβ is also the most potent inducer of unwanted collagen gene expression by fibroblasts resulting in excessive tissue deposition and eventually fibrosis [190]. Fibrosis is characterized by a high level of the pyridinoline collagen cross-links [191] and the formation of these cross-links is induced by the presence of either of the three isoforms of TGFβ [192]. Fibrosis is also seen in vivo in articular joints where injection of TGFβ leads to fibrosis of the synovium [193]. In addition, blockade of the TGFβ-pathway in murine experimental OA prevents against synovial fibrosis [184].

Taken together, TGFβ has differential effects on cell behavior and extracellular matrix production and cross-linking. It seems that the effect is depending on cell phenotype, the physical environment determined by culture set-up or a combination of these factors. The contribution of these factors to the differential effects of TGFβ has to be elucidated to be able to apply TGFβ to improve cartilage tissue engineering. This information will be useful for the choice of biomaterials to be used for cartilage tissue engineering. Our goal was to compare the effect of TGFβ on collagen biochemistry and especially collagen cross-linking in different cells cultured in different physical environments. To

evaluate the influence of cell phenotype, we used different cells ranging from differenti-
ated chondrocytes to fibroblasts. To evaluate the effect of the physical environment,
the cells were cultured in two different environments: attached to plastic in monolayer,
where they are under continuous cellular contractile stress, or embedded in alginate gel,
where they cannot adhere and thus are free from mechanical loading.

Materials and Methods

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged
6–12 months. Primary fibroblasts were obtained from split skin biopsies of the skin cov-
ering the bovine joints. To isolate chondrocytes and fibroblasts, both the full-thickness
slices of noncalcified articular cartilage and the split skin biopsies were subjected to
pronase (2 mg/ml, Sigma, St. Louis, MO, USA) digestion for two hours followed by
overnight collagenase B (1.5 mg/ml, Roche Diagnostics) digestion. Depending on the
culture conditions, the primary P0 or expanded P3 cells were either resuspended in
1.2% (w/v) low viscosity alginate (Keltone) in 0.9% NaCl (Sigma) at a concentration of
4×10^6 cells/ml to make alginate beads or cultured as monolayer on plastic in a concen-
tration of 7.500 cells per cm^2. Beads were made as described previously [194]. Beads were
cultured in 75 µl/bead and monolayers in 2 ml/monolayer Dulbecco's Modified Eagle
Medium (DMEM) high glucose (GibcoBRL, Gaithersburg, MD, USA) supplemented with
5% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50
µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). Cells were cultured with or
without 2.5 ng/ml Transforming Growth Factor beta 2 (TGFβ2). TGFβ1 and TGFβ2 have
similar effects on matrix production [189]. TGFβ3 is an important factor in chondrogenic
differentiation of mesenchymal stem cells and less used when primary chondrocytes
are cultured [195, 196]. No differences were found in our previous study about the three
isoforms and their effect on collagen cross-linking in fibroblast cultures [192]. Therefore,
TGFβ2 was chosen based on previous results [188]. Culture medium was replaced three
times a week.

Biochemical assays

Alginate beads or monolayers were digested overnight at 56°C in papain buffer (200
µg/ml papain in 50 mM Ethylene Diamintetraacetate (EDTA) and 5 mM L-cystein).
The amount of DNA in each RNAse (Sigma) pre-treated papain-digested sample was
determined using ethidium bromide (GibcoBRL) dye with calf thymus DNA (Sigma) as
a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxy-
proline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP and lysylpyridinoline,
LP) was performed as described previously [162, 163].. The quantities of cross-links were

expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

Gene expression analysis

For total RNA isolation, alginate beads were dissolved in 150 µl/bead 55 mM sodium citrate acid. Cell pellets of the alginate beads and monolayers were suspended in 1000µl RNA-Bee™ (TEL-TEST, Inc.; Friendswood, TX, USA), and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). For Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Forward: GTCAACGGATTTGGTCGTATTGGG, Reverse: TGCCATGGGTG-GAATCATATTGG, Probe: Fam-TGGCGCCCCAACCAGCC-Tamra) and SRY-box containing gene 9 (SOX9, Forward: CAACGCCGAGCTCAGCA, Reverse: TCCACGAAGGGCCGC, Fam-TGGGCAAGCTCTGGAGACTTCTGAACG-Tamra), each cDNA sample was amplified using specific primers and specific probe (Eurogentec, Seraing, Belgium). Amplifications were done as described previously [188]. Data were analyzed using Sequence Detector version 1.7 (Applied Biosystems) software and normalized for GAPDH expression.

Immunohistochemical staining for alpha Smooth Muscle actin, type I Collagen and type II Collagen

Staining for the presence and organization of alpha smooth muscle actin (αSMA) was performed after 1 week of culture of primary chondrocytes and fibroblasts, to examine differences in mechanical stress between the monolayer and alginate beads. The additional effect of TGFβ2 on the organization of the αSMA fibers was also investigated. The presence of collagen types I and II was investigated after 4 weeks of culture. For all three stainings, three alginate beads were dissolved in 55 mM sodium citrate acid to prepare cytospins. Cells in monolayer were cultured on glass culture chambers (BD Biosciences San Jose, CA, USA) to stain them immunohistochemically. Cells with surrounding pericellular matrix from alginate beads were centrifuged on glass slides and fixed in cold acetone, monolayers in cold 70% ethanol. The samples were incubated with monoclonal antibodies against αSMA (clone number 1A4, Sigma), type I collagen (ab6308, Abcam, Cambridge, UK), or type II collagen (1:100, II-II6B3; Developmental Studies Hybridoma Bank) at room temperature for 1 h. This was followed by incubation with link and label from the link-label kit (BioGenex, San Ramon, CA, USA) for 30 minutes. Freshly prepared neo-fuchsin substrate was used to achieve staining.

The collagen staining was scored for the number of cells positive for collagen types I and II per 100 cells in four fields of sight per specimen, resulting in a percentage of positive cells. An arbitrary scoring system from 1 to 5 was used, with 1 representing less

than 10% of the cells positive, 2 representing 10-40% positive, 3 representing 40-60% positive, 4 representing 60-90% positive and 5 representing more than 90% of the cells positive. A cell was considered positive, when the matrix around the cell or the cytoplasm was stained pink.

Data analyses

The experiments were performed three times with 3 samples of 7 beads or with 3 monolayers per experimental condition for biochemical analyses and one sample of 10 beads or one monolayer per experimental condition for gene expression analysis. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) software. All data are presented as mean ± standard deviation. Control groups without TGFβ2 and groups supplemented with TGFβ2 were compared with a Kruskall-Wallis H test with a post hoc Mann–Whitney U test. The effect of adding TGFβ2 in the alginate bead condition versus the monolayer for each cell type was compared with a two-way ANOVA with a post-hoc Bonferroni test. The relative difference between the control group (set at 100%) and the TGFβ2-group was used for these calculations.

Results

Cell features

The amount of DNA was determined at day 0 and after 28 days of culture, to be able to compare matrix deposition between the conditions and examine the effect of the culture environment on cell proliferation. At day 0, 0.12 ± 0.03 μg DNA /bead and 0.23 μg DNA /monolayer was present. In all conditions, the amount of DNA increased or remained stable after 28 days of culture. This indicates that there was no cell death and even cell proliferation in most of the conditions. Overall, addition of TGFβ2 increased the total DNA amount ($p<0.005$), although not always significantly and with exception of fibroblasts in alginate beads (Figure 3.1A).

The total amount of isolated RNA was 101 ± 11 ng RNA per bead and 24890 ± 4904 ng RNA per monolayer. This increased with the presence of TGFβ2 in every condition ($p<0.05$) except for the fibroblasts in beads. Here, the amount of RNA was not significantly changed in the presence of TGFβ2.

Collagen matrix deposition

Addition of TGFβ2 to the primary chondrocytes in alginate beads had no effect on collagen deposition. A low amount of collagen was deposited in cultures of dedifferentiated chondrocytes, as well as primary and dedifferentiated fibroblasts in alginate beads. Addition of TGFβ2 increased the collagen deposition in P3 chondrocytes and fibroblasts
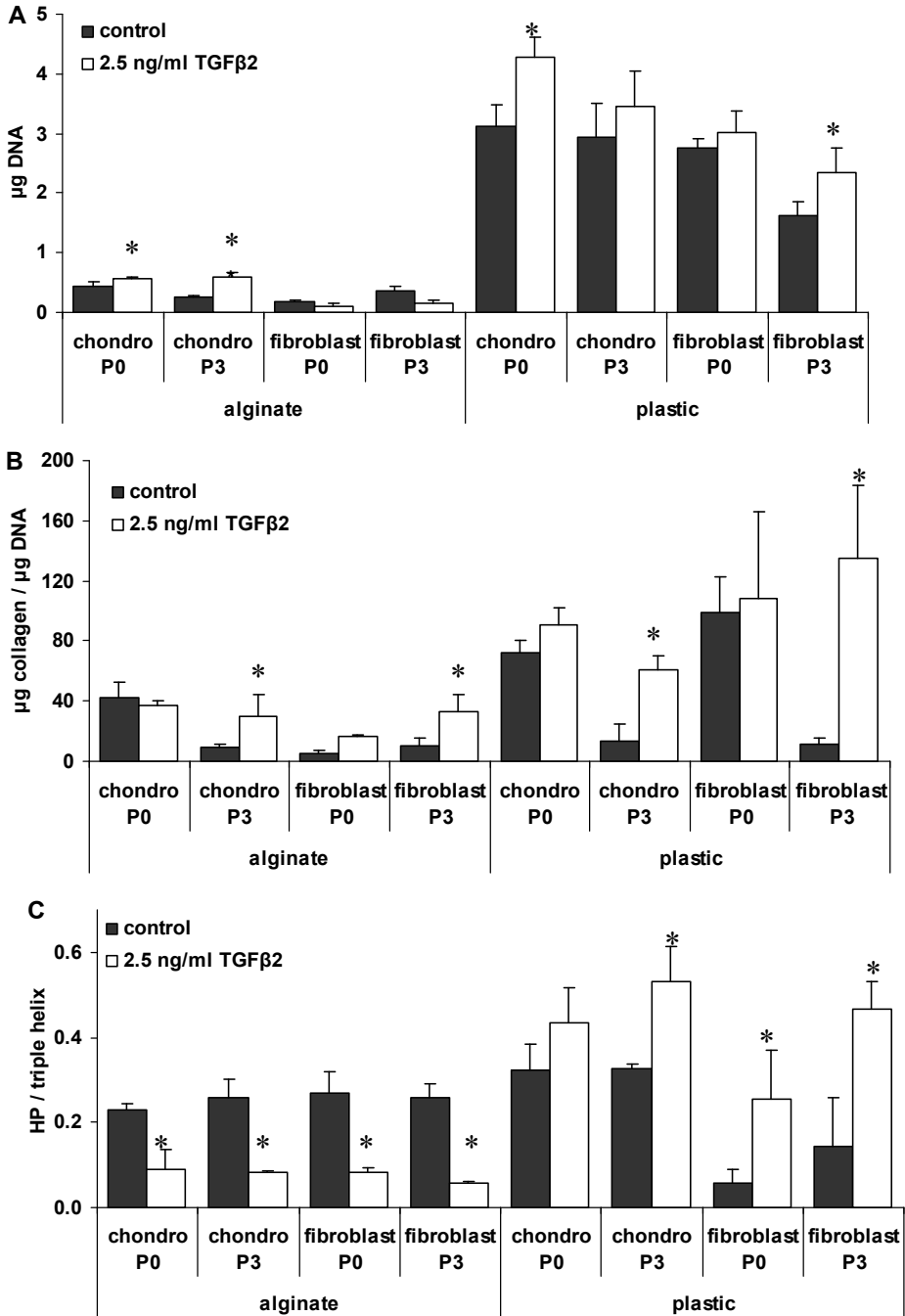
Figure 3.1. The effect of 2.5 ng/ml TGFβ2 on (A) DNA content, (B) collagen deposition and (C) number of collagen cross-links in cultures of primary (P0) and expanded (P3) chondrocytes and fibroblasts cultured in alginate beads or as monolayer on plastic for 28 days (n = 6). * indicates significant difference (p<0.05) from control without TGFβ2. Data are shown as mean ± SD.

in alginate beads (p<0.005). Collagen deposition by primary chondrocytes and fibroblasts on plastic was high but not influenced by the presence of TGFβ2. Low amount of collagen was deposited in dedifferentiated P3 chondrocytes and P3 fibroblasts on plastic where addition of TGFβ2 increased collagen deposition (p<0.005, Figure 3.1B).

Matrices deposited by primary and expanded chondrocytes and fibroblasts in alginate beads had an average of 0.22 HP cross-links per collagen molecule. Matrices deposited by chondrocytes on plastic had a similar number of cross-links per collagen molecule in the control condition. Matrices deposited by fibroblasts on plastic had a relatively low number of HP cross-links per collagen molecule in the control condition. Addition of TGFβ2 to the culture medium resulted in a decrease of collagen cross-links in all alginate bead cultures and an increase of collagen cross-links in all cultures on plastic, independent of cell type (p<0.001 for alginate bead versus plastic, Figure 3.1C).

Type of collagen produced

Chondrocytes cultured in alginate beads deposited mainly type II collagen (arbitrary score 4.25 ± 0.5, Figure 3.2A) and less type I collagen (score of 1.5 ± 0.6, Figure 3.2B)
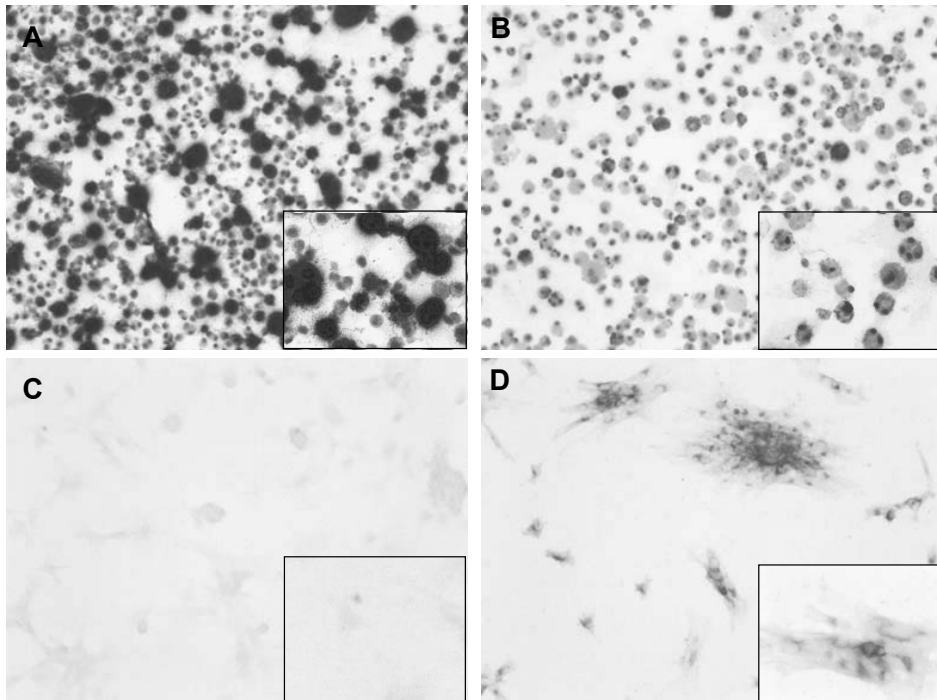
Figure 3.2. Immunohistochemical staining for collagen type II (A and C) and collagen type I (B and D) of chondrocytes cultured for 28 days in alginate beads (A and B, here shown as cells with pericellular matrix on glass slides) and as monolayer on plastic (C and D). Positive cells are shown in red. Magnification is 100×.

as determined by immunohistochemistry. Expanded chondrocytes in alginate beads had a trend towards more type I collagen than primary chondrocytes (score of 2 ± 0.5, not significant). However, after 28 days, type II collagen was the main type of collagen deposited (score 3.25 ± 1, p<0.05 versus collagen type I production) by the expanded chondrocytes as well. The primary and expanded fibroblasts in beads deposited almost no type II collagen and more type I collagen than the chondrocytes. Addition of TGFβ2 only increased the type II collagen deposition (score of 3.75 ± 0.5, p<0.05 versus control without TGFβ2) in the expanded chondrocytes, and no effect of TGFβ was seen in the other alginate bead conditions. Chondrocytes cultured on plastic deposited very little type II collagen (score 1.5 ± 0.6) and more type I collagen (score 3.75 ± 0.5) than the chondrocytes cultured in alginate beads. Dedifferentiated chondrocytes and both primary as well as expanded fibroblast deposited only type I collagen (score 4.75 ± 0.5, Figure 3.2C) and almost no type II collagen (score 0.5 ± 0.6, Figure 3.2D). After TGFβ addition, a trend towards more type II collagen was present in the primary chondrocytes on plastic (1.25 ± 0.5, not significant). No effect of TGFβ was seen in the other conditions.

Phenotype of the cells

After culture for 28 days, the gene expression for SRY-box containing gene 9 (SOX9) was examined to provide an indication of the phenotype of the cells. SOX9 gene expression was the highest in primary chondrocytes cultured in alginate beads (P0 chondro beads). Chondrocytes cultured for three passages and subsequently in alginate beads (P3 chondro beads) and the primary chondrocytes cultured on plastic (P0 chondro monolayer) also expressed some SOX9, but not as much as the primary chondrocytes in alginate beads. The passage 3 chondrocytes on plastic and all the fibroblast conditions almost did not
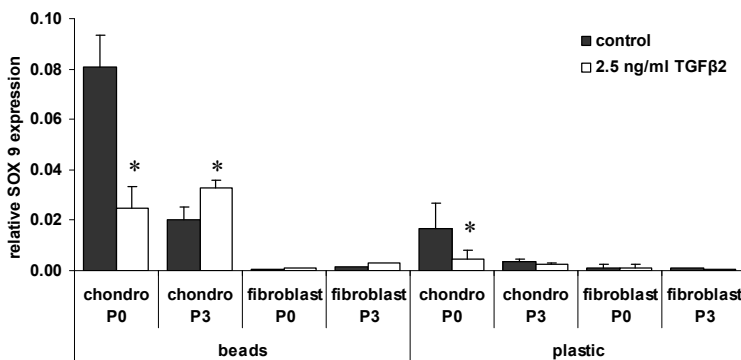
Figure 3.3. Relative gene expression of SOX9 by primary (P0) and expanded (P3) chondrocytes and fibroblasts cultured in alginate beads or in monolayer on plastic for 28 days (n = 6). The gene expression analysis was used to determine the phenotype of the cells after culture. * Indicates significant difference (p<0.05) from control without TGFβ2. Data are shown as relative expression ± SD.
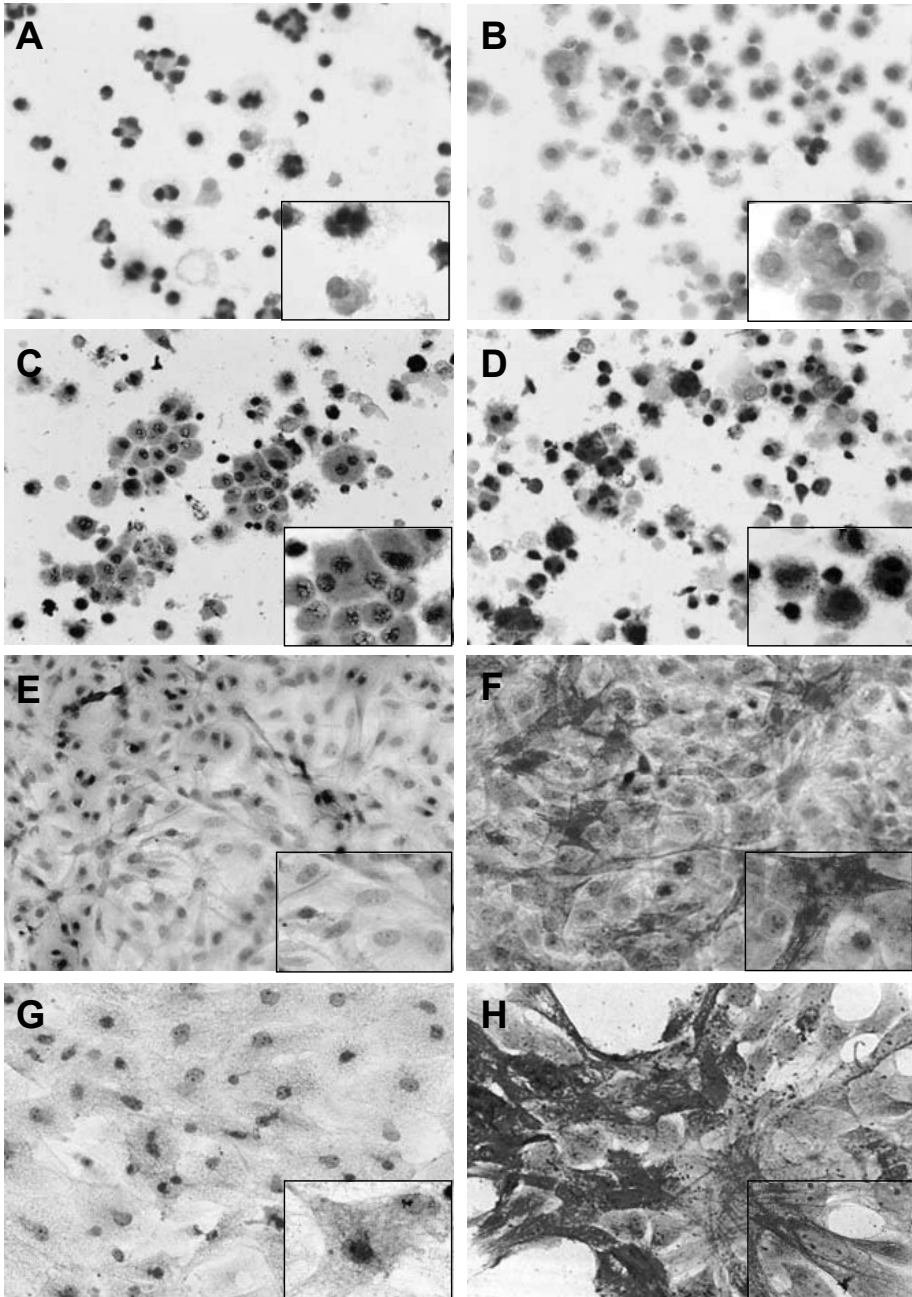
Figure 3.4. Immunohistochemical staining for α smooth muscle actin (αSMA) of primary P0 chondro-cytes (A, B, E, F) and fibroblasts (C, D, G, H) cultured in alginate beads (A-D, here shown as cells with pericellular matrix on glass slide) or in monolayer on plastic (E-H) for 28 days. αSMA shown in red is upregulated in response to TGFβ2 (B, D, F, H) although not organised in spindles in the cells cultured in alginate beads (B and D). Cells cultured in monolayer in the presence of TGFβ2 have organised αSMA fibres (F and H). Magnification is 200× with a 1000× inset.

express the SOX9 gene (Figure 3.3). Addition of TGFβ2 reduced SOX9 expression in the alginate bead and monolayer cultures of primary chondrocytes (p<0.005) but increased it in the expanded chondrocytes in alginate beads (p<0.005).

Alpha Smooth Muscle Actin organization

In alginate beads, none of the chondrocytes had detectable alpha smooth muscle actin (αSMA) but some fibroblasts were positive for αSMA (Figures 3.4A and 4C). In monolayer culture, positive αSMA staining without specific stress fibers was detectable in the majority of the chondrocytes (Figure 3.4E). All fibroblasts were positive for αSMA as shown with a red staining, although also no clear stress fibers were visible (Figure 3.4G). Addition of TGFβ2 to chondrocytes and fibroblasts in alginate beads resulted in more αSMA and the intensity of staining was higher. However, no intracellular stress fibers of αSMA were visible (Figure 3.4B and 4D). When the cells (chondrocytes and fibroblasts) were cultured on plastic, the presence of TGFβ2 induced αSMA with clearly visible organized stress fibers in all the cells as shown in figures 3.4F and 3.4H.

Discussion

This study demonstrates that the effect of TGFβ on cross-linking of collagen is mostly determined by the physical environment determined by the culture substrate of the cells and less by the cell phenotype or the type of collagen produced. When chondrocytes are cultured in alginate beads, TGFβ2 leads to less collagen cross-links in the deposited matrix. In the monolayers on plastic on the other hand, addition of TGFβ2 leads to more collagen cross-links in the deposited matrix.

The results elucidate the discrepancy in our previous results. A study with skin fibroblast cultured on plastic showed that TGFβ increased collagen crosslink formation [192]. However, in a study with differentiated chondrocytes in alginate gel, we surprisingly found an inhibition of collagen crosslink formation by TGFβ [188]. These studies differed in cell type used as well as physical environment used. In the present study eight different combinations of cell type and physical environment were compared to evaluate what determines the effect of TGFβ on collagen crosslink formation.

One of the main physical differences between plastic and alginate culture is the contractile stress on the cell. The culture systems used were investigated for their effect on the contractile stress on chondrocytes and fibroblasts by staining the cells for the presence and organization of αSMA. It has previously been shown that when the culture substrate exerts force on a cell, more αSMA is present in the cell [197-199]. Inhibition of αSMA assembly reduces contraction of a collagen-glycosaminoglycan scaffold, indicating the importance of αSMA in the interaction between the cell and

its environment [200]. This corresponds to our findings where αSMA is absent or poorly organized in the cells cultured in alginate beads, but clearly present in attaching cells cultured on plastic. It also confirms that cells cultured on plastic are under continuous contractile stress as a result of anchorage to the substrate and cell-cell contact. Cells cultured in alginate beads do not experience this stress because of less or no cell-cell contact and the absence of anchorage to the culture substrate. In the culture systems we used, especially the monolayer on plastic, it is difficult to uncouple cell-cell contact and anchorage to the culture substrate and their effect on the organization of αSMA. The effect of other differences between alginate and plastic, like for instance differences in nutrient diffusion or cell viability, cannot be completely excluded. Considering the cell viability, the amount of DNA did not decrease from day 0 to day 28 for any alginate condition. Together with the presence of RNA after 28 days of culture, this indicates the absence of cell death also in the fibroblasts cultured in alginate beads. We therefore believe that the effect seen in the alginate beads depends on the culture environment and not on cell viability and therefore a selection in the type of collagen deposited or phenotype. We can conclude that the culture environment greatly influences the effect of TGFβ on the collagen matrix.

To study the relation with cell phenotype, we determined gene expression for SOX9 and Immunohistochemical staining for collagen type I and II. SOX9 gene expression and the type of collagen deposited are related to the phenotype of the cells but not to the effect of TGFβ on collagen cross-linking: In case of the alginate bead cultures, no collagen type II was present in the fibroblast cultures. Here, addition of TGFβ decreased the number of collagen cross-links. Collagen type II was also present in the P0 chondrocytes cultured on plastic. The deposition of collagen type II increased slightly together with an increase in collagen cross-links with the presence of TGFβ. On the other hand, our data indicate that the cellular contractile stress on the cells, as determined by αSMA production and organization, is related to the effect of TGFβ. We conclude from these data that the effect of TGFβ on collagen cross-linking is dependent on the physical environment of the cells. It was recently found that αSMA is a mechanosensitive protein that is recruited to stress fibers under high tension. This recruitment allows cells to exert more stress on the culture scaffold [201]. TGFβ further upregulates αSMA deposition and condensation. Previously, it was found that TGFβ induces αSMA in fibroblasts [202, 203]. More recently, it is also seen in chondrocytes [200, 204] which corresponds with our findings that cells incubated with TGFβ have more αSMA staining. We hypothesize that in conditions of high tensile stress on the cell, the presence of TGFβ further increases αSMA organization and leads to collagen cross-link formation in order to better resist the stress. Moreover, addition of TGFβ does not inevitably has to result in fibrosis. If tensile stress is prevented, TGFβ can be used to increase collagen and proteoglycan production without leading to fibrosis. Therefore, the intended effect of TGFβ in cartilage repair will depend

on environmental conditions, such as mechanical environment and the scaffold used. Because collagen cross-linking has important implications on mechanical properties of the repair tissue, this should be seriously considered when choosing a scaffold in tissue engineering procedures where TGFβ is often used to stimulate tissue formation.

Acknowledgements

4

Contribution of collagen network features to functional properties of engineered cartilage

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Abstract

Background

Damage to articular cartilage is one of the features of osteoarthritis. Cartilage damage is characterized by a net loss of collagen and proteoglycans. The collagen network is considered highly important for cartilage function but little is known about processes that control composition and function of the cartilage collagen network in cartilage tissue engineering. Therefore, our aim was to study the contribution of collagen amount and number of cross-links on the functionality of newly formed matrix during cartilage repair.

Methods

Bovine articular chondrocytes were cultured in alginate beads. Collagen network formation was modulated using the crosslink inhibitor β-aminopropionitril (BAPN; 0.25 mM). Constructs were cultured 10 weeks with/without BAPN or 5 weeks with BAPN followed by 5 weeks without. Collagen deposition, number of cross-links and susceptibility to degradation by MMP-1 were examined. Mechanical properties of the constructs were determined by unconfined compression.

Results

BAPN for 5 weeks increased collagen deposition accompanied by increased construct stiffness, despite the absence of cross-links. BAPN for 10 weeks further increased collagen amounts. Absence of collagen cross-links did not affect stiffness but ability to hold water was lower and susceptibility to MMP-mediated degradation was increased. Removal of BAPN after 5 weeks increased collagen amounts, allowed crosslink formation and increased stiffness.

Discussion

This study demonstrates that both collagen amounts and its proper cross-linking are important for a functional cartilage matrix. Even in conditions with elevated collagen deposition, cross-links are needed to provide matrix stiffness. Cross-links also contribute to the ability to hold water and for the resistance against degradation by MMP-1.

Introduction

The functioning of articular cartilage depends on the structure, the composition and the integrity of its extracellular matrix (ECM). With cartilage degeneration in osteoarthritis (OA), changes in this structure and composition of the tissue occur. Fewer proteoglycans are present in OA cartilage [205] even though the rate of synthesis is increased [94]. Enhanced cleavage of collagen type II is seen in OA cartilage [143] accompanied by an increase in collagen type II gene expression and protein synthesis in the middle and deep layers of the cartilage but not in the superficial layer [95, 96]. Together, these biochemical changes in OA suggest an activated repair mechanism that is however ineffective in repairing or maintaining the ECM homeostasis.

The mechanical stiffness of cartilage obtained from OA patients decreases in relation to the extent of its degeneration [206]. The same is seen in cartilage from OA animal models, where the mechanical properties are also changed in addition to, or as a consequence of the above-mentioned biochemical changes in OA. These changes in mechanical properties include a decrease of the cartilage compression stiffness and an increase in the tendency to swell compared to healthy cartilage [207]. Recent experiments attribute the mechanical properties of cartilage to collagen [77, 78]. For example, an increase in matrix stiffness is associated with an increase in collagen content and to a lesser extent with the proteoglycan content in cartilage explants [79]. Conversely, the hydraulic permeability of cartilage is more associated with the proteoglycan content of the explants [79]. In a recent study, it was found that the increased swelling and deformation of osteoarthritic cartilage is highly associated with the amount of degraded collagen molecules [86]. As such, collagen properties contribute largely to the mechanical, hence functional characteristics of the tissue and induction of collagen repair may thus be highly relevant for maintaining or restoring cartilage integrity.

To study cartilage repair mechanisms, endogenous molecules such as growth factors are often used to promote regeneration. From these studies it has become clear that cartilage repair is hampered by low collagen production whereas proteoglycans are formed in higher amounts [169, 208]. Consequently, because collagen is important to withstand mechanical stress, the mechanical properties in these studies were not optimal. This is shown in cartilage tissue engineering as well when the effect of matrix components on mechanical functioning is examined. Both proteoglycans and collagen are associated with increasing mechanical strength. In one study in agarose constructs, collagen associated strongly with the tensile stiffness whereas proteoglycans correlated more strongly with the permeability of the matrix [209]. In other studies, the stiffness and permeability of the agarose constructs increased in time when the proteoglycan content was mainly constant and collagen content still increased [169, 210].

The results of both the OA cartilage studies and the tissue engineering studies point out the importance of targeting collagen in cartilage repair. However, this repair mechanism seems to be ineffective in the case of osteoarthritis. Understanding mechanisms of repair of the collagen network is therefore key for functional repair of cartilage. The present study was designed to investigate the contribution of the collagen network on the functionality of the newly formed matrix during cartilage repair processes. Specifically, we investigated the effect of the amount of collagen and number of collagen cross-links (two key characteristics of the collagen network) on mechanical properties and the resistance towards proteolysis of the newly formed cartilage matrix in chondrocyte alginate cultures.

Materials and Methods

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged 6–12 months. To isolate chondrocytes, full-thickness slices of noncalcified articular cartilage were subjected to pronase (2 mg/ml, Sigma, St. Louis, MO, USA) digestion for two hours followed by overnight collagenase B (1.5 mg/ml, Roche Diagnostics) digestion. The chondrocytes were resuspended in 1.2% (w/v) low viscosity alginate (Keltone) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/ml, and beads were made as described previously [157, 158, 211]. Beads that appeared smaller or larger than the average size after visual inspection were not included in the experiment. After transfer to six-well plates (BD Falcon, Bedford, MA, USA), beads were cultured in 75 µl/bead Dulbecco's Modified Eagle Medium (DMEM)/F12 (GibcoBRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). Cells were cultured under three conditions: without β-aminopropionitrile (BAPN) for 70 days (-/-), with 0.25 mM BAPN for 35 days followed by 35 days without BAPN (+/-) and with BAPN for 70 days (+/+). BAPN blocks collagen cross-linking by inhibition of the enzyme involved in collagen cross-linking, lysyl oxidase (LOX) [212]. Samples were taken after 21, 35, 41, 60 and 70 days of culture for biochemical analysis.

Isolation of cell associated matrix

Alginate beads cultured for 35 and 70 days were dissolved by adding 75 µl per bead of 55 mM sodium citrate and 20 mM Ethylene Diamintetraacetate (EDTA) in 150 mM NaCl for 20 min at room temperature. The suspension was centrifuged 10 min at 1000 rpm (Eppendorf), to separate the cells surrounded by its cell associated matrix (CM, the pellet) from components originating predominantly from the 'interterritorial' or further

removed matrix (FRM; the supernatant) from the cells as described previously [163, 187, 194]. However, we cannot completely rule out the possibility that matrix constituents from the other matrix component were present in smaller amounts.

Biochemical assays

Alginate beads or separated CM and FRM were digested overnight at 56°C in papain buffer (200 µg/ml papain in 50 mM EDTA and 5 mM L-cystein). Glycosaminoglycan (GAG) amount in the digest was quantified using dimethylmethylene blue (DMB) assay as described previously [160, 211]. The amount of DNA in each papain-digested sample was determined using Hoechst 33258 dye [213] with calf thymus DNA (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP and lysylpyridinoline, LP) was performed by the methods of Bank [86, 162, 211]. The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

Gene expression analysis

For total RNA isolation, alginate beads cultured for 35 and 70 days were dissolved in 150 µl/bead 55 mM sodium citrate acid. Each cell pellet was suspended in 1000µl RNA-Bee™ (TEL-TEST, Inc.; Friendswood, TX, USA) per million cells according to manufacturer's guidelines, and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using Ribogreen™ reagent (R-11490, Molecular Probes Europe BV, Leiden, the Netherlands) according to manufacturer's instructions, and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Collagen type 1 (COL1) and SRY-box containing gene 9 (SOX9), each cDNA sample was amplified using specific primers and specific probe (Table 1, Eurogentec, Seraing, Belgium). For Collagen type 2 (COL2), each cDNA sample was amplified using specific primers and specific molecular beacons (Table 4.1, Biolegio, Nijmegen, The Netherlands). Amplifications were performed as described previously [211].

Table 4.1: sequences of primers and probes or beacons for real-time PCR.

	Primers	Probe
GAPDH	Forward: GTCAACGGATTTGGTCGTATTGGG Reverse: TGCCATGGGTGGAATCATATTGG	Fam-TGGCGCCCCAACCAGCC-Tamra
COL1	Forward: CAGCCGCTTCACCTACAGC Reverse: TTTTGTATTCAATCACTGTCTTGCC	Fam- CGGTGTGACTCGTGCAGCCATC-Tamra
SOX9	Forward: CAACGCCGAGCTCAGCA Reverse:TCCACGAAGGGCCGC	Fam-TGGGCAAGCTCTGGAGACTTCTGAACG-Tamra
COL2	Forward: AAGGTTTTCTGCAACATGGAGACT Reverse: AGATGTGTTTCTTGTCCTTGCTCTT	FAM-cgtgcGTCTACCCCAACCCGGCCAGCgcacg-Dabcyl

Data were analyzed using Sequence Detector version 1.7 (Applied Biosystems) software and normalized for GAPDH expression [164].

Immunocytochemical staining for collagen type I and II

To prepare cytospins, three alginate beads cultured for 35 days were dissolved in 55 mM sodium citrate acid. After fixation in cold acetone, the cytospins were incubated with monoclonal antibodies against either type I collagen (1:2000, ab6308 ABCAM, Cambridge, UK), or type II collagen (1:100, II-II6B3; Developmental Studies Hybridoma Bank) at room temperature for 1 h. This was followed by incubation with link and label from the link-label kit (BioGenex, San Ramon, CA, USA) each for 30 minutes. Freshly prepared neo-fuchsin substrate was used to achieve staining. After staining, cytospins were scored for the number of cells positive for collagen types I and II per 100 cells in four fields of sight per specimen, resulting in a percentage of positive cells. A cell was considered positive, when the matrix around the was stained red.

Mechanical testing

For mechanical characterization, we used 4×10^6 cells/ml in 1.2% (w/v) alginate constructs of 3 mm thick and 6 mm in diameter. The constructs were prepared as previously described by Wong [166]. After 35 days and 70 days of culture, constructs were mounted on a materials testing machine, the DMA Q800 Dynamicmechanical Analyzer (TA Instruments, New Castle, DE, USA), in a radially unconfined stress relaxation test with the construct between impermeable platens, and hydrated in 0.9% saline with the protease inhibitor Complete™ (Sigma). A 20% uniaxial compressive strain was applied within 10 seconds at a ramped displacement strain rate of 2% s^{-1}, based on the measured thickness. The strain was maintained constant for 30 min, the load was recorded at a sampling rate of 10 Hz using a 18N load cell. The applied load recorded by the load cell was divided by the cross-sectional area of the construct to calculate the applied stress. The secant modulus was calculated as stress/strain at 10 seconds, where the stress had its peak response and the strain reached its maximum. At the end of the test, the equilibrium aggregate modulus was determined as stress/strain at 30 minutes. The secant modulus is related to the interaction between the solid and the liquid phase and is therefore an indication for the ability to hold water. The modulus measured at equilibrium depends strongly on the compressive stiffness of the (cartilaginous) solid matrix [83].

In vitro extracellular matrix degradation

After 70 days of culture, a sample of four alginate beads from each group was washed with 1 ml 0.9% NaCl, in order to remove the serum used in the culture medium. Washed beads were incubated for 24 h at 30°C in 250 µl 50 mM Tris [pH 7.5], 5 mM $CaCl_2$, 150 mM NaCl, 1 µM $ZnCl_2$, 0.01% Brij-35, 0.02% NaN_3 containing the general proteinase

inhibitor Complete™ (EDTA-free, one tablet in 25 ml) in the presence or absence of 100 nM recombinant human MMP-1 (R&D systems, Oxon, United Kingdom), which was activated by 4-aminophenyl mercuric acetate (APMA; Sigma) and calibrated using an MMP activity assay [165]. MMP-1 is one of the proteases involved in the physiological turnover of healthy cartilage and therefore chosen as collagenase in this assay to demonstrate the susceptibility of the matrix to degradation [214]. The released collagen (soluble and present in the supernatant) was separated from the insoluble collagen matrix by centrifugation. The amount of collagen in the soluble fraction represents the amount of collagen directly released into the medium by MMP-1. Finally, all fractions were hydrolyzed in 6 M HCl and Hyp levels were determined.

Data analysis

The experiments were repeated three times. Each experiment consisted of 3 times 7 beads per experimental condition for biochemical analyses, 3 times 2 beads for matrix degradation by MMP-1 and one sample of 10 beads per experimental condition for gene expression analysis. The experiments for mechanical testing were repeated two times with a total of 8 samples per group. Statistical analysis was performed using SPSS 11.5 (SPSS Inc. Chicago, IL) software. All data are presented as mean ± standard deviation. Control groups without BAPN and groups supplemented with BAPN were compared with a Kruskall-Wallis H test followed by a post hoc Mann–Whitney U test.

Results

Composition of the matrix: collagen deposition and cross-linking

The number of HP-cross-links increased in time in the control condition without BAPN, up to 0.72 ± 0.1 HP cross-links per collagen molecule after 70 days of culture (Figure 4.1a). When chondrocytes were cultured in the presence of BAPN, either for 35 days or 70 days, almost no cross-links were detectable in the newly deposited collagen (0.07 ± 0.1 HP cross-links per collagen molecule). When BAPN was removed after 35 days (BAPN +/-), collagen cross-links were formed from approximately day 40 onwards, reaching a number similar to the control condition at day 70 (Figure 4.1A).

The inhibition of cross-linking as a consequence of BAPN was accompanied by increased collagen deposition. In the control condition without inhibiting the crosslink formation with BAPN, 8.9 ± 0.7 µg collagen was deposited per bead after 35 days and 12.7 ± 0.5 µg collagen per bead after 70 days of culture. Culturing chondrocytes in the presence of BAPN for 35 days resulted in 1.5 times more collagen (13.6 ± 1.4 µg/bead µg/bead) than in the control condition. This increased to 1.6 times more collagen

A

B
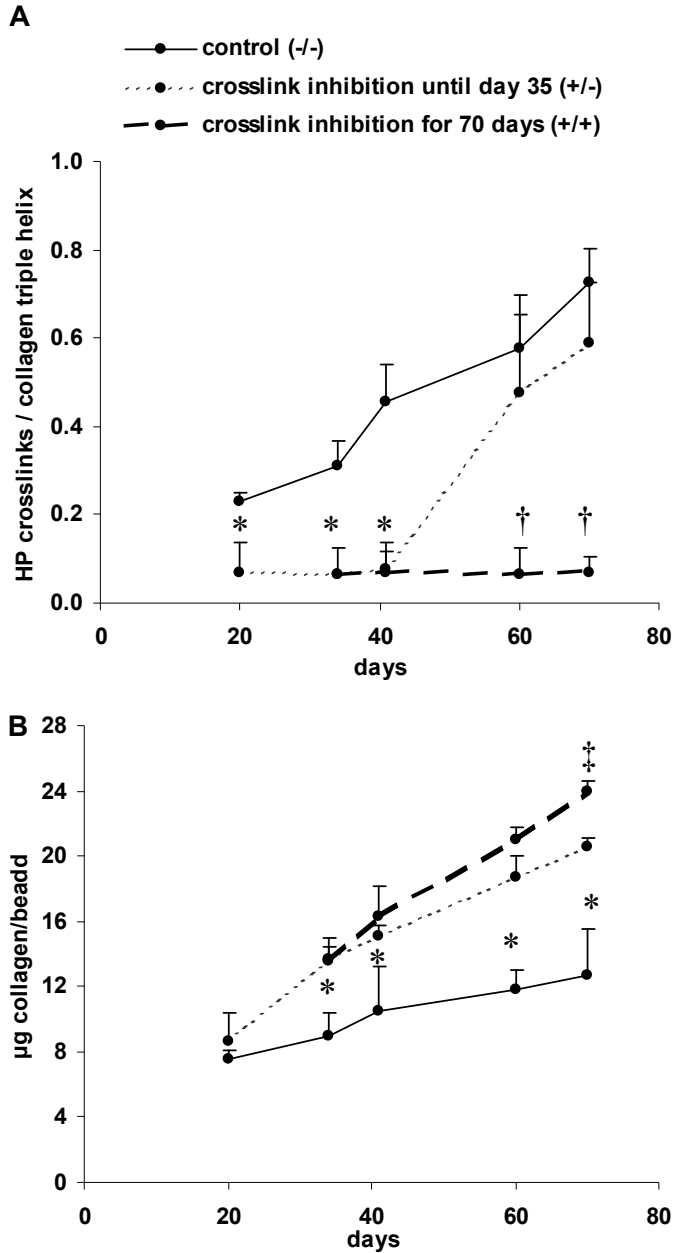
Figure 4.1. Effect of (transient) collagen crosslink inhibition on A) collagen crosslinking and B) collagen deposition during 10 weeks of culture (n = 9). Collagen crosslinking was inhibited by BAPN until day 35 in the +/- condition. From day 35 onwards, two conditions were cultured without the presence of BAPN and crosslinks could be formed. In one condition, crosslinks were inhibited during the full 70 days of culture. * Indicates P<0.002 between the control and the BAPN +/- condition at the same time point. † Indicates P<0.001 between BAPN +/+ and the other two conditions. ‡ Indicates P<0.005 between BAPN +/+ and the other two conditions. Data are shown as mean ± SD.

than in the control condition after 70 days of culture (20.6 ± 2.8 µg/bead), even though the crosslink inhibitor was not present anymore from day 35 onwards. Culturing chondrocytes in the presence of BAPN for 70 days (BAPN +/+) resulted in 1.9 times more collagen (24.0 ± 0.6 µg/bead) (Figure 4.1B).

Proteoglycan deposition and DNA content were not influenced by the presence of BAPN. The amount of DNA was determined in the alginate beads, to give an indication of the number of chondrocytes present in the beads. In all three the conditions, the DNA content and the deposition of the proteoglycans increased until day 20 and reaching a plateau thereafter at 0.32 ± 0.03 µg DNA /bead and 32.7 ± 1.7 µg proteoglycans/bead (data not shown). The data above are shown per bead because we are interested in the total collagen amount and number of cross-links and how this affects the functioning of the tissue engineered construct.

Quality of the matrix: mechanical properties

The equilibrium aggregate modulus at day 35 was two times higher in the condition with more collagen but without collagen cross-links (BAPN condition) than in the control condition; 897 Pa ± 277 Pa versus 451 Pa ± 165 Pa (Figure 4.2A). The peak force
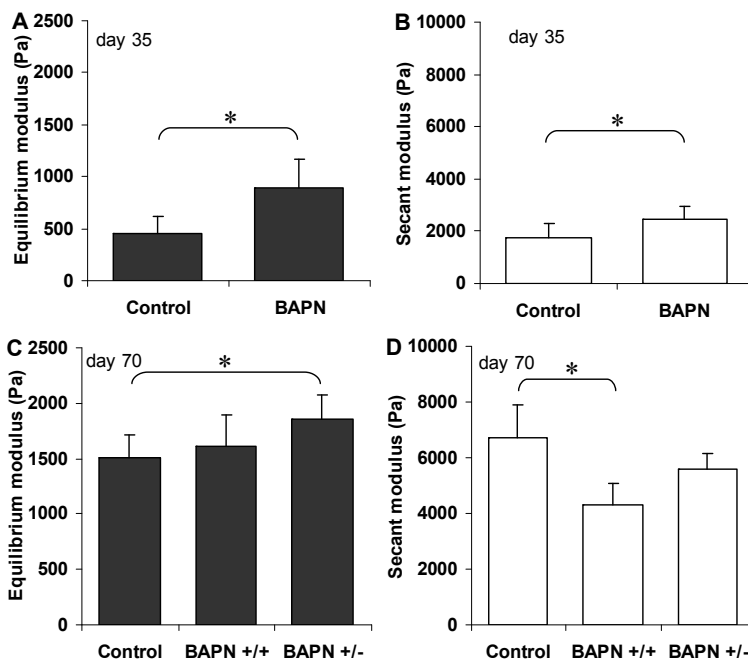

Figure 4.2. Mechanical properties of alginate discs cultured for 5 weeks (A&B) and 10 weeks (C&D). Both equilibrium aggregate modulus (A&C) and secant modulus (B&D) were examined (n = 8). * Indicates P< 0.002 compared to the control condition without inhibition of crosslink formation. Data are presented as mean modulus ± SD.

and subsequently the ability to hold water described by the secant modulus were not significantly different between the two culture conditions at day 35 (Figure 4.2B).

At day 70, the increase in collagen deposition in the BAPN +/+ condition was not sufficient by itself to increase the equilibrium modulus compared to the control condition that contained less collagen. At this time point, the equilibrium aggregate modulus only significantly increased when the increased collagen deposition was also cross-linked as was the case in the BAPN +/- condition (Figure 4.2C). The secant modulus and therefore the ability to hold water was significantly lower than the control in the BAPN +/+ condition whereas in the BAPN +/- condition, where cross-links were present, the secant modulus was not significantly different from the control (Figure 4.2D).

Quality of the matrix: susceptibility for degradation

The effect of modulating the cartilage collagen network on the degradability of the collagen was examined by subjecting alginate beads cultured for 70 days to the collagenase MMP-1. When cultured alginate beads were incubated with MMP-1, 6.2 ± 0.16 µg collagen was released in the control condition (which corresponds to 16.2% of the total amount in the alginate beads) and 7.0 ± 0.35 µg in the BAPN +/- condition (11.5% of total). This is not significantly different from each other and only 1.2 times more than when these beads were incubated without MMP-1. When beads from the BAPN +/+ condition were incubated with MMP-1, 14.9 ± 1.23 µg of collagen was released from the beads, which is 21.1% of the total collagen amount in the beads. This is 2.5 times as much as the control condition and almost twice as much as when no MMP-1 was present during incubation (Figure 4.3).
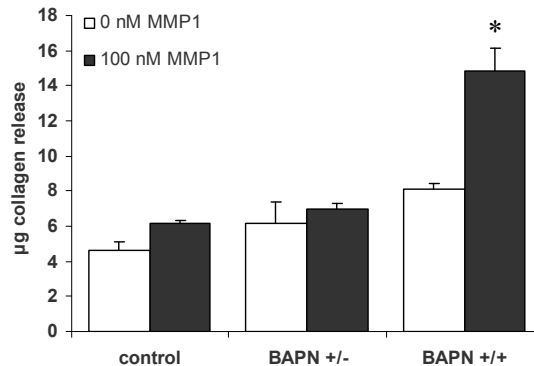
Figure 4.3. Susceptibility of the matrix for MMP-1 degradation after 10 weeks of culture. Amount of collagen in the soluble fraction directly after incubation with 100 nM MMP-1 is higher for the matrix without sufficient collagen crosslinks as represented by the +/+ condition than for matrices with collagen crosslinks (-/- and +/-), even when crosslink formation was transiently inhibited (+/-). * Indicates P< 0.001 compared to the -/- and +/- condition. Data are presented as mean percentage of the total amount of collagen in the beads ± SD (n = 6).

Structure of the matrix: Cell associated Matrix versus Further Removed Matrix

The distribution of the collagen within the alginate bead was determined by measuring the amount of collagen in the cell-associated matrix (CM) and the further removed matrix (FRM). The percentage of collagen in the CM was 76% of the total collagen in the control condition, 82% in the BAPN +/- condition and 81% in the BAPN +/+ condition. Thus, slightly more collagen seems to be present in FRM of the control condition than in FRM of the two BAPN conditions, although not reaching statistical significance (Figure 4.4).
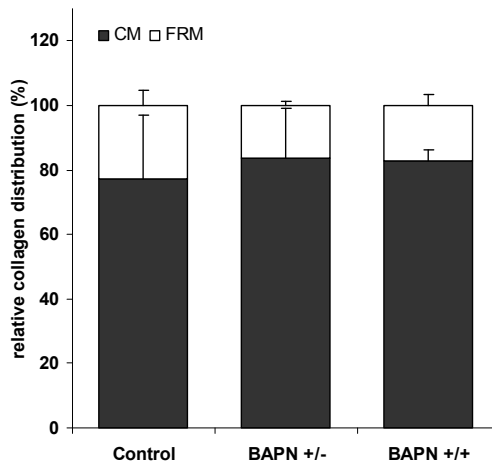
Figure 4.4. Distribution of collagen between the cell associated matrix (CM) and the further removed matrix (FRM). No significant differences are found in distribution between the three conditions. Data are shown as mean percentage per compartment ± SD (n=6).

Phenotype of the chondrocytes

Whether the phenotype of the chondrocytes was influenced by the addition of BAPN was investigated by the SRY-box containing gene 9 (SOX 9) gene expression [215] and the collagen type II/collagen type I gene and protein expression ratio (COL2/COL1 ratio). At day 35 and day 70, the COL2/COL1 ratio was a two-fold higher in the conditions where BAPN was present than in the control conditions (Figure 4.5A), due to decreased COL1 expression and increased COL2 expression. When BAPN was removed after 35 days in the +/- condition, the COL2/COL1 ratio at day 70 was comparable to the control condition, whereas it was still increased in the +/+ condition. To support the change in collagen gene expression, semi-quantative protein expression was determined by immunocytochemistry. Without the presence of BAPN, the ratio between collagen type II and collagen type I positive cells is 90%:60% as shown by immunocytochemistry at day 35 in Figure 4.6A and B. When BAPN was present for 35 days, less chondrocytes
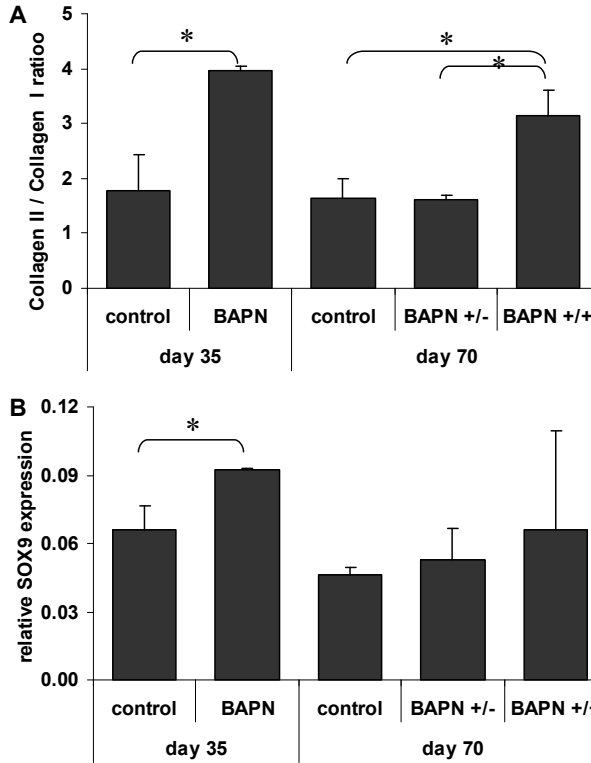
Figure 4.5. Gene expression of collagen type I, collagen type II and SOX 9, shown as A) collagen type II-collagen type I gene expression ratio and B) relative SOX 9 gene expression. Both parameters are indicative for the phenotype of the chondrocytes. * Indicates P< 0.001 compared to the -/- and +/- condition. Data are presented as mean ± SD (n = 3).

were positive for collagen type I which changed the collagen type II/collagen type I protein ratio to 90%:40% positive cells (Figure 4.6C and D). The collagen type II staining is also more intense in the presence of BAPN (Figure 4.6C).

SOX9 gene expression was only significantly higher when BAPN was present at day 35. No significant effect of BAPN addition was observed in the SOX9 gene expression after 70 days of culture (Figure 4.5B).

Discussion

The present study demonstrates that modulation of the number of cross-links and amount of collagen deposited during cartilage tissue regeneration has an effect on the functional properties of the construct depending on the amount of collagen deposited up till then. To study the involvement of collagen and collagen cross-links in the functioning of cartilage, the alginate culture system was used. In contrast to explants, the

Figure 4.6. Immunocytochemical analysis of produced matrix after 35 days of culture. Control chondrocytes stained for A) collagen type II and B) collagen type I and chondrocytes cultured in the presence of BAPN for 35 days stained for C) collagen ty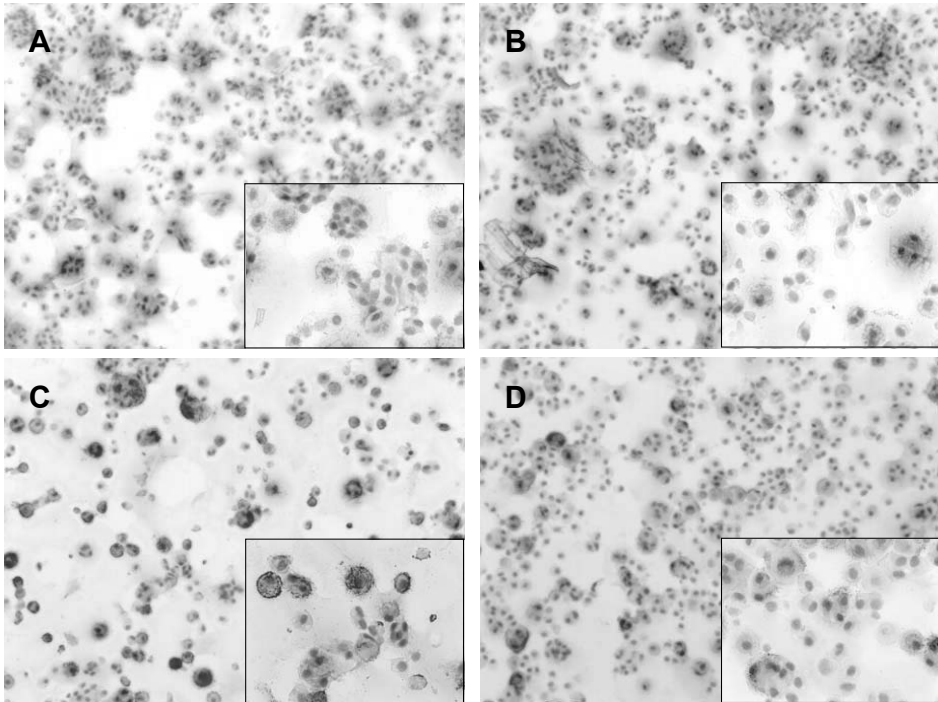pe II and D) collagen type. Magnification is 100 times, with a 400 times inset. Positive cells are indicated in red with haematoxylin counter stain (purple).

collagen deposition in an alginate culture system can be modulated from the beginning and effects of collagen modulation are not influenced by collagen already present, as is the case in explants.

We found that more collagen deposition in the BAPN condition after 35 days of culture contributed to the increase in equilibrium aggregate modulus of the newly formed matrix even though there are no cross-links present in the collagen molecules. The fact that a higher collagen deposition contributes to an improvement of the equilibrium aggregate modulus is in agreement with previous studies, although collagen cross-links were probably present [169, 210]. At 70 days, the increase in collagen deposition alone was no longer sufficient to increase the equilibrium aggregate modulus: proper cross-linking was also required. This is shown by the increase in collagen deposition in the BAPN +/+ condition at day 70 without an increase of the equilibrium modulus, while the initial increase in collagen deposition with subsequent collagen cross-linking did increase the equilibrium modulus (the BAPN +/- condition). These findings corroborate studies performed in other tissues such as skin and bone, where mechanical tissue strength and stiffness are also associated with collagen crosslink levels [216, 217]. Next to enzymatic cross-links,

adult human articular cartilage also contains non-enzymatically formed cross-links, the so-called advanced glycation endproducts (AGEs). However, because of the low glucose concentration in our culture medium (5mM), it is unlikely that these AGEs are formed during the 70 days of culture [218, 219]. Differences between culture conditions in levels of non-enzymatic cross-links and subsequently altered mechanical properties are therefore not expected. We hypothesize that the effect of collagen deposition on mechanical stiffness of the matrix depends on the amount deposited: when a low amount of collagen is deposited in the first five weeks of the chondrocyte culture, a small increase is able to improve matrix stiffness. When more collagen is deposited, cross-linking of the collagen is needed to further improve the matrix stiffness. The same hypothesis is applicable for the secant modulus. After five weeks of culture, the secant modulus was not significantly affected by the increase in collagen without cross-links being present. However, when collagen deposition increased even more during an additional five-week culture without the ability to crosslink, the secant modulus was lower than in the control condition with cross-links. This indicates that collagen cross-links are needed in addition to proteoglycans (that have previously been shown to be important [209]) to regulate the secant modulus and thus the ability of the matrix to hold water. The ability of the matrix to hold water is important for cartilage to resume its original shape and texture after deformation and is mainly attributed to proteoglycans. However, we see a decrease in the ability to hold water even though the amount of proteoglycans in the alginate beads is comparable in each condition. We therefore hypothesize that collagen cross-links might be important in maintaining a dense structure in order for the proteoglycans to retain the water in the cartilage matrix.

The role of cross-links in cartilage collagen catabolism was examined by subjecting the alginate constructs to collagenase (MMP-1) digestion. The data demonstrate that the presence of pyridinoline cross-links reduces the susceptibility to MMP-1 degradation. These observations are in concordance with what was recently found by our group, where matrices with more pyridinoline cross-links produced by skin fibroblasts were less susceptible for MMP-1 degradation [220].

To examine whether the modulation of collagen cross-linking and subsequently the collagen deposition had an effect on the matrix distribution, we determined the collagen distribution between the cell associated matrix (CM) and the further removed matrix (FRM). In contrast to a previous study where collagen was equally present in both compartments after BAPN treatment [163], in the present study more collagen was deposited in the CM after BAPN treatment with no difference in relative amounts between the conditions. Since a difference in mechanical properties is observed when the control condition is compared with the BAPN +/- condition, we conclude that this effect is mainly caused by the difference in collagen cross-links and that the distribution over the CM versus FRM does not affect this mechanical property in this test system.

Whether the addition of BAPN to the culture medium adversely affected the phenotype and behavior of the chondrocytes was examined with SOX9 gene expression and the ratio between Collagen type II and Collagen type I gene and protein expression. In a previous study with BAPN, a positive affect of BAPN on the chondrocyte phenotype was found. This was shown by the shape of the chondrocytes and the production of collagen types II, IX, X and XI [221]. In concordance to this study, BAPN had no detrimental effects on the chondrocyte phenotype, here shown by the ratio between collagen type II and collagen type I gene and protein expression and SOX9 gene expression. In fact, it appears that addition of BAPN leads to an increase in SOX9 gene expression and a higher Collagen type II/Collagen type I gene expression ratio indicating a possible positive effect of BAPN on chondrocyte phenotype. The fact that the protein and gene expression data are not exactly similar can be explained by the fact that immunocytochemistry is semi-quantitative and that only the number of cells expressing collagen type I or II is determined and not the absolute amount of protein.

After ten weeks of culture, 0.7 HP cross-links per collagen triple helix were found in the control and the BAPN +/- condition. This closely resembles the number found in immature calf cartilage [79]. The observed increase in collagen deposition can be explained by a so-called 'feedback-loop'. Normally, specific integrins on the surface of chondrocytes are occupied by collagen molecules leading to a feed back loop. In case of crosslink inhibition, collagen can diffuse further away from the cell resulting in less occupation of the intregrin receptors and less or no feed back, stimulating the cell in collagen production [163]. Based on our results, the matrix still remains in the CM. The collagen deposition remains increased after the removal of BAPN, which can be explained by the fact that the enzyme lysyl oxidase (LOX) needs to be produced and that the formation of mature pyridinoline cross-links takes approximately 3 weeks [222].

In conclusion, our data imply a significant role for both the amount of collagen and the number of collagen cross-links in the mechanical properties of the formed cartilage matrix. Initially an increase in amount of collagen is sufficient to increase the mechanical stiffness but at a certain collagen level, also cross-links are needed to increase the mechanical stiffness to a higher level. Furthermore, collagen cross-links also play an important role in matrix integrity by making the matrix less susceptible for degradation. These data suggest that in reparation of the collagen network, collagen amount but especially cross-linking is important for the mechanical properties of the newly formed cartilage, as well as the susceptibility of the new cartilage for enzymatic degradation. Successful modulation of collagen synthesis and cross-linking could potentially greatly benefit cartilage repair. Several approaches could possibly be used in future to achieve this, like application of growth factors as well as the use of gene therapy techniques.

Acknowledgements

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5

Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartilage matrix production

Yvonne M. Bastiaansen-Jenniskens, Wendy Koevoet, Kaspar M.B. Jansen, Jan A.N. Verhaar, Jeroen DeGroot, Gerjo J.V.M. VanOsch

Abstract

Objective

To understand cartilage degenerative diseases and improve repair procedures, we investigate the influence of glycosaminoglycans (GAGs) on cartilage matrix biochemistry and functionality.

Methods

Bovine articular chondrocytes were cultured in alginate beads with(out) para-nitrophenyl-beta-d-xyloside (PNPX) to inhibit GAG incorporation into newly formed proteoglycans.

Results

As expected, GAG deposition in alginate beads decreased with increasing PNPX concentration. Next to GAGs, collagen deposition and cross-linking also decreased. In the presence of PNPX, GAGs and collagen were deposited further away from the chondrocyte than in the control and increased amounts were found in the culture medium. These changes resulted in decreased functional properties of the construct.

Discussion

We conclude that in our culture system, intact proteoglycans play a role in deposition of collagen and thus the formation of a functional matrix. The effect of less proteoglycans on the collagen network could explain why cartilage repair is ineffective in osteoarthritis and help us with development of new therapies.

Introduction

The function of articular cartilage depends on structure, composition and integrity of its extracellular matrix (ECM). The main ECM molecules present in cartilage are collagens and proteoglycans. Collagens are organized into a fibrous network, which defines the basic tissue architecture and provides tensile strength [223]. Together with type IX and XI, collagen type II forms fibrils [25, 224], which are stabilized by the formation of intermolecular cross-links. A high concentration of proteoglycans (PGs) is embedded in the collagen network giving the cartilage the ability to hold water and to reverse deformation [83]. Proteoglycans consist of a protein core with carbohydrate side chains called glycosaminoglycans (GAGs). Aggrecan is the most common PG in articular cartilage with chondroitin and keratan sulphate side chains [36]. Other smaller proteoglycans and present to a lesser extent include decorin, biglycan and perlecan [225, 226]. These small proteoglycans have one or two GAG side chains such as dermatan sulphate or heparan sulphate. For all proteoglycans, these GAG side chains are covalently joined to the protein core by a glycosidic bond between xylose and the hydroxyl group of a serine residue [39]. After xylosylation of serine, linkage region synthesis occurs by addition of two galactosyl moieties by galactosyltransferase I [40] and glucuronic acid by glucuronosyltransferase I [41]. For chondroitin sulphate, alternate transfer of N-acetylgalactosamine and glucuronic acid [42] results in elongation of side chain.

With cartilage degeneration in osteoarthritis (OA), changes in structure and composition of the tissue occur. Fewer proteoglycans are present in OA cartilage [205] together with enhanced collagen degradation [143]. However, the rate of synthesis of both matrix components is increased as well [94-96]. This suggests an activated repair mechanism that is however ineffective in repairing or maintaining the ECM homeostasis. In OA, the mechanical stiffness of cartilage decreases in relation to the extent of its degeneration [206]. Changes in mechanical properties include a decrease of the cartilage compression stiffness and an increase in the tendency to swell compared to healthy cartilage [83, 86].

Understanding matrix assembly and interaction between collagens and proteoglycans is necessary to understand the disease process in OA and develop solutions for the repair of articular cartilage damage. The purpose of this study was therefore to examine the effect of GAGs on matrix production and distribution and consequently matrix functionality, by dose-dependently inhibiting GAG incorporation in the newly formed cartilage matrix using the exogenous substrate acceptor of galactosyl transferase para-nitrophenyl-beta-d-xyloside (PNPX).

Methods

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged 6–12 months. Chondrocytes were isolated, suspended in 1.2% (w/v) alginate in a concentration of 4 x 10^{6} cells per ml of alginate, and alginate beads were made as described previously [211]. Beads were cultured in 75 µl/bead DMEM/F12 with GlutaMAX™ supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). Chondrocytes were cultured for the entire culture period with or without 0.025 mM or 0.25 mM para-nitrophenyl-beta-d-xyloside (PNPX) based on previous results [227,228]. PNPX is an exogenous acceptor for galactosyl transferase I, which competes with endogenous xylosylated core proteins for glycosaminoglycan chain production and thus prevents glycosaminoglycans being incorporated in the matrix [229]. Culture medium was changed three times a week. Alginate beads were harvested after 21 days of culture for RNA and 35 days for matrix production and distribution. Gene expression was analyzed at day 21, matrix composition, collagen cross-linking, and functionality at day 35 since it takes time for genes to be translated and assembled into functional (glyco)proteins, especially collagen [222].

RNA isolation and quantitative RT-PCR

For total RNA isolation, alginate beads were dissolved in 150 µl/bead 55 mM sodium citrate and 20 mM Ethylene Diamintetraacetate (EDTA) in 150 mM NaCl. Cell pellets of the alginate beads were suspended in 1000µl RNA-Bee™ (TEL-TEST, Inc.; Friendswood, TX, USA), and precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA). Primer sequences for genes were as follows: for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, reference gene) forward: GTCAACGGATTTGGTCGTATTGGG, reverse: TGCCATGGGTGGAATCATATTGG, and probe: Fam-CCATCACCATCTTCCAGGAGCGAGA-Tamra. Aggrecan core protein (AGCN) forward: GGACACTCCTTGCAATTTGAGAA, reverse: CAGGGCATTGATCTCG-TATCG, probe: Fam-TGGCGCCCCAACCAGCC-Tamra. Collagen type 2 (COL2) forward: GGCAATAGCAGGTTCACGTACA, reverse: CGATAACAGTCTTGCCCCACTT, probe: FAMCCGGTATGTTTCGTGCAGCCATCCT- Tamra. SRY-box containing gene 9 (SOX9), Forward: CAACGCCGAGCTCAGCA, reverse: TCCACGAAGGGCCGC, probe: Fam-TGGGCAAGCTCTGGAGACTTCTGAACG-Tamra. PCR conditions and analysis were as described previously [230]

Isolation of cell associated matrix

Alginate beads were dissolved by adding 75 µl sodium citrate buffer per bead for 20 min at room temperature. The suspension was centrifuged 10 min at 1000 rpm (Eppendorf), to separate the cells surrounded by their cell associated matrix (CM, the pellet) from components originating predominantly from the 'interterritorial' or further removed matrix (FRM; the supernatant) from the cells as described previously [194, 230].

Biochemical assays

Alginate beads or separated CM and FRM were digested overnight at 56°C in buffer with papain (250 µg/ml papain in 50 mM EDTA and 5 mM L-cystein). Glycosamino-glycan (GAG) amount in the digest was quantified using dimethylmethylene blue (DMB) assay [160]. The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio A530:A590 was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. The Blyscan sulfated glycosaminoglycan assay (Biocolor Ltd, Northern Ireland) was used to determine the GAGs in the culture medium. In both assays, the cationic dye DMB binds to negatively charged GAGs. PNPX prevents these GAGs from binding to the core protein resulting in elongation of the GAG chain without the formation of a proteoglycan. These unbound GAGs can still be detected with the assays described above. The amount of DNA in each papain-digested sample was determined using ethidium bromide with calf thymus DNA (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP) was performed as described previously [162, 163]. The quantities of cross-links were expressed as number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

Mechanical testing

For mechanical characterization, we used 4×10^6 cells/ml in 1.2 % (w/v) alginate constructs of 3 mm thick and 6 mm in diameter. Constructs were prepared as previously described [166, 230]. After 35 days of culture, constructs were mounted on a materials testing machine, the DMA Q800 Dynamicmechanical Analyzer (TA Instruments, New Castle, DE, USA), in a radially unconfined stress relaxation test with the construct between impermeable platens, hydrated in 0.9% saline with the protease inhibitor Complete™ (Sigma). A 20% uniaxial compressive strain was applied within 10 seconds at a ramped displacement strain rate of 2% s^{-1}, based on the measured thickness. Strain was maintained constant for 30 min, load was recorded at a sampling rate of 10 Hz using a 18N load cell. The applied load recorded by the load cell was divided by the cross-sectional area of the construct to calculate the applied stress. The secant modulus was calculated as stress/strain at 10 seconds, where the stress had its peak response

and the strain reached its maximum. At the end of the test, the equilibrium aggregate modulus was determined as stress/strain at 30 minutes. The secant modulus is related to the interaction between the solid and the liquid phase and is therefore an indication for the ability to hold water. The modulus measured at equilibrium depends strongly on the compressive stiffness of the (cartilaginous) solid matrix [83].

Statistical analysis

Experiments were repeated three times, with two pooled animal donors per experiment. Per experiment, 3 samples were taken with 7 beads per experimental condition for biochemical analyses and two samples of 10 beads per experimental condition for gene expression analysis. The experiments for mechanical testing were repeated two times, with two pooled animal donors per experiment and a total of 6 samples per group per experiment. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) software. Control groups without PNPX and groups supplemented with PNPX were compared with a Kruskall-Wallis test followed by a post hoc Dunn's multiple comparison tests.

Results

Gene expression

Aggrecan core protein (AGCN) gene expression, Collagen type 2 (COL2) gene expression and SOX9 gene expression as indication for chondrocyte phenotype were not
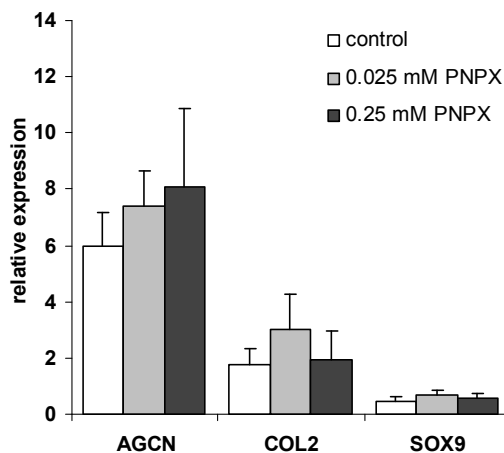
Figure 5.1. Relative gene expression of aggrecan (AGCN), collagen type 2 (COL2) and SOX9 at day 21 with or without 0.025 mM or 0.25 mM PNPX. Gene expression is normalised to GAPDH. N = 6, data are shown as mean + sd.

significantly changed by the presence of PNPX at day 21 of culture. Although AGCN had a trend towards upregulation with increasing PNPX concentration, it did not reach statistical significance (Figure 5.1).

Matrix production and distribution

After 35 days of culture, no difference was seen in the amount of DNA between the conditions, being on average 0.23 ± 0.07 µg DNA per bead (Figure 5.2A)

The absolute amount of glycosaminoglycans (GAGs) deposited in the alginate bead during the 35 days of culture was significantly lower in the presence of 0.25 mM PNPX, 21.3 ± 0.5 µg GAG per bead in the control condition versus 9.2 ± 0.6 µg GAG per bead in the presence of 0.25 mM PNPX. The distribution of GAGs and collagen in the culture system was examined by analyzing separately the cell-associated matrix (CM), the further removed matrix (FRM) and the culture medium of the alginate bead. In the control condition, 43% of the glycosaminoglycans were present in the CM, 52% present in the FRM. The culture medium collected during the 35 days of culture contained 5% of the total GAG. This distribution was not changed in the presence of 0.025 mM PNPX but shifted to the FRM and the culture medium with 0.25 mM PNPX. Here, 19% of the GAGs were present in the CM, 35% in the FRM, and 46% in the culture medium (Figure 5.2B).

PNPX decreased the total amount of collagen deposited in the alginate bead from 25.5 ± 3.6 µg collagen per bead in the control condition to 19.3 ± 0.6 µg collagen per bead in the presence of 0.25 mM PNPX. The high concentration of PNPX also changed the collagen distribution towards the FRM and the culture medium. In the control condition, 60% of the collagen was present in the CM, 23% present in the FRM, and 17% was present in the culture medium. With 0.25 mM PNPX, 41% of the collagen was further removed and 26% was present in the culture medium. Only 32% of the produced collagen was cell associated. However, total collagen production (in CM, FRM and culture medium) was not changed when PNPX was present (Figure 5.2C). Less collagen cross-links were present with 0.25 mM PNPX than in the control condition and the low concentration of PNPX; 0.16 ± 0.01 HP per collagen triple helix versus 0.48 ± 0.01 HP per collagen triple helix in the control condition (Figure 5.2D)

Matrix functionality

The ability to hold water of the alginate constructs, indicated by the secant modulus, was 6.3 ± 1.0 kPa in the control condition. Addition of 0.25 mM PNPX decreased this to 5.1 ± 0.6 kPa (Figure 5.3A). The stiffness indicated by the equilibrium modulus decreased from 1.4 ± 0.5 kPa in the control condition to 0.7 ± 0.4 Pa in the condition with 0.25 mM PNPX (Figure 5.3B).
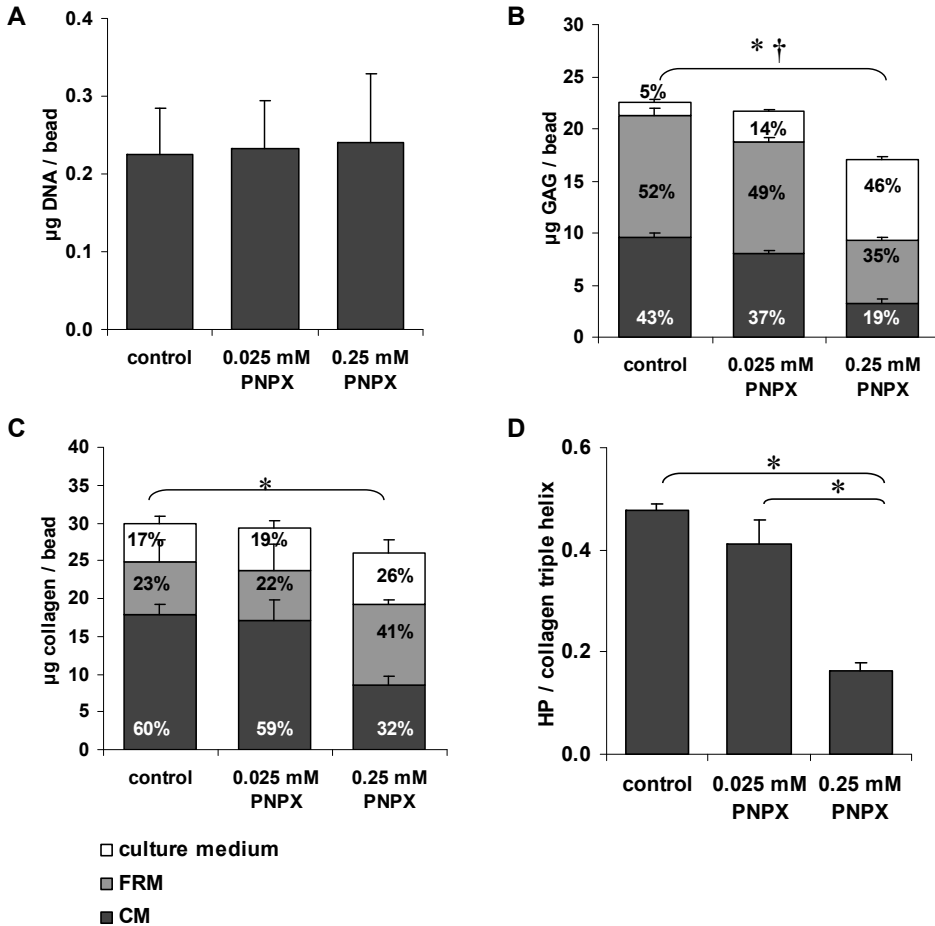
Figure 5.2. DNA content, matrix deposition and matrix distribution after 35 days of culture. A) DNA content shown as µg DNA per alginate bead. Distribution is shown between the cell associated matrix (CM, dark grey), further removed matrix (FRM, light grey) and the culture medium (white) in the presence of PNPX after 21 days of culture for B) GAG and C) collagen. The bars represent the absolute quantity with the relative distribution shown as percentages. D) HP cross-links shown as HP per collagen triple helix. N = 9, data are shown as mean + sd, * indicates significant difference in relative distribution (p<0.05), † indicates significant difference in absolute deposition in the alginate bead.

Discussion

Once damaged, adult articular cartilage has a poor repair capacity even though the loss of proteoglycans is considered reversible [142, 231]. Although collagen turnover is also increased in osteoarthritis, this does not lead to the formation of a functional network [96, 232]. In this study, the effect GAGs on matrix assembly and distribution and the subsequent mechanical functioning of the matrix was examined. We found that by

Figure 5.3. Matrix functionality after 35 days of culture, with or without 0.025 mM or 0.25 mM PNPX: A) The ability to hold water of the newly formed matrix defined by the secant modulus and B) the stiffness of the matrix defined by the equilibrium modulus. N = 12, data are shown as mean + sd, * indicates significant difference (p<0.01)

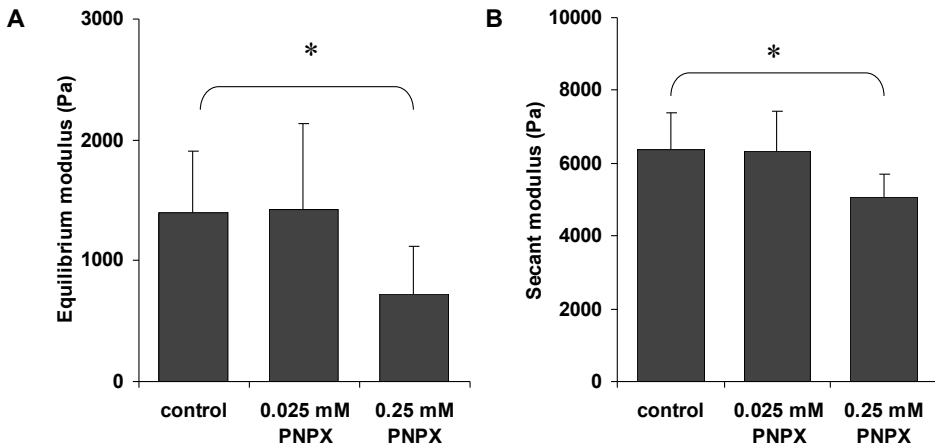inhibiting GAG incorporation in the newly formed cartilage matrix, not only the synthesized GAGs diffused further away from the chondrocyte but also collagen was less cross-linked and diffused further away from the chondrocyte and even into the culture medium. As a logical consequence, the stiffness and the ability to hold water were lower. We therefore conclude that in our culture system, GAG incorporation in proteoglycans contribute to the formation of a functional collagen network. To our knowledge, this is the first study where the effect of modulation of proteoglycan synthesis on cartilage collagen network formation is examined.

Total GAG incorporation in the matrix was inhibited by addition of PNPX. When CM, FRM and medium are added up, fewer GAGs are produced in the 0.25 mM PNPX condition than in the control condition without PNPX. This is in contrast to previous studies where inhibition of GAG incorporation lead to more free GAGs as a possible compensation mechanism [233, 234]. Aggrecan core protein gene expression had a trend towards upregulation, possibly indicating that increasing aggrecan core protein production is an attempt to compensate the lack of intact proteoglycans.

Collagen synthesis was not changed by the presence of PNPX as shown on gene expression level for collagen type II and on total collagen protein level in alginate bead and culture medium. Surprisingly, collagen deposition in the alginate bead was lower when GAG incorporation was inhibited. The produced collagen was partly excreted into the culture medium.

The number of cross-links per collagen molecule was downregulated in the presence of PNPX. This suggests that the lower amount of incorporated GAGs influences the

diffusion of collagen and subsequently collagen cross-linking. This could solely be a result of the absence of an intact proteoglycan network, which normally inhibits collagen from diffusion out of the alginate bead. However, the main proteoglycan present in articular cartilage and produced during chondrocyte alginate culture, aggrecan [194], also binds to COMP [56] and COMP is believed to interact with collagen type II [96] and IX [55]. Other proteoglycans present in articular cartilage are small proteoglycans such as decorin, fibromodulin and biglycan [235]. These small proteoglycans also interact with collagen, mainly by binding to the surface of the collagen fibril influencing the fibril diameter [236, 237]. By influencing collagen fiber interaction and thus collagen fibril diameter, collagen cross-linking can also be influenced. Small diameter fibrils result in a lower concentration of collagen cross-links, which might also explain the reduced number of cross-links seen after PNPX treatment.

An intact proteoglycan network might thus be very important for the integrity and assembly of the functional cartilage matrix. This might also explain the absence of cartilage repair in OA. In OA, proteoglycan synthesis is altered [94, 238] possibly leading to a different or less functional proteoglycan network that might contribute to the ineffective matrix assembly resulting in limited cartilage repair even though collagen synthesis is upregulated.

We therefore conclude that the decrease in collagen deposition and cross-linking in alginate beads, the increase in collagen excretion into the medium and the decrease in mechanical properties is a result of an incomplete proteoglycan network. This incomplete proteoglycan network influenced matrix properties directly and also indirectly via its effect on the collagen network. The effect of less GAG on the collagen network seen here could explain why cartilage repair is ineffective in OA and help with the development of new repair or tissue engineering strategies. Stimulating collagen cross-linking to prevent collagen diffusing away or stimulate production of specific proteoglycans for a functional proteoglycan network are possible therapeutic options derived from this study.

6

Stimulation of **COMP** production in cartilage matrix generation decreases collagen fibril diameter

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Abstract

Objective

Cartilage Oligomeric Matrix Protein (COMP) is a protein present in the cartilage matrix and is expressed more abundantly in OA cartilage than in healthy cartilage. The present study was designed to investigate the effect of growth factors on COMP deposition and the influence of COMP on collagen biochemistry.

Methods

Bovine chondrocytes in alginate beads were cultured with or without 25 ng/ml IGF1, TGFβ2 or FGF2. Human COMP (hCOMP) was overexpressed in bovine chondrocytes using lentiviral transfection. COMP gene expression, COMP protein production, collagen and proteoglycan deposition, and collagen fibril thickness were determined.

Results

Addition of TGFβ2 resulted in more COMP mRNA and protein than the control condition without growth factors or with addition of IGF1. FGF2 resulted in less and partially degraded COMP. Lentiviral transduction with hCOMP resulted in elevated gene expression of hCOMP and increased COMP levels in the alginate bead and culture medium compared to untransfected cells. Overexpression of COMP did not affect the deposition of collagen, collagen cross-linking, proteoglycan deposition or the mechanical properties. Stimulating COMP production by either TGFβ2 or lentivirus resulted in collagen fibrils with a smaller diameter.

Discussion

Taken together, COMP deposition can be modulated in cartilage matrix production by addition of growth factors or by overexpression of COMP. Inducing COMP protein expression resulted in collagen fibrils with a smaller diameter. Since it has been demonstrated that the collagen fibril diameter is associated with mechanical functioning of the matrix, modulating COMP levels could therefore contribute to successful cartilage regeneration strategies.

Introduction

Cartilage oligomeric matrix protein (COMP) is a homo-pentameric protein of 520-550 kDa with subunits of 100-120 kDa [239]. COMP was originally found in cartilage [50] but is also present in a large variety of other tissues such as tendon [51], synovium and skin [52]. The function of COMP is not completely understood. It is known that COMP interacts with several components of the extracellular matrix such as collagen type I, II[54], and IX [55]. and the non-collagenous proteins matrilin (MATN) 1, 3, and 4 and aggrecan [56, 62].

A large number of COMP mutations has been identified, some causing phenotypes ranging from severe pseudoachondroplasia (PSACH) to mild multiple epiphyseal dysplasia (MED) [240] depending on the location of the mutation. Because COMP interacts with other matrix proteins, the mutations in the comp gene resulting in retention in the endoplasmatic reticulum of the cell often also lead to altered excretion of collagen type IX and matrilin 3. Excretion of collagen type II is less affected by COMP mutations [55, 63, 241]. In addition, COMP mutations result in alterations in cartilage matrix assembly and reduced interaction between chondrocytes and COMP which could also contribute to the PSACH or MED phenotype [242]. Surprisingly, complete absence of COMP does not result in morphological or anatomical changes and does not lead to any signs of PSACH or MED [237]. Recently, COMP was found to influence the fibril formation of collagen type I and II leading to more and organized collagen fibrils in vitro. However, COMP was not associated with mature collagen fibrils suggesting a role as catalyst in fibrillogenesis for COMP [57].

The distribution of COMP within healthy cartilage changes in time. In young cartilage, COMP is uniformly present in the superficial layers of the cartilage whereas in the middle layer the location is more territorially (centered around the chondrocytes), shifting with age towards a more interterritorial distribution [243]. In osteoarthritic (OA) cartilage as a result of reactivation of COMP synthesis, COMP is mainly present in the pericellulair matrix of cell clusters [244, 245]. Here COMP is mainly located on the collagen fibers, whereas in healthy cartilage almost no COMP is associated with collagen fibers [244]. Besides the shift in matrix distribution, gene expression and protein expression are also increased in OA when compared to healthy cartilage [67, 244]. The interactions between COMP and other matrix components and the shift of COMP distribution in OA cartilage to a more immature distribution pattern in a possible attempt to repair the cartilage, suggest that COMP may have a role in the development of articular cartilage.

Because of the absence of spontaneous functional cartilage repair in OA, cartilage regeneration strategies include the use of growth factors to induce a chondrogenic cell phenotype or enhance extracellular matrix production. Because of the possible role of COMP in the cartilage matrix production, assembly, and regeneration, the goal of the present study was to examine the influence of Insulin-like Growth Factor (IGF)-1,

Transforming Growth Factor (TGF)β and Fibroblast Growth Factor (FGF)-2 on COMP gene expression and protein production by isolated chondrocytes and the consequent effect of COMP on extracellular matrix assembly and functional properties of the generated matrix. Because of the diverse effects of the used growth factors on matrix biochemistry and functionality earlier described by us [211], we also overexpressed COMP in primary chondrocytes using lentivirus for stable integration to examine the role of COMP during cartilage matrix generation.

Methods

Cell culture

Articular cartilage was harvested from metacarpophalangeal joints of calves aged 6–12 months. Full-thickness slices of non-calcified articular cartilage were subjected to pronase (2 mg/ml, Sigma, St. Louis, MO, USA) digestion for two hours followed by overnight collagenase B (1.5 mg/ml, Roche Diagnostics) digestion. Chondrocytes were resuspended in 1.2% (w/v) low viscosity alginate (Keltone) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/ml and beads were made as described previously[157]. After transfer to a six-well plate (BD Falcon, Bedford, MA, USA) they were cultured in 75 µl/bead DMEM/F12 supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). For growth factor experiments, cells were cultured with and without 25 ng/ml FGF2 (Serotec, Oxford, UK), TGFβ2 (recombinant human, R&D systems, Abington, UK) or IGF1 (Sigma) and harvested after 11 and 21 days of culture. Growth factors and concentrations were chosen based on previous results [211]. For experiments with lentivirus, chondrocytes were incubated with or without virus for four hours prior to suspension in alginate, and cultured for 11, 21 or 35 days.

COMP and GFP lentivirus production

A four-plasmid expression system was used to generate lentiviral vectors by transient transfection. The four plasmids were (i) the expression plasmid (pLV) in which human cartilage oligomeric matrix protein (COMP) or green fluorescent protein (GFP) cDNA fused, (ii) the packaging plasmid pLP1 encoding Gag and Pol (iii) the packaging plasmid pLP2 encoding Rev and (iv) the envelope plasmid, pLP/VSVG for surface proteins. Vectors were produced by transfection of plasmid DNA into 293FT cells using the transfection agent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfections were done in 75cm² flasks using 9 µg expression plasmid, 6.4 µg of pLP1, 4.8 µg of pLP2 and 3.4 µg of pLP/VSVG. Medium was changed after 24 hours and collected after an additional 48 hours. The medium was filtered and concentrated by ultracentrifugation. The p24 ELISA

(Gentaur, Brussels, Belgium) was used according to the manual to determine the number of infectious units. Cell transduction was performed by incubating bovine chondrocytes with COMP or GFP lentivirus at an MOI of 0.75 for 4 hours prior to suspending the chondrocytes in alginate and making beads.

Determination of transfection efficiency

Chondrocytes infected with GFP were recovered from alginate beads using 75 µl per bead of 55 mM sodium citrate and 20 mM Ethylene Diamintetraacetate (EDTA) in 150 mM NaCl for 20 min at room temperature. After washing twice in PBS, chondrocytes were resuspended in PBS with 0.1% FCS. Analysis of transfection efficiency was performed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA). A minimum of 10,000 cells per sample was collected. Data acquisition and analysis were performed using CellQuest 3.3 (Becton Dickinson).

RNA isolation and quantitative RT-PCR

For total RNA isolation, alginate beads were dissolved in 150 µl/bead sodium citrate buffer. After centrifugation, cell pellets were suspended in 1000µl RNA-Bee™ (TEL-TEST, Inc.; Friendswood, TX, USA), and subsequently precipitated with 2-propanol and purified with lithium chloride. Total RNA was quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 500 ng total RNA of each sample was reverse transcribed into cDNA using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Quantitative real-time PCR was performed using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA) for quantification using Taqman and SYBR Green and melting curve analysis. Primer sequences for the genes were as follows: for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH reference gene) forward: GTCAACGGATT TGGTCGTATTGGG, reverse: TGCCATGG GTGGAATCATATTGG, and probe: Fam-TGGCGCCCCAACCAGCC-Tamra. For the COMP assay specific for bovine, reverse: TCTGATCTGAGTTGGGCACCTT, forward: CCAGAAGAACGACGACCAGAA. For the COMP assay specific for human, a TaqMan® Gene Expression Assay was used (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection during the last step. For GAPDH and human COMP, Taqman 2x Universal PCR Mastermix (Roche) was used in the reaction. For bovine COMP, qPCR Mastermix Plus SYBR Green I (Eurogentec) was used in the reaction. All PCRs were performed in a total volume of 25 µl. Relative quantification of PCR signals was performed by comparing the threshold cycle value (C_t), in duplicate, for the gene of interest in each sample with the C_t value for the reference gene GAPDH. Quantitative PCR analysis of each sample was performed in duplicate.

Isolation of cell associated matrix

Alginate beads were dissolved by adding 75 µl per bead of sodium citrate buffer for 20 min at room temperature. The suspension was centrifuged 10 min at 1000 rpm (Eppendorf), to separate cells surrounded by its cell associated matrix (CM, the pellet) from components originating predominantly from the 'interterritorial' or further removed matrix (FRM; the supernatant) from the cells as described previously[157, 187].

Biochemical assays

Alginate beads or separated CM and FRM were digested overnight at 56°C in papain buffer (250 µg/ml papain in 50 mM EDTA and 5 mM L-cystein). Glycosaminoglycan (GAG) amount in the digest was quantified using dimethylmethylene blue (DMB) assay as described previously[160]. The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio $A_{530}:A_{590}$ was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP) was performed by the methods of Bank[162]. The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix.

COMP detection with ELISA and Western blotting

To analyze the produced extracellular matrix and the culture medium for COMP, alginate beads were dissolved in the sodium citrate buffer. To block proteolysis, the broad-spectrum protease inhibitor Complete (Roche Diagnostics) dissolved in phosphate buffered saline (PBS) was added to the alginate and culture medium samples. The human and animal COMP ELISA (Anamar, Göteborg, Sweden) were used to quantify the COMP content according to the manual. For qualitative Western blotting, protein separation was performed with SDS-PAGE. The system consisted of 4% acrylamide (Roth, Karlsruhe, Germany) in the stacking gel with a pH of 6.8 and 0.5M Tris-HCl, 6% acrylamide in separation gel for the non-reducing gels and 10% for the reducing gels, both 8.8% and 1.5M Tris-HCl. Reducing and non-reducing sample buffer (Roth) were used to load samples on the gel. Roti®-Mark Western Marker (Roth) was used as reference. Tris-glycine was used as electrophoresis buffer, and separation was carried out at 150 V for 1 hour. After electrophoresis, proteins were blotted onto PVDF membranes (Roth). Membranes were washed in PBS and blocked for 1 hour in PBS/5% (w/v) milk powder at room temperature. Immunoreactions were performed applying the anti-COMP antibody 12-C4[246] for 1 hour, diluted 1:500 in PBS/2.5% milk. The secondary goat-anti-mouse antibody coupled to horse reddish peroxidase was diluted 1:1500 and co-incubated with Roti®-Mark Western HRP conjugate (Roth) diluted 1:2000 for 1 hour at room temperature. Three 5-minute washes with PBS-Tween 0.1% were carried

out between all incubation steps. Visualization was achieved using the Immun-Star HRP chemiluminescent kit (Bio-Rad, Hercules, CA, USA).

Mechanical testing

For mechanical characterization, we used 4×10^6 cells/ml in 1.2% (w/v) alginate constructs of 3 mm thick and 6 mm in diameter. Constructs were prepared as previously described[166]. After 35 days of culture, constructs were mounted on the Dynamic Mechanical Analyzer DMA Q800 (TA Instruments, New Castle, DE, USA) and tested in a radially unconfined stress relaxation test as described previously[230]. The secant modulus is related to the interaction between the solid and the liquid phase and is therefore an indication for the ability to hold water. The modulus measured at equilibrium depends strongly on the compressive stiffness of the (cartilaginous) solid matrix[83].

Electron microscopy and fibril measurements

Beads were rinsed three times in PBS and fixed for 2 hours at room temperature in 0.1 M sodium cacodylate-buffered 1.5% glutaraldehyde (EM grade, Sigma) and 1% paraformaldehyde, pH 6.7, then rinsed 3 times in 0.15 M sodium cacodylate. After post-fixation for 2 hours in 0.1 M sodium cacodylate buffered 1% osmium tetroxide (OsO4), pH 6.7, beads were dehydrated in a series of graded acetone and embedded in LX 112 (Epon). Ultrathin sections (LKB ultratome IV), were mounted on copper grids (300 mesh) and contrasted with 2% uranyl acetate (10 minutes at 45° C) and lead citrate. They were examined with a Zeiss 902 electron microscope. Pictures were taken at 2800 times, 18000 times, and 89000 times magnification. Four pictures per sample (89000 times magnification) were used to measure collagen fibril diameter with ImageJ 1.40g (NIH).

Statistical analysis

The experiments were repeated three times. Each experiment consisted of 3 times 7 beads per experimental condition for biochemical analyses and two samples of 10 beads per experimental condition for gene expression analysis. Experiments for mechanical testing were repeated two times with a total of 6 samples per group. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) software. All data are presented as mean ± standard deviation. Control groups without growth factors or lentivirus and groups supplemented with growth factors or transfected with COMP lentivirus were compared with a Kruskall-Wallis test and a Dunn's multiple comparison test.

Results

COMP expression in the presence of growth factors

IGF1 had no effect on COMP gene expression, the presence of TGFβ2 increased the expression 14-fold and FGF2 decreased it more than 5-fold as determined after 11 days of culture (Figure 5.1A). COMP protein deposition in the alginate bead, quantified with ELISA, was not influenced by the presence of IGF1 on day 11 and day 21. TGFβ2 increased the deposition approximately 2-fold and approximately 6 times less COMP was deposited in the presence of FGF2 (Figure 6.1B). A reducing Western blot was performed to assess the integrity of the COMP in the alginate beads at day 11 and 21. The control, IGF1, and TGFβ2 condition had a signal at approximately 110 kDa with increased intensity at day 21 compared to day 11. Almost no signal was seen in the FGF2 condition (Figure 6.1C). COMP protein was also excreted into the culture medium of the alginate beads as shown on the reducing Western blot of the medium. Approximately the same amount of COMP was present in the control, IGF1 and FGF2 condition. The presence of TGFβ2 resulted in more COMP in the culture medium (Figure 6.1D).

Distribution of matrix components after addition of growth factors

The distribution of COMP, collagen and proteoglycans within the alginate bead at day 21 was determined by separation of the cell-associated matrix (CM) and further removed matrix (FRM). In the control condition, 27 ± 2.5% of the COMP was located in the CM and 73 ± 2.6 % of the COMP in the FRM without differences between growth factor conditions (Figure 6.2A). The same distribution for the proteoglycans was seen in the control condition; 28 ± 3.2 % of the proteoglycans in the CM and 72 ± 1.8 % of the proteoglycans in the FRM. However in the case of the proteoglycans, there is a shift in this distribution when IGF1 or FGF2 is present resulting in an equal distribution over CM and FRM. Addition of TGFβ2 resulted in a slight shift towards the FRM, although this is not significantly different from control (Figure 6.2B). In case of collagen, 66 ± 8.2 % was cell associated and 34 ± 4.1 % was further removed from the cell in the control condition. This distribution was changed to 36% in the cell-associated matrix in the presence of TGFβ2, but not in the presence of IGF1 or FGF2 (Figure 6.2C).

COMP gene expression after lentiviral transfection

A lentiviral vector was used to more selectively increase COMP gene expression, using human COMP gene in order to distinguish endogenous bovine COMP from lentivirally transfected COMP. A GFP lentivirus was used in parallel to determine transfection efficiency. In time, the percentage of GFP positive cells did not decrease indicating that
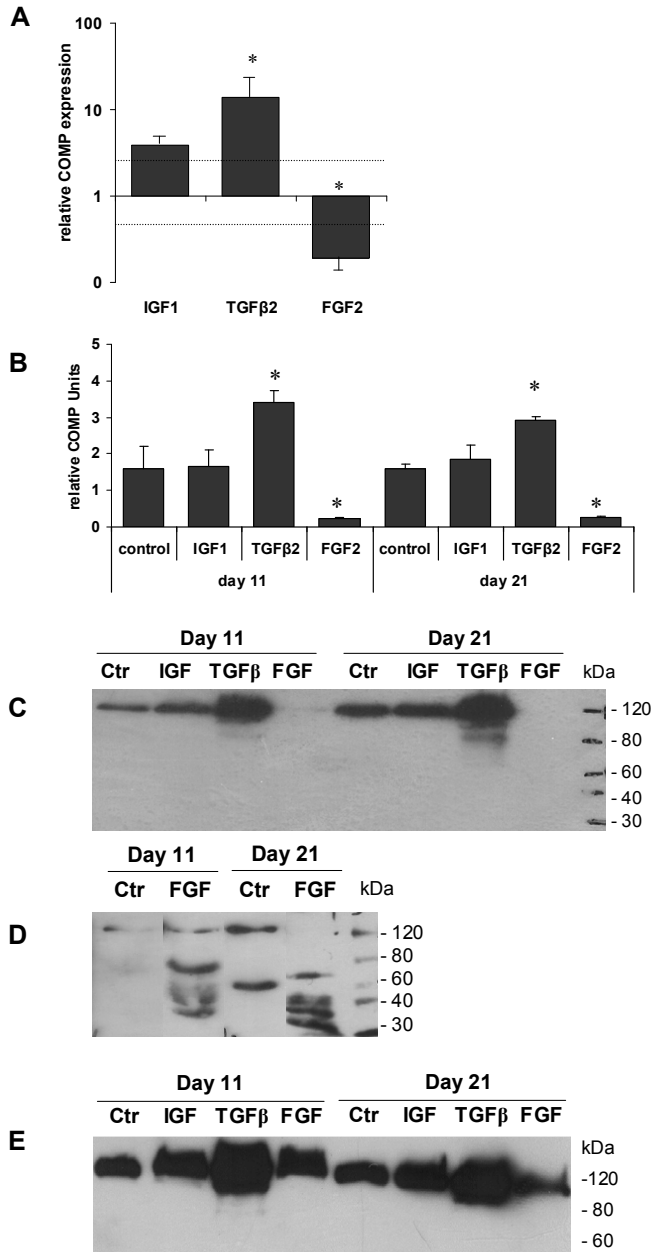
Figure 6.1. The effect of 25 ng/ml IGF1, TGFβ2 and FGF2 on COMP gene and protein expression. Gene expression of bovine COMP is shown in (A) by expression after culturing for 11 days relative to the control without growth factors, which is set at 1 (n = 6). Variation in the control condition is indicated by dotted lines. B) Protein deposition of COMP in the alginate beads is shown in relative COMP units as determined with ELISA (n = 9) and C) reducing Western blot after 11 and 21 days. D) The COMP release in the culture medium after 11 and 21 days is also determined by reducing Western blot. * Indicates significant difference ($p<0.05$) between growth factor and control without growth factors.
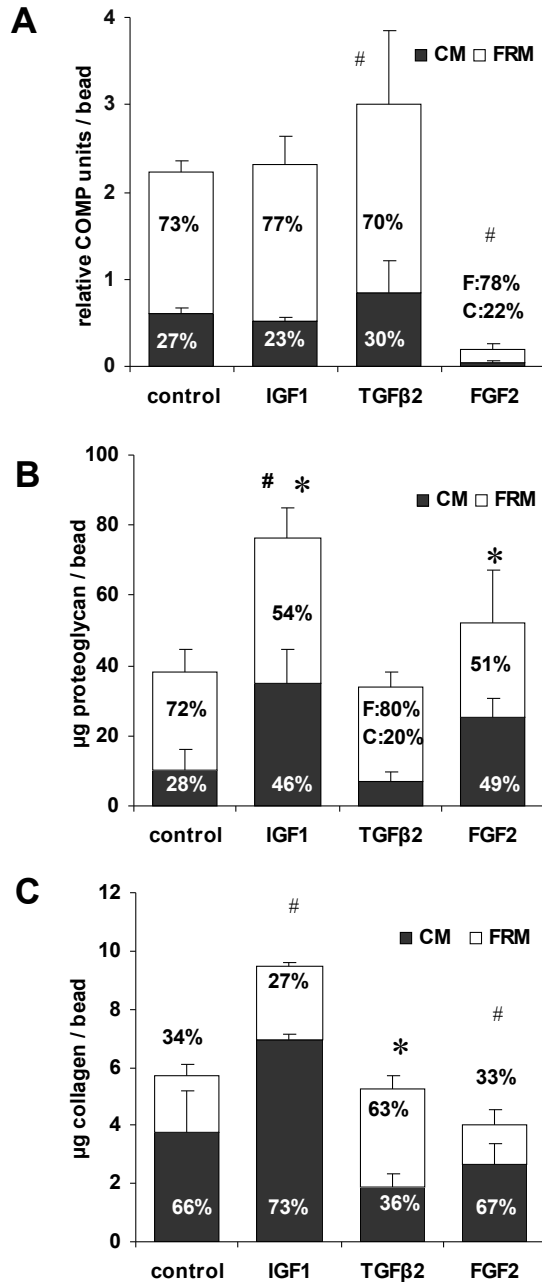
Figure 6.2. The effect of 25 ng/ml IGF1, TGFβ2 and FGF2 on absolute matrix distribution with relative percentages after 21 days of culture as shown by the distribution of A) COMP, B) proteoglycans, and C) collagen between the cell associated matrix (CM, grey bars) and the further removed matrix (FRM, white bars). N = 9, * indicates significant difference (p<0.05) in relative distribution between growth factor condition and control without growth factor, # indicates significant difference (p<0.05) in absolute values between growth factor condition and control condition.

A

B
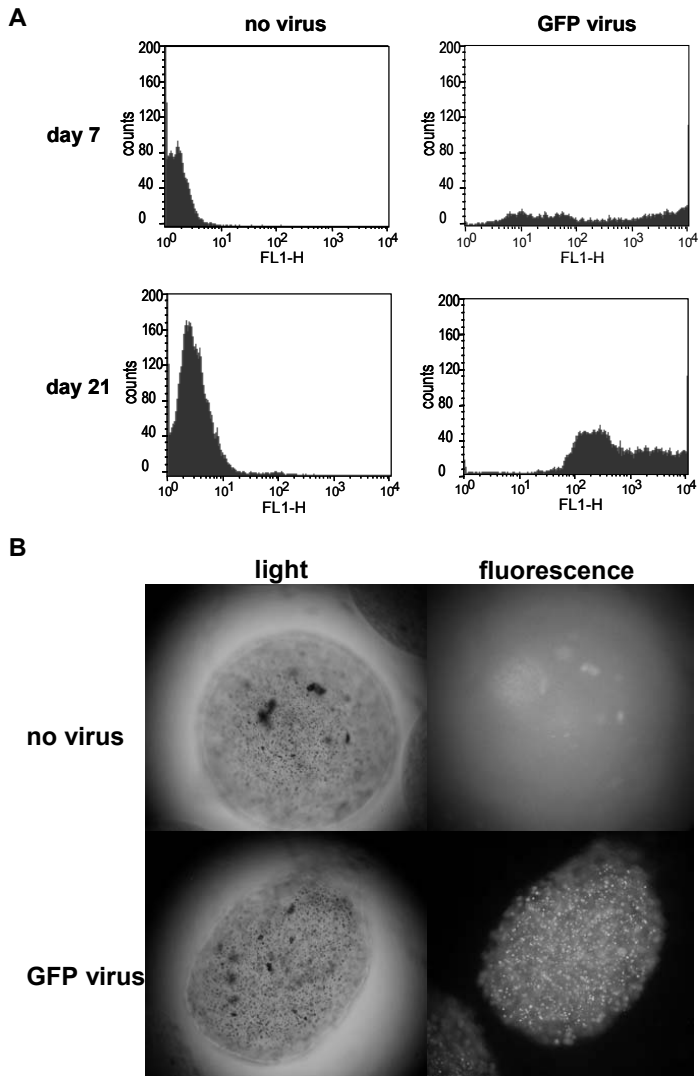
Figure 6.3. The effect of lentiviral transfection of GFP as a control for transfection efficiency. A) The percentage of positive cells in the GFP transfected condition increased from 92% at day 7 to 99% at day 21. No GFP positive cells were present in the untransfected control condition. N = 2. B) Cells with or without GFP lentivirus in alginate analysed with a fluorescence microscope. No green fluorescent cells were present in the control condition after 21 weeks of culture, green cells were visible in the GFP lentivirus condition.

a stable transfection was accomplished (Figure 6.3A and B). The human COMP gene expression increased in time when bovine chondrocytes were transfected with the COMP lentivirus. In the control conditions without virus or an empty virus, no human COMP gene expression was seen (Figure 6.4A). Bovine COMP gene expression was not influenced by transfection with lentivirus (Figure 6.4B).
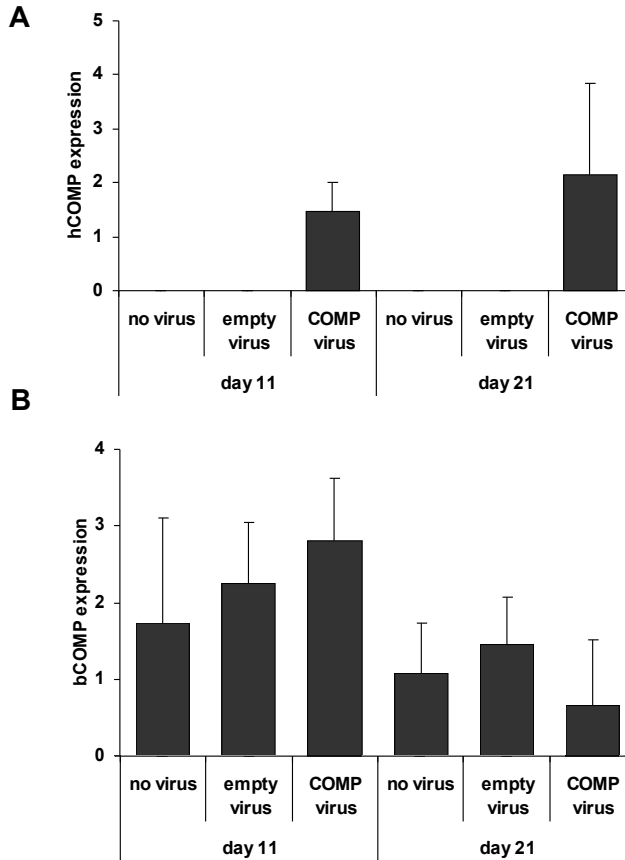
Figure 6.4. COMP gene expression in bovine chondrocytes, transfected with or without lentivirus containing the human COMP gene or an empty virus. A) Relative human COMP gene expression corrected for GAPDH after lentiviral transduction of bovine chondrocytes. No human COMP gene expression was detected in the bovine chondrocytes in the control conditions. B) Relative endogenous bovine COMP gene expression after transduction with human COMP. N = 6.

COMP protein deposition in response to overexpression

As quantified by ELISA, COMP protein deposition was increased 1.5-fold after lentiviral transfection at day 21. COMP excretion into the culture medium was also increased (Figure 6.5A). To investigate whether COMP produced in these experiments had a pentameric structure, a non-reducing Western blot was performed. COMP protein of approximately 550 kDa was detectable in alginate beads of the control condition and the lentivirally transfected condition, with an increase in quantity after lentiviral transfection. In addition, smaller fragments were present in the alginate beads of the COMP lentivirus condition. In the culture medium, more COMP protein was present than in the alginate beads. A small increase of COMP excretion into the medium is seen with COMP overexpression. This is however not as clear as detected with the ELISA (Figure 6.5B).
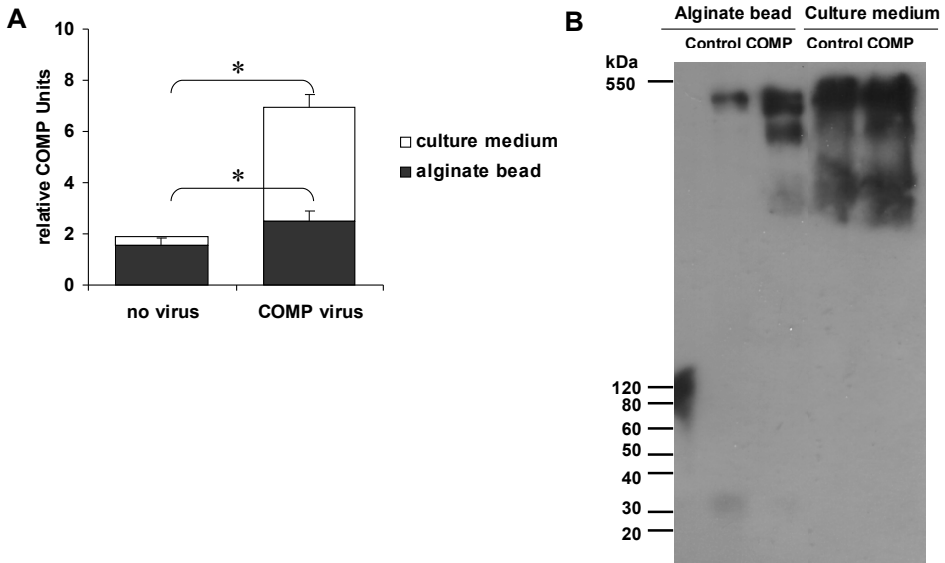
Figure 6.5. The effect of lentiviral COMP overexpression on COMP production. After transfection with or without lentivirus containing the human COMP gene, alginate beads (grey bars) and the culture medium (white bars) were analysed after 21 days of culture for the presence of COMP with ELISA (A). Alginate beads and culture medium were also analysed with a non-reducing Western blot at day 21 (B).

Matrix deposition and functionality in response to COMP overexpression

The amount and distribution of collagen deposited was not influenced after COMP transfection (Figure 6.6A and B). The number of HP cross-links per collagen triple helix was 0.57 ± 0.02 in the control condition and not significantly changed after COMP transfection (Figure 6.6C). The proteoglycan production (Figure 6.6D) and distribution (Figure 6.6E) were also not influenced by COMP overexpression. The ability to hold water (defined by the secant modulus) and the stiffness of the matrix (defined by the equilibrium modulus) were also not significantly different in the control and COMP transfected condition (Figure 6.6F and G).

Collagen fibril diameter in response to increased COMP production

To further investigate the effect of increasing COMP production on the newly formed matrix we measured collagen fibril diameter on an electron microscopic level. The matrix of chondrocytes cultured in the presence of TGFβ2 appears more organized than the matrix produced in the corresponding control condition (Figure 6.7A) since collagen fibrils are aligned more parallel to the chondrocyte surface in the presence of TGFβ2. In addition, collagen fibril diameter decreased when TGFβ2 was present (Figure 6.7B). The induction of COMP production by chondrocytes using a lentivirus also resulted in a less
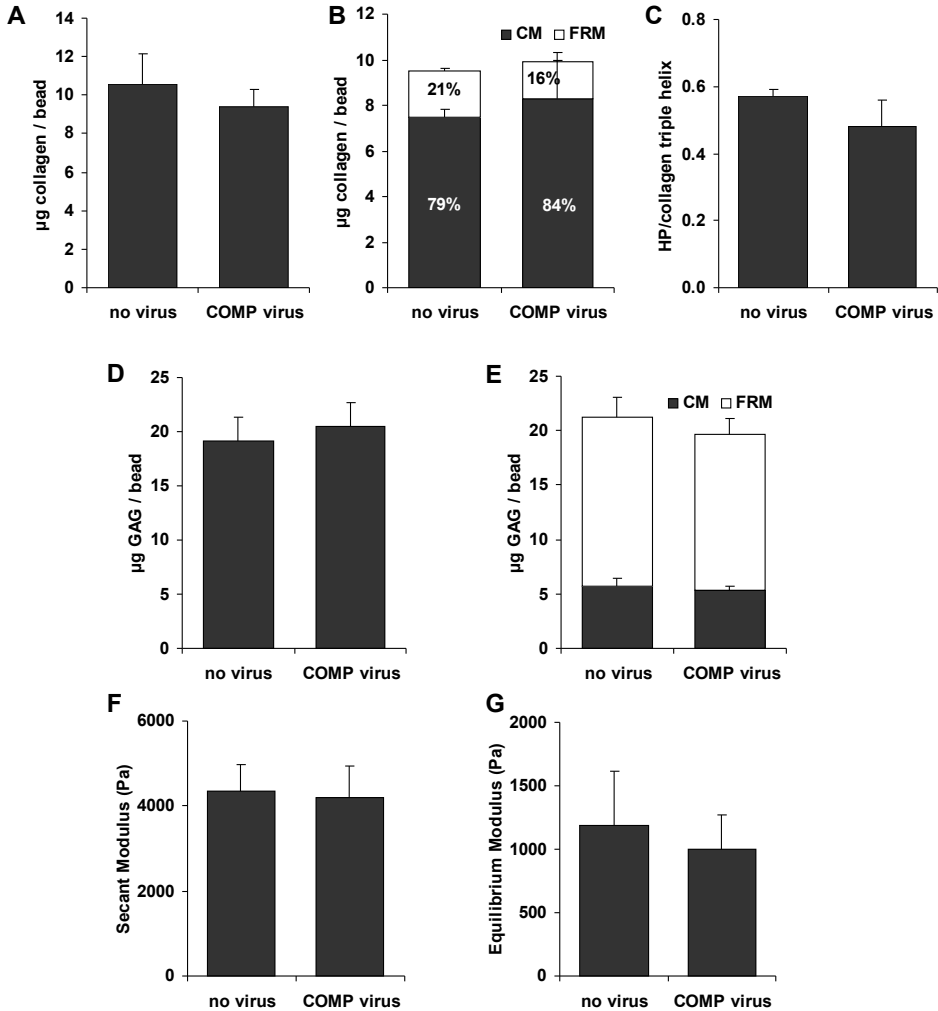
Figure 6.6. Collagen biochemistry and matrix functionality in lentiviral transfected chondrocytes. A) Collagen deposition, B) collagen distribution between cell associated matrix (CM, grey) and further removed matrix (FRM, white), C) collagen crosslinking, D) Proteoglycan deposition, and E) Proteoglycan distribution between cell associated matrix (CM, grey) and further removed matrix (FRM, white) (all N=9), F) Secant modulus and G) equilibrium modulus of newly formed matrix after 35 days of culture, N=12.

organized matrix compared to the corresponding control condition (Figure 6.7C). This effect however was less obvious than the effect of TGFβ2 because of EM processing artefacts (indicated by arrowheads). Again, overexpressing COMP resulted in smaller diameters of the collagen fibrils (Figure 6.7D).

Figure 6.7. Representative examples of electron microscopic analysis of alginate beads cultured for three weeks in the presence of TGFβ2, after COMP lentiviral transfection, and their corresponding control conditions. A) Newly formed matrix in the presence of TGFβ2 and its control condition without growth factors. Arrows point out collagen fibrils parallel to the cell surface. Arrows point out organised collagen fibrils. Used magnifications are 18000 times (upper row) with the bar 2 μm and 89000 times (lower row) with the bar indicating 200 nm. B) Diameter of collagen fibrils with or without TGFβ showed as diameter of individual fibrils with the average diameter ± SD. C) Newly formed matrix after lentiviral transfection and its control without virus. Arrows point out organised collagen fibrils, arrowheads point out processing artefacts. Used magnifications are 1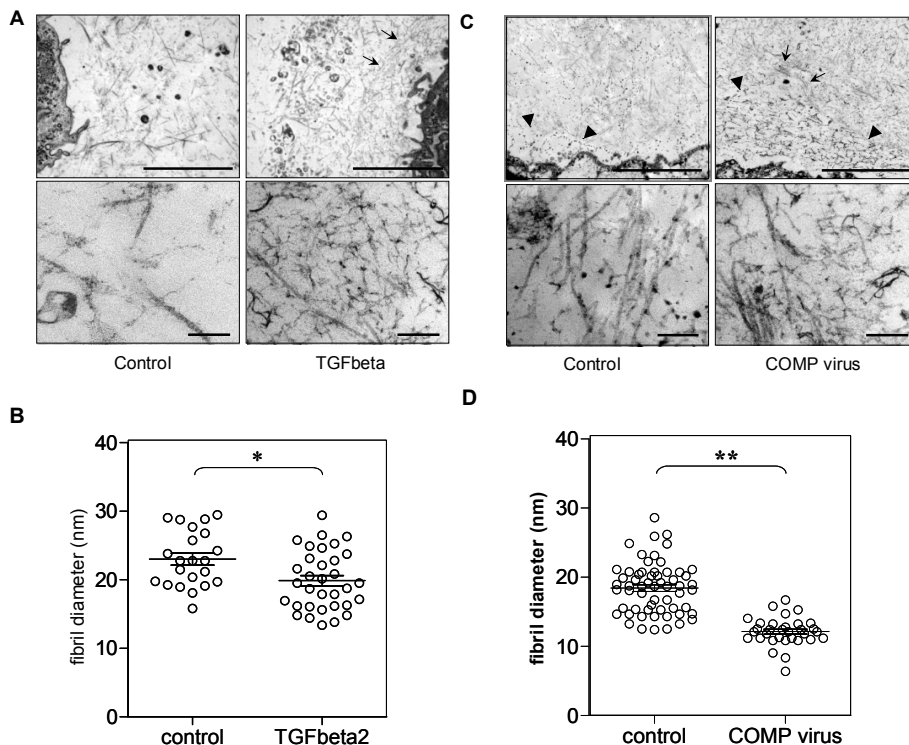8000 times (upper row) with the bar indicating 2 μm and 89000 times (lower row) with the bar indicating 200 nm. D) Diameter of collagen fibrils with or without lentiviral overexpression of COMP showed as diameter of individual fibrils with the average diameter ± SD. * indicates significant difference, $p < 0.05$, ** indicates significant difference, $p < 0.001$.

Discussion

COMP interacts with several other cartilage matrix molecules and is involved in matrix-chondrocyte interaction [247]. It is also up-regulated during the progression of OA [67] and therefore used as a marker for OA [248-251]. This study shows that COMP gene expression and protein deposition by chondrocytes in alginate beads were upregulated in the presence of TGFβ2. We previously examined collagen and glycosaminoglycan deposition as well as mechanical properties of these samples [211]. There we found that TGFβ2 slightly influenced collagen deposition by bovine chondrocytes in alginate and severely

reduced the number of collagen cross-links without affecting functionality of the newly formed matrix. Because of the pleiotropic effects of TGFβ, it is not possible to determine whether the increase of COMP deposition in this condition leads to a better integrity of the newly formed matrix and whether COMP is involved in cartilage development as seen previously [244]. With lentiviral overexpression we increased COMP gene expression and protein production. This appeared to have no effect on absolute matrix deposition and functional properties. However, increasing COMP production resulted in a more organized collagen matrix and collagen fibrils with a smaller diameter, independent of the approach used to induce COMP production (i.e. lentiviral overexpression of COMP or addition of TGFβ to the culture medium).

COMP interacts with collagen type 2 (COL2) [54] and COMP could function as a catalyst in collagen fibrillogenesis. When COMP and collagen are co-incubated, most of the COMP is not associated with the newly formed collagen fibrils even though the absence of COMP slows the process of fibrillogenesis [57]. Co-expression of transgenic mutant COMP and endogenous COL2 leads to a disruption of the fibrillar collagen network. In addition, more mutant COMP is localized on the formed COL2 aggregates then when wild type COMP is overexpressed [242]. This might be explained by the fact that mutant COMP cannot act as catalyst in fibrillogenesis and remains on the collagen aggregates (personal communication with dr. F. Zaucke). The catalyst function of COMP might explain why COMP overexpression only resulted in a mild elevation of COMP in the alginate bead, but did result in more COMP in the culture medium as determined with ELISA. Possibly, the extra COMP is not necessary and therefore directly excreted into the culture medium. Future experiments might include induction of collagen deposition as shown previously [211, 230] in combination with COMP overexpression to examine COMP and collagen interaction more closely.

Interestingly, inducing COMP production by both TGFβ stimulation and lentiviral transduction resulted in more organized collagen matrix and a decrease of collagen fibril diameter. To our knowledge, two other studies describe the relation between COMP amount and collagen fibril diameter. In equine tendons, a positive correlation between high COMP levels and the percentage of small collagen fibrils was present [252], which corresponds to our findings. Halázs et al concluded more recently that COMP is involved in collagen fibrillogenesis affecting intermediate forms of collagen fibrils and increasing rate of fibril formation. Without the presence of COMP, collagen fibrils with a larger diameter were found [57]. Next to altered fibril diameter, the latter study also reports about altered collagen organization when COMP is absent. In our study, we also found an effect of COMP on collagen fibril organization.

The diameter of the collagen fibrils plays a significant role in determining the mechanical properties of the tissue. Deformation of tissues is related to the proportion of small diameter fibrils and as the diameter increases the flexibility of the tissue

decreases [253, 254]. We did not observe altered mechanical properties when COMP production was stimulated, despite the small diameter of the collagen fibrils. Immaturity of the newly formed matrix might explain this. This was also seen for collagen cross-links that became important when the matrix was more mature [230]. This suggests that a more mature cartilage matrix where high levels of COMP had been present during formation can be of less quality because of the small diameter collagen fibrils.

Small bands in the non-reducing Western blot indicate the presence of the monomeric form of COMP and more COMP subunits are present after lentiviral transfection. This could indicate increased degradation in the overexpression condition after excretion into the culture medium. A minor discrepancy present between the ELISA and the Western blot in case of the overexpression experiments can be explained by the difference in used antibody and the recognized epitope [255]. The ELISA is based on a sandwich principle where one monoclonal and one polyclonal antibody capture COMP. For Western blotting, a different monoclonal antibody was used [246].

Regulation of COMP gene expression and protein production by chondrocytes with growth factors has been shown before[256]. In that study, TGFβ addition also resulted in increased synthesis of COMP and therefore confirms our results. However, monolayer cultures of chondrocytes were used and expression was analyzed after a short-term culture. In our study, chondrocytes were cultured in a 3D environment in order to maintain their phenotype resulting in proper matrix production. COMP distribution over the matrix is not affected by any of the growth factor conditions, in contrast to collagen and proteoglycan distribution. This suggests that COMP localization is not dependent on collagen or proteoglycans. Overexpression of COMP did not influence collagen or proteoglycan production and distribution. This confirms earlier results for COMP and collagen [244].

Taken together, COMP production and deposition is increased by TGFβ and lentiviral overexpression both leading to smaller diameters of collagen fibrils and changed organization of collagen. From the overexpression experiments we conclude that induction of COMP production has no effect on other biochemical parameters. Increased production of COMP is one of the features of OA [67, 244], next to increased synthesis of other cartilage matrix molecules [96, 142] although repair is ineffective. Increased COMP production in OA could lead to decreased stiffness of the cartilage via decreasing collagen fibril diameter therefore negatively affecting the quality of the matrix. Modulation COMP levels could therefore contribute to successful cartilage regeneration strategies.

Acknowledgements

7

Combined role of collagen IX and COMP in cartilage matrix assembly – COMP counteracts collagen IX limitation of cartilage collagen fibril growth

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Abstract

Objective

Defects in assembly and composition of the cartilage extracellular matrix are likely to result in impaired matrix integrity and increased susceptibility for cartilage degeneration. This study was undertaken to determine the functional interaction of the collagen fibril associated proteins collagen IX and cartilage oligomeric matrix protein (COMP) during cartilage matrix formation.

Methods

Primary chondrocytes from collagen IX and COMP double deficient mice were cultured in monolayer or alginate beads. Anchorage of matrix proteins, proteoglycan and collagen content, collagen cross-links, matrix metalloproteinase activity, and mechanical properties of the matrix were measured. Electron microscopy was employed to study the formation of fibrillar structures.

Results

In cartilage lacking both collagen IX and COMP, matrilin-3 showed an increased solubility. Less matrilin-3 was deposited in the matrix of double deficient chondrocytes, while larger amounts were secreted into the medium. Also proteoglycans were less well retained in the matrix formed in alginate cultures, while collagen deposition was not significantly affected. Electron microscopy revealed similar cartilage collagen fibril diameters in cultures of double deficient and wildtype chondrocytes. In contrast, a larger fibril diameter was observed in the matrix of chondrocytes deficient in only collagen IX.

Discussion

Our results show that collagen IX and COMP are involved in matrix assembly by mediating anchorage and regulating distribution of other matrix macromolecules such as proteoglycans and matrilins and have counteracting effects on collagen fibril diameter. Loss of collagen IX and COMP leads to matrix aberrations that may make cartilage more susceptible for degeneration.

Introduction

The cartilage extracellular matrix (ECM) is a complex alloy of proteins and proteogly-cans, the composition of which defines tissue structure and physiological functions [257]. Changes in matrix composition are likely to result in impairment of matrix integrity and lead to an increased susceptibility for cartilage degeneration. Human genetics studies have indeed shown that mutations in matrix proteins can predispose for disease like chondrodysplasia and/or osteoarthritis [258, 259]. The role of many of the matrix proteins is still uncertain and it is often not known how deficiencies in specific proteins predispose for cartilage degeneration.

Cartilage collagen fibrils provide stiffness to the cartilage matrix. Proteoglycans, mainly aggrecan, account for the osmotic pressure and are therefore important to maintain the high water content of cartilage [79]. Collagen IX, in combination with collagen II and XI, is a key component in the cartilage collagen fibril [223] and is thought to limit its lateral growth by binding to the fibril surface [63, 224]. Collagen IX can also act as a molecular bridge between fibrillar collagens and other extracellular matrix components, such as matrilin-3 [63] and cartilage oligomeric matrix protein (COMP, thrombospondin-5) [55, 260]. COMP in turn interacts with matrilins and aggrecan as well as with collagen I and II and is known to influence collagen I and II fibrillogenesis [57].

A number of knockout mouse models have been generated to gain further insight into the role of single ECM proteins, including collagen IX and COMP, in cartilage matrix assembly and function. COMP deficient mice lack an obvious phenotype [237], maybe due to a functional compensation by other matrix proteins. Mice homozygous for the inactivated col9a1 allele display degenerative changes in articular cartilage, first detected at the age of 6 months [224, 261]. These mice have cartilage collagen fibrils with a larger diameter [63]. They also show alterations in the expression of matrix metalloproteases and in mechanical properties, similar to what is seen during development of human osteoarthritis [207]. The loss of proteoglycans and adapter proteins (e.g. matrilin-3), which mediate interactions between collagen fibrils and their environment, has been suggested to be the molecular basis for the altered biomechanical properties [63, 207, 262].

Thus, mice lacking collagen IX have an increased collagen fibril diameter and show a loss of matrilin-3 anchorage [63], whereas COMP deficient mice exhibit no similar defects in matrix assembly or fibril diameter [237]. It has been unclear whether the loss of both collagen IX and COMP enhances the matrix assembly phenotype observed in collagen IX deficient animals and whether collagen IX and COMP play compensatory or antago-nistic roles during matrix assembly. Recently, we and others have established mouse lines deficient in collagen IX and COMP or in collagen IX and multiple thrombospondins (TSPs) [263, 264]. In these mice, a disorganized growth plate architecture and alterations in matrix deposition were observed both in newborn animals and at later stages of

development [263]. Loss of other TSPs in addition to COMP, results in an even more pronounced phenotype. When subjected to running exercise, flattening of the articular surface and an increased susceptibility for osteoarthritis was reported for both single and double deficient mice. However, only in mice lacking both collagen IX and COMP, fibrillation of the surface was seen [264].

To date, the molecular events leading to these phenotypes are not well understood. In this study, we set out to analyze the contribution of collagen fibril associated proteins to matrix assembly by using our previously described mouse model lacking both collagen IX and COMP, and culturing primary chondrocytes derived from these mice in both monolayer and in an alginate-based three-dimensional culture system. Analyses of proteoglycan and collagen expression as well as matrix deposition, together with the study of the fibrillar structures formed, provided further insight into the functional interaction of collagen IX and COMP.

Material and Methods

Animals

Collagen IX deficient mice [261] were kindly provided by the group of Peter Bruckner, Münster, and were bred onto a C57/BL6 background for at least five generations. To generate mice deficient in both collagen IX and COMP [263], collagen IX deficient mice were intercrossed with mice lacking COMP ([237]; kindly provided by Åke Oldberg, Lund) on a C57/BL6 background. C57/BL6 mice were used as wildtype controls.

Antibodies

For immunohistological stainings the following primary antibodies were used: a monoclonal mouse antibody directed against human collagen II (1:1000; Calbiochem), an affinity-purified polyclonal rabbit antibody against the NC4 domain of mouse collagen IX (1:2000, [63], a polyclonal rabbit antibody against bovine COMP (1:1000, [265]) and an affinity-purified polyclonal rabbit antibody against mouse matrilin-3 (1:1000, [266]). As secondary antibodies, Alexa Fluor 488 conjugated goat anti rabbit IgG and Cy3 conjugated goat anti mouse IgG immunoglobulins (both obtained from Molecular Probes) were applied.

Histology and immunohistochemistry

For histochemical and immunohistochemical stainings, 4% paraformaldehyde fixed, paraffin embedded tissue sections (5 μm) were dewaxed in xylol and rehydrated in isopropanol, 96%, 70% and 50% ethanol and water. To analyze the general tissue morphology and proteoglycan deposition, a combined haematoxylin/eosin/ alcian

blue staining (0.015% in 80% ethanol and 20% glacial acetic acid, 20 minutes.) was performed. For immunohistological stainings on sections, these were digested with testicular hyaluronidase (10 mg/ml) for 30 minutes at 37°C, followed by a permeabilisation with 0.1% Triton X-100 and blocking in 10% fetal calf serum. Cultured monolayer cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes, permeabilised with 0.1% Triton X-100 and blocked with 2% normal goat serum. Both sections and cells were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour at room temperature.

Sequential protein extraction from rib cage cartilage

Rib cages of less than one week old double deficient and wildtype animals were isolated, freed from surrounding noncartilaginous tissue, and frozen at -80°C. Proteins were extracted as described previously [267].

Western blotting

For immunoblot detection of matrilin-3, samples were electrophoresed on 8% SDS-polyacrylamide gels, electrotransferred onto nitrocellulose membranes, blocked with 5% skim milk, and incubated overnight at 4°C with an affinity-purified polyclonal rabbit antibody directed against matrilin-3 (1:1.000) [266]. After washing, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:2.000, Amersham Biosciences) for 1 hour at room temperature. Antibody detection was performed using 2.5 mM luminol, 0.4 mM p-coumaric acid, 0.01% H_2O_2 as a luminescent agent (Fluka) and exposure on X-ray films.

Isolation and culture of primary chondrocytes

Primary chondrocytes were isolated from rib cages and cultured in monolayer as described previously [268]. For alginate culture, isolated chondrocytes were resuspended in 1.2% (w/v) low viscosity alginate (Keltone) in 0.9% NaCl (Sigma) at a concentration of 4×10^6 cells/ml. The cell-alginate suspension was passed through a 22-gauge needle into 105 mM $CaCl_2$. Beads were washed with 0.9% NaCl and DMEM/F12 (GibcoBRL) and inspected visually. Beads that were unusually small or large were excluded from the experiment. After transfer to a 6-well plate beads were cultured in 75 μl/bead DMEM/F12 supplemented with 10% fetal bovine serum (GibcoBRL), 50 μg/ml L-ascorbic acid 2-phosphate (Sigma), 50 μg/ml gentamicin and 1.5 μg/ml fungizone (both GibcoBRL). Medium was changed three times per week and at the end of the experiment the medium of the final change was analyzed. Alginate beads were harvested and analyzed after three weeks of culture.

Isolation of cell associated and further removed matrix

Alginate beads were dissolved by adding 75 µl per bead of 55 mM sodium citrate, 20 mM EDTA in 150 mM NaCl for 20 minutes at room temperature. The suspension was centrifuged for 10 minutes at 1000 rpm (Eppendorf) to separate the cells surrounded by its cell associated matrix (CM, the pellet) from components originating predominantly from the 'interterritorial' or further removed matrix (FRM; the supernatant) as described previously [163, 187, 194].

Biochemical assays

Alginate beads or samples of separated CM and FRM were digested overnight at 56°C with papain (200 µg/ml papain in 50 mM EDTA, pH 5.3, and 5 mM L-cystein). Glycosaminoglycans were quantified using the dimethylmethylene blue (DMB) assay [160]. The metachromatic reaction with DMB was monitored with a spectrophotometer and the ratio $A_{540}:A_{595}$ used to determine the glycosaminoglycan amount with chondroitin sulfate C (Sigma) as standard. The amount of DNA in each papain-digested sample was determined using ethidium bromide with calf thymus DNA (Sigma) as standard. For high-performance liquid chromatography of amino acids (hydroxyproline) and collagen cross-links (hydroxylysylpyridinoline and lysylpyridinoline), the papain digest was hydrolyzed (108°C, 18-20 hours) in 6 M HCl. The hydrolyzed samples were dried and redissolved in 200 µl water containing 2.4 mM homoarginine as an internal standard (Sigma). Samples were diluted 25-fold with 50% (v/v) acetic acid for cross-link analysis and diluted 250-fold with 0.1 M sodium borate buffer, pH 8.0, for amino acid analysis. To determine hydroxyproline, amino acids were labeled with 9-fluorenylmethyl chloroformate. Reversed-phase high-performance liquid chromatography of amino acids and cross-links were performed as described previously [162, 163]. The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 hydroxyproline residues per triple helix.

To determine total MMP activity, culture medium was analyzed as described earlier [165], using 5 µM fluorogenic substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys[Fluorescein]-Ala-Lys-NH$_2$) in the presence or absence of 12.5 µM BB94 (a general MMP inhibitor). Medium samples were diluted with one volume of MMP buffer (50 mM Tris, pH 7.5, 5 mM CaCl$_2$, 150 mM NaCl, 1 µM ZnCl$_2$, 0.01% Brij-35, 0.02% NaN$_3$) containing a proteinase inhibitor cocktail (Complete, Roche). The MMP activity in each sample was calculated as the difference in the initial rate of substrate conversion (linear increase in fluorescence in time, expressed as relative fluorescence units per second) between samples with and without BB94 addition. Fluorescence was measured for 6 hours at 30°C using a Cytofluor 4000 (Applied Biosystems).

Mechanical testing

For mechanical characterization, 4×10^6 cells/ml in 1.2% (w/v) alginate disks, 3 mm thick and 6 mm in diameter, were used. The disks were prepared as previously described [166, 211]. To ensure that enough matrix is produced and to test its functionality, beads were mounted after 35 days of culture on a materials testing machine (DMA Q800 Dynamic Mechanical Analyzer, TA Instruments) in a radially unconfined stress relaxation test with the beads between impermeable platens, and hydrated in 0.9% saline containing protease inhibitors (Complete, Sigma). A 20% uniaxial compressive strain was applied within 10 seconds at a ramped displacement strain rate of 2% s^{-1}, based on the measured thickness. The strain was maintained constant for 30 minutes, the load was recorded at a sampling rate of 10 Hz using a 18 N load cell. The applied load recorded by the load cell was divided by the cross-sectional area of the construct to calculate the applied stress. The secant modulus was calculated as stress/strain at 10 seconds, where the stress had its peak response and the strain reached its maximum. At the end of the test, the equilibrium aggregate modulus was determined as stress/strain at 30 minutes. The secant modulus is related to the interaction between the solid and the liquid phase and is therefore an indication for the ability to retain water. The modulus measured at equilibrium depends strongly on the compressive stiffness of the (cartilaginous) solid matrix [83].

Electron microscopy

Beads were rinsed three times in PBS and fixed for 2 hours at room temperature in 0.1 M sodium cacodylate-buffered 1.5% glutaraldehyde (EM grade, Sigma) and 1% paraformaldehyde, pH 6.7, then rinsed three times in 0.15 M sodium cacodylate. After post-fixation for 2 hours in 0.1 M sodium cacodylate-buffered 1% osmium tetroxide, pH 6.7, the beads were dehydrated in a series of graded acetone and embedded in LX 112 (Epon). Ultrathin sections (LKB ultratome IV), were mounted on copper grids (300 mesh), contrasted with 2% uranyl acetate (10 minutes at 45ºC) and lead citrate and examined with a Zeiss 902 electron microscope. Pictures were taken at 2800 times, 18000 times, and 89000 times magnification. Four pictures per sample (89000 times magnification) were used to measure collagen fibril thickness with ImageJ 1.40g (NIH).

Data analysis

Each alginate culture experiment was repeated two times, each time using at least four pooled cartilage donors. Each experiment consisted of three times seven beads per experimental condition for biochemical analyses, three beads for electron microscopic analysis, and six disks for mechanical testing. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software) software. All data are presented as mean ± standard deviation. Samples from the wildtype and double deficient groups were compared with a Kruskall-Wallis test followed by post hoc Dunn's multiple comparison tests.

Results

Deposition of matrix constituents

Immunofluorescence microscopy on rib and sternum sections revealed the deposition of collagen IX and COMP in wildtype rib cartilage, while the lack of staining in double deficient animals confirmed the absence of both proteins (Figure 7.1A-D). Antibodies directed against collagen II stained all cartilaginous areas in both control and knockout animals, suggesting that collagen II deposition is not substantially influenced by the absence of collagen IX and COMP (Figure 7.1E-F). In contrast, matrilin-3 staining was lost in most cartilaginous areas when collagen IX and COMP were missing. Matrilin-3 was detected neither in ribs nor in the sternal growth plate, whereas small amounts of the protein were present in the zone between adjacent sternal growth plates, where it is normally not detected, and in calcified cartilage (Figure 7.1H). Control animals showed a strong matrilin-3 staining in most cartilage elements (Figure 7.1G). Staining with alcian blue, a dye that binds to negatively charged glycosaminoglycans and thus to the major cartilage proteoglycan aggrecan, was not altered between genotypes and revealed no obvious malformations (Figure 7.1I-J).

Extraction of matrilin-3 and proteoglycans

The amount and solubility of matrilin-3 was examined by sequentially extracting cartilage. In wildtype tissue, matrilin-3 was extracted only with buffers containing EDTA (buffer II) or the chaotropic agent GuHCl (buffer III), whereas in double deficient animals some protein could be extracted with TBS alone (buffer I). In addition to the increased solubility, the total amount of matrilin-3 that could be extracted was reduced when both collagen IX and COMP were ablated (Figure 7.2A). This, together with the results from immunohistochemistry (Figure 7.1H), points to a decrease in the pool of matrilin-3 anchored in the matrix.

We also analyzed cartilage proteoglycans by the same extraction approach. Neither the solubility nor the total amount of extracted proteoglycans differed significantly between genotypes (Figure 7.2B).

Matrix deposition in short-term chondrocyte monolayer cultures

Chondrocytes were isolated from newborn wildtype and double deficient mice and cultured in monolayer for five days. In cultures of wildtype cells, large quantities of both collagen IX and COMP were integrated into an extracellular matrix, forming networks surrounding the chondrocytes (Figure 7.3A,C). A similar staining pattern was obtained for collagen II, revealing a deposition throughout the chondrocyte pericellular matrix regardless of the genotype (Figure 7.3E-F). Interestingly, matrilin-3 was completely
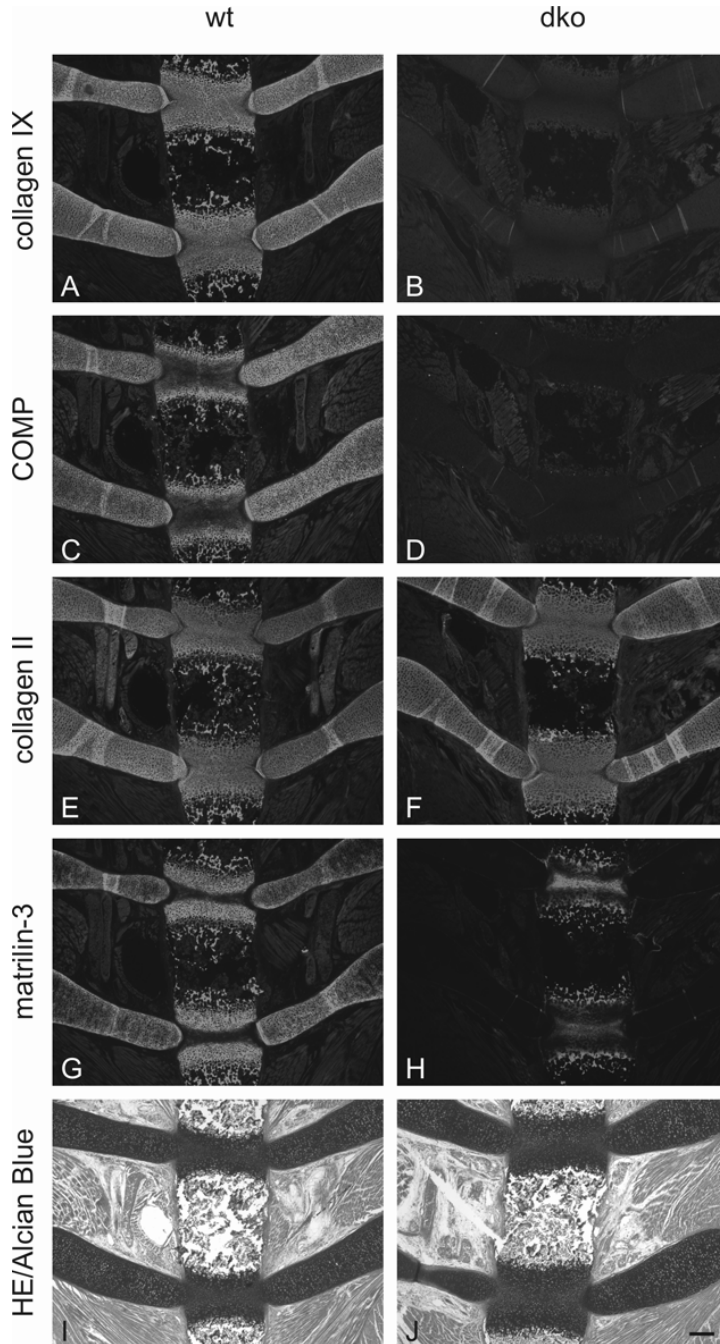
Figure 7.1. Immunofluorescence microscopy of rib cage cartilage. Sections through the rib cage of wildtype (wt) and collagen IX/COMP double deficient (dko) mice were incubated with antibodies directed against collagen IX (A-B), COMP (C-D), collagen II (E-F), and matrilin-3 (G-H). Sections were also stained with hematoxylin/eosin and alcian blue (I-J). The bar represents 100 μm.
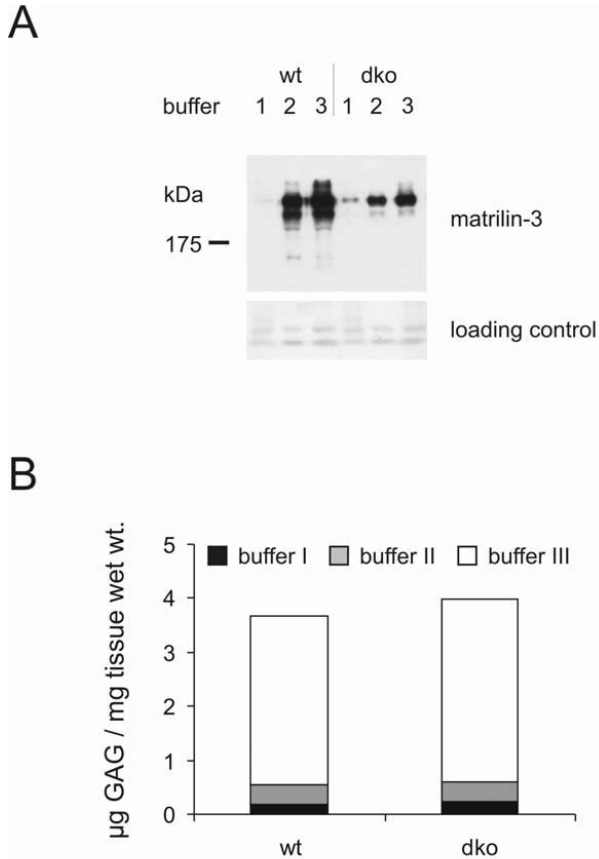
Figure 7.2. Solubility of matrilin-3 and proteoglycans from rib cartilage. Matrilin-3 and proteoglycans were sequentially extracted in TBS alone (buffer I), and TBS containing 10mM EDTA (buffer II) or 4 M GuHCl (buffer III). (A) Immunoblots were used to detect matrilin-3 and Ponceau staining as a loading control. (B) Glycosaminoglycan concentrations were determined in extracts obtained with the different buffers from 1 mg tissue wet weight .wt, wildtype; dko, collagen IX/COMP double deficient.

lost from extracellular fibrillar structures, when these lacked collagen IX and COMP. However, matrilin-3 was still secreted and, in these cultures, unspecifically coated the surface of the culture dish.

We determined the amount of proteoglycans deposited in the extracellular matrix after up to seven days. Alcian Blue binding assays revealed no significant difference between double deficient and control chondrocytes at these early time points in mono-layer cultures (results not shown).

Figure 7.3. Immunofluorescence microscopy of monolayer cultures of chondrocytes. Primary chondrocytes isolated from rib cartilage of wildtype (wt) and double deficient (dko) animals were cultured and stained with antibodies directed against collagen IX (A-B), COMP (C-D), collagen II (E-F) and matrilin-3 (G-H). The bar represents 20 μm.
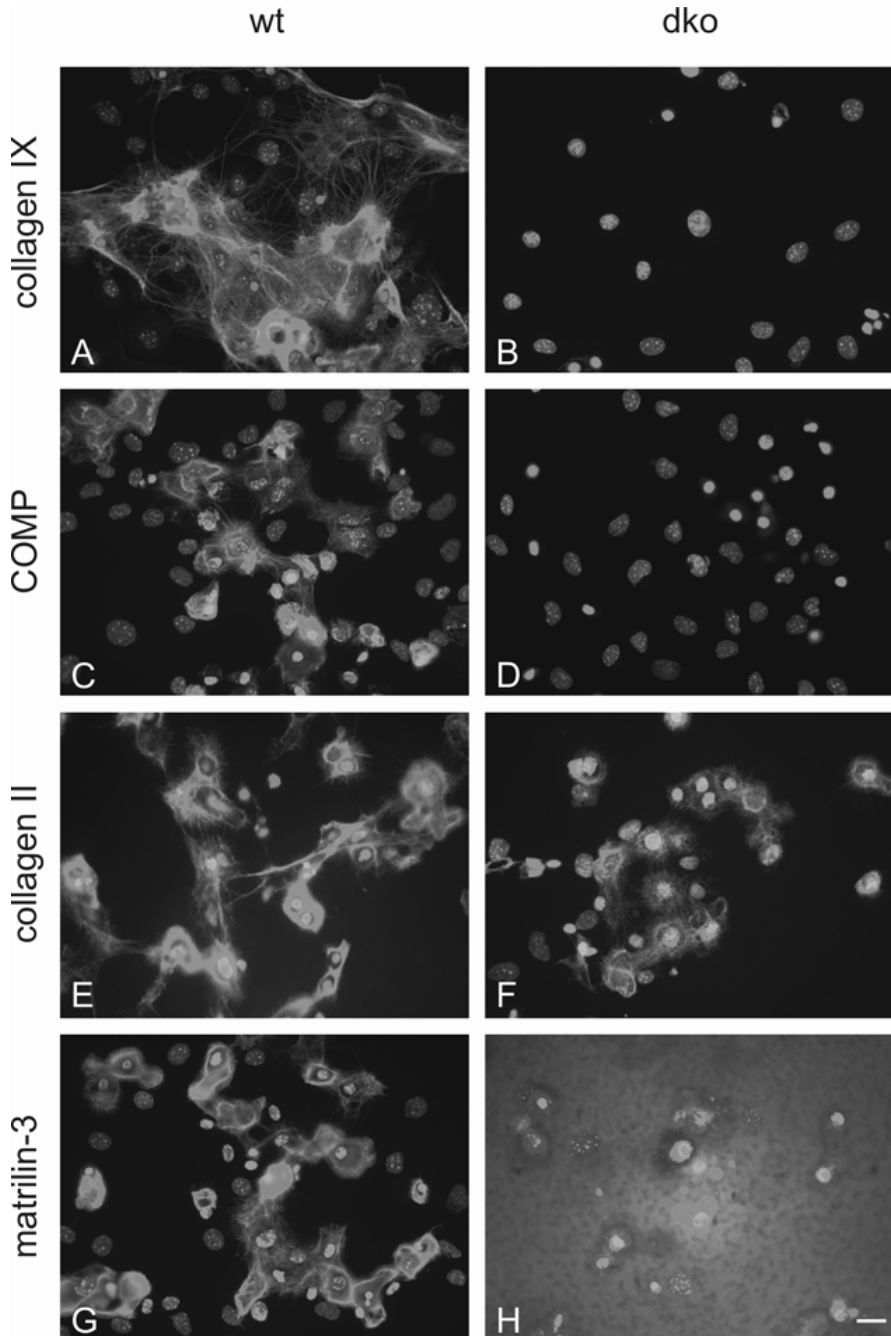
Protein and proteoglycan deposition in the extracellular matrix of long-term chondrocyte cultures in alginate

To analyze the involvement of collagen IX and COMP in longer term matrix assembly and maintenance and to allow the quantitative analysis of different matrix compartments, isolated chondrocytes were cultured in alginate beads. Also under these conditions the chondrocytes synthesized collagen II and not collagen I during the three-week period studied (results not shown).

Western blot analyses of CM and FRM from both wildtype and double deficient chondrocyte cultures showed less matrilin-3 deposition in the matrix of deficient cells (Figure 7.4A). Instead, more matrilin-3 was detected in the culture medium of these cells than in that from wildtype controls.
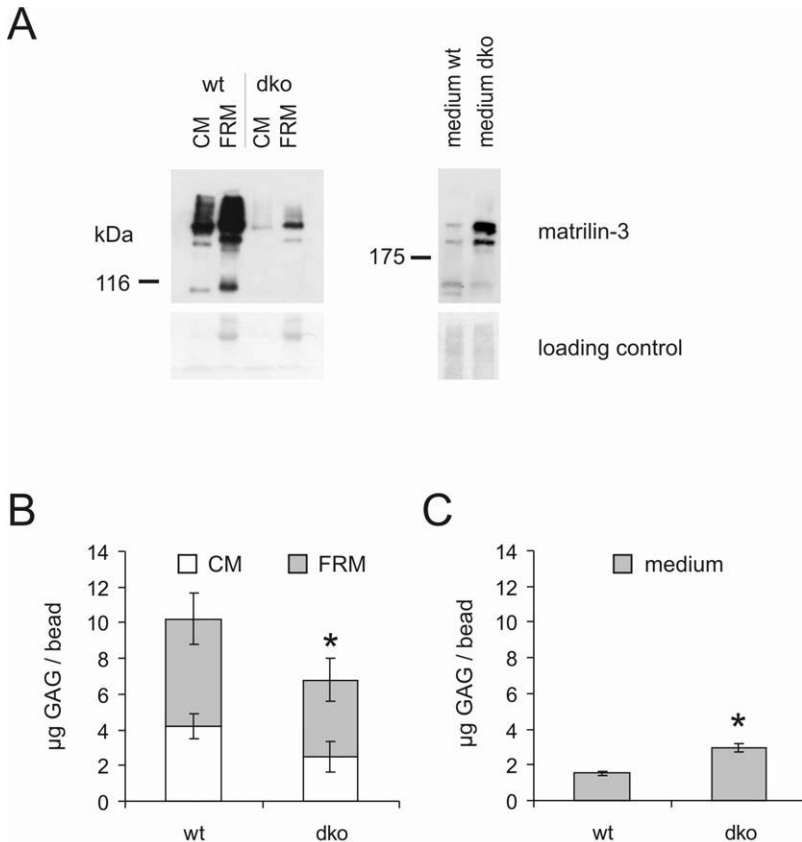
Figure 7.4. Amount and compartmental distribution of (A) matrilin-3 and (B) proteoglycans after three weeks of chondrocyte culture in alginate beads. (A) Immunoblot analysis of cell associated matrix (CM), further removed matrix (FRM) and culture medium using antibodies directed against matrilin-3. Ponceau staining was used as a loading control. (B) Glycosaminoglycan distribution between the CM (open bars), the FRM (grey bars) and (C) the culture medium. n = 6, * indicates significant difference in absolute values (p<0.05). wt, wildtype; dko, collagen IX/COMP double deficient.

Wildtype chondrocytes produced large amounts of proteoglycans over the three-week period, resulting in proteoglycan deposition in the alginate bead of 10.2 ± 2.1 µg/bead. In cultures of double deficient chondrocytes, significantly less proteoglycans (6.8 ± 2.1 µg/bead) were deposited. However, the relative distribution between CM and FRM was not significantly different when compared to wildtype cultures (Figure 7.4B). We also analyzed the culture medium harvested during the experiment. More proteoglycans were released into the culture medium of deficient chondrocytes (3.0 ± 0.2 µg/bead, 30% of the total proteoglycan production) in comparison to wildtype cells (1.5 ± 0.1 µg/bead, 13% of the total proteoglycan production), indicating less retention of proteoglycans in a collagen IX/ COMP deficient matrix (Figure 7.4C).
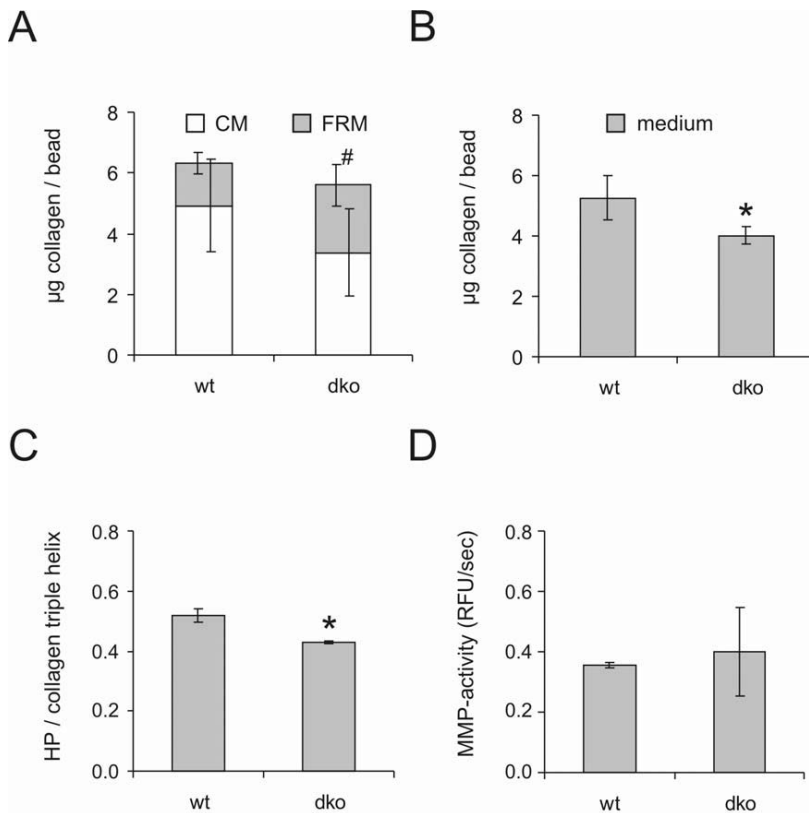
Figure 7.5. Distribution of collagen, collagen cross-links and MMP activity after three weeks of chondrocyte culture in alginate beads. (A). Collagen distribution between the cell associated matrix (CM, open bars), the further removed matrix (FRM, grey bars) and (B) the culture medium. n = 6, # indicates significant difference in distribution, * indicates significant difference in absolute values (p<0.05). (C) Hydroxylysylpyridinoline (HP) crosslinks in newly formed collagen (n = 4). * indicates p<0.01, data are presented as mean ± SD. (D) Overall MMP acitivity was measured in the culture medium after 21 days of culture using a fluorescent substrate. Data are presented as mean relative fluorescent units (RFU) per second ± SD (n = 4). wt, wildtype; dko, collagen IX/COMP double deficient.

The total collagen deposition was not significantly different in beads containing wildtype and double deficient chondrocytes (6.3 ± 1.9 µg/bead for wildtype and 5.6 ± 2.1 µg/bead for double deficient cells). Interestingly, the relative distribution within the alginate bead was altered, with 23% of the total collagen being deposited in the FRM of chondrocytes isolated from double deficient animals and 12% in the FRM of wild type chondrocytes (Figure 7.5A). Less collagen was found in the culture medium collected during the experiment with double deficient chondrocytes (4.0 ± 0.3 µg/bead) than in the medium of the wildtype cells (5.26 ± 0.7 µg/bead) (Figure 7.5B). This difference in absolute release did not result in a significant relative difference when compared to the total collagen production. Collagen was less cross-linked when collagen IX and COMP were absent, as determined by measuring the number of hydroxypyridinoline cross-links per collagen triple helix (Figure 7.5C).

A decreased amount of collagen in the medium can result from less synthesis or less degradation. MMPs are major matrix degrading enzymes that influence matrix quality and quantity. We determined the overall MMP activity in the culture media, but found comparable activity irrespective of the genotype (Figure 7.5D).

Mechanical properties

The secant modulus and the equilibrium aggregate modulus were examined to test whether changes in the amount and distribution of produced extracellular matrix components affect mechanical properties of the alginate beads containing chondrocytes. The peak force and eventually the ability to retain water, described by the secant modulus, were not altered in three-week cultures of chondrocytes lacking collagen IX and COMP when compared to wildtype (5070 ± 1623 Pa for wildtype, 4588 ± 890 Pa for double deficient cultures). Also the stiffness, indicated by the equilibrium aggregate modulus, did not differ significantly between genotypes (767 ± 232 Pa for wildtype, 970 ± 283 Pa for double deficient cultures).

Collagen fibril assembly

After three weeks of culture, complete alginate beads were processed for electron microscopy. The matrix deposited by wildtype and collagen IX/COMP double deficient chondrocytes appeared similar with some collagen fibrils positioned parallel to the cell surface and the fibrils being relatively closely spaced (Figure 7.6A-I). In cultures of collagen IX/COMP double deficient chondrocytes the fibril diameter was comparable to that found in cultures of wildtype cells (Figure 7.6J).

The result that neither appearance nor diameter of fibrils produced by double deficient cells differed compared to wildtype cultures was surprising, as we previously detected an increased collagen fibril diameter in cartilage from collagen IX single knockout mice [63]. We therefore analyzed the matrix produced by chondrocytes lacking expression of only
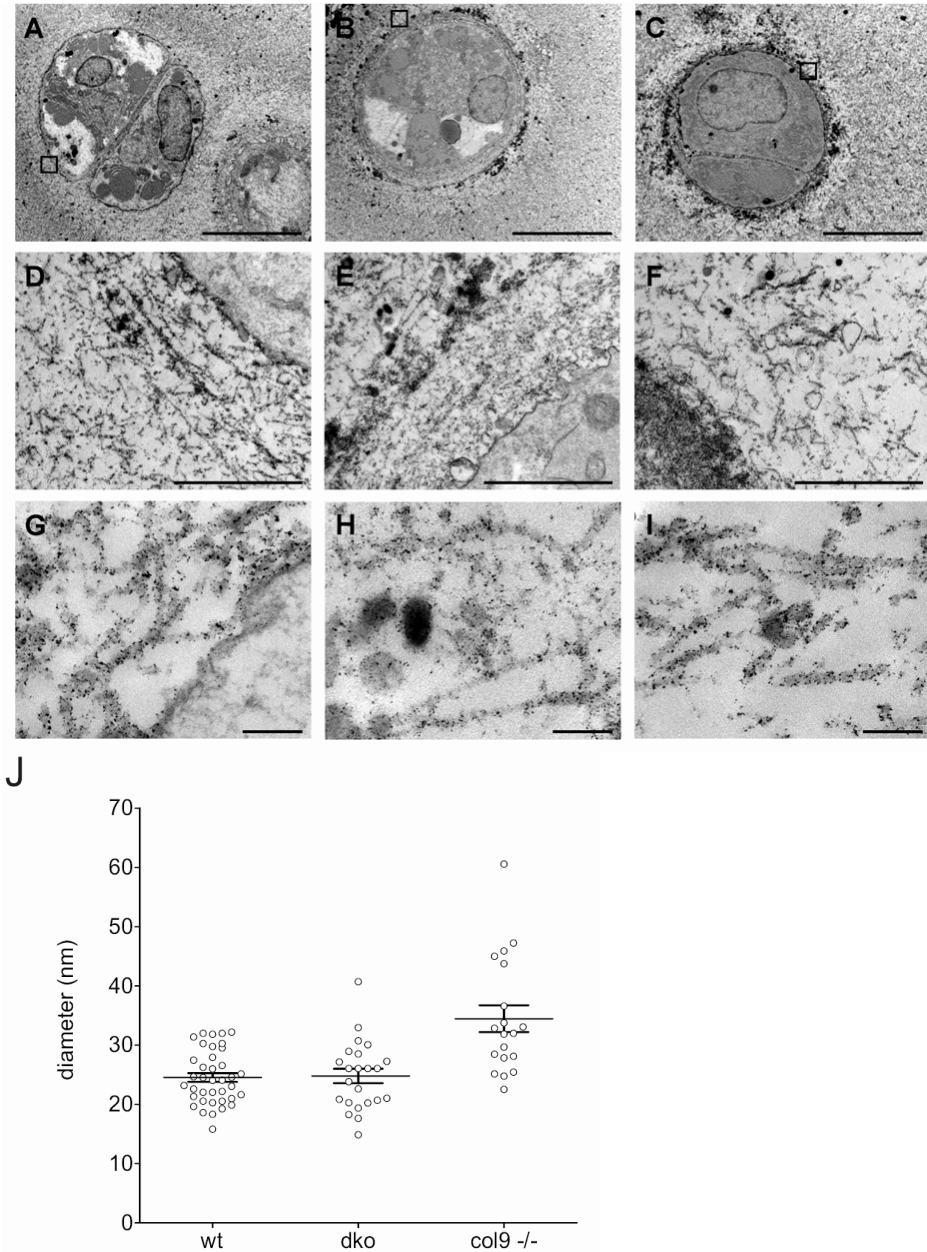
Figure 7.6. Electron microscopy of chondrocytes and collagen fibrils in alginate beads cultured for three weeks. Alginate beads containing wildtype (A, D, G), collagen IX/COMP double-knockout (B, E, H), or collagen IX single knockout chondrocytes (C, F, I) were submitted to electron microscopy and micrographs taken of different magnifications (A-C, bar = 10μm; D-F, bar = 2μm; G-I, bar = 200nm). The rectangles in the low-magnification electron micrographs indicate the areas analysed in higher magnifications. (J) The diameter of individual collagen fibrils formed by wildtype, collagen IX/COMP double deficient or collagen IX deficient chondrocytes, represented as mean ± SD.

collagen IX in alginate beads under identical conditions. In agreement with earlier studies on intact cartilage, electron microscopy of such cultures showed a less organized matrix and a significantly larger fibril diameter than in either wildtype or collagen IX/COMP double deficient cultures.

Discussion

Even though the exact functions of collagen fibril-associated proteins, such as collagen IX COMP and matrilins, are far from clear, as a group these proteins are thought to be critically important for skeletal development and presumably for the development of musculoskeletal diseases. Mice lacking collagen IX have an increased collagen fibril diameter and a loss of matrilin-3 anchorage [268], whereas COMP deficient mice have no similar defects in matrix assembly or fibril diameter [269]. Until now it has been unclear whether the loss of both collagen IX and COMP enhances the matrix assembly phenotype observed in collagen IX deficient animals and if collagen IX and COMP play compensatory or antagonistic roles during matrix assembly.

First, we addressed the anchorage of matrix proteins *in vivo*. Immunofluorescence staining confirmed the absence of collagen IX and COMP in double deficient cartilage. Collagen II deposition was not visibly altered in the rib cage of double deficient mice. In contrast, matrilin-3 was lost from most cartilaginous tissues of double deficient animals, similar to what was previously reported for mice deficient in only collagen IX [268]. Retention of matrilin-3 was found in the zone between adjacent sternal growth plates as well as in calcified cartilage, indicating the presence of new interaction partners within these regions of collagen IX/COMP deficient tissue. In agreement with the lost matrilin-3 staining, the protein was more easily extracted from the tissue. Differences in the deposition of aggrecan were not detected by glycosaminoglycan analysis of mouse cartilage extracts and histological sections through the rib cage of wildtype and double deficient animals revealed no obvious malformations. This is different from the tibial growth plate cartilage, where hypocellular areas and impaired chondrocyte alignment was observed [263]. Apparently, different types of cartilage have different requirements for collagen fibril-associated proteins.

To analyze matrix assembly and protein deposition in greater detail, we employed both short- and long-term cell culture systems. In short-term chondrocyte cultures, matrilin-3 deposition was clearly influenced by the loss of collagen IX and COMP. In earlier studies employing collagen IX deficient cells, matrilin-3 was secreted into the medium instead of being incorporated into the matrix [268]. However, if also COMP is ablated, amorphous matrilin-3 aggregates bind to the surface of the culture dish. A strong interaction between matrilin-3 and COMP has been described [270]. While matrilins

show poor solubility, COMP is highly hydrophilic. It is likely that complex formation between matrilin-3 and COMP increases matrilin-3 solubility in the cell culture supernatant and prevents deposition on the surfaces. In tibiae of collagen IX/COMP deficient mice [263], matrilin-3 was amorphously deposited in the central region of the epiphyseal cartilage, a phenomenon that was not seen when only collagen IX was lacking. This indicates that COMP may act as a carrier for matrilin-3 also *in vivo*. An intense collagen II staining was detected in the matrix of both wildtype and double deficient chondrocytes, showing the assembly of pericellular collagen fibrils regardless of genotype. Matrilin-3 binds with low affinity directly to collagen II *in vitro* [270], but apparently collagen II alone can not provide adequate tissue anchorage for matrilin-3 if collagen IX and COMP are missing. In agreement with the unchanged *in vivo* proteoglycan solubility, the amount deposited in monolayer culture did not differ between wildtype and double deficient cells. In monolayer cultures, the amount of matrix formed is limited and differences in aggrecan retention within this matrix may be difficult to detect.

We also employed a three-dimensional cell culture model. This culture system can be used for longer-term experiments as it ensures phenotypic stability of chondrocytes [271-273]. Again, matrilin-3 was less well anchored in the matrix produced by double deficient cells and was instead released into the supernatant. Similarly, less proteoglycans were present within the matrix. As COMP can bind to aggrecan [56], the main aggregate-forming proteoglycan in cartilage, the loss of COMP might result in decreased aggrecan anchorage, an effect that could be enhanced by the secondary loss of matrilin-3, as matrilins may link aggrecan to microfibrillar networks [274]. This effect was not detected *in vivo*, probably due to the high density of collagen fibrils that limits aggrecan diffusion and thereby compensates for the decreased matrix anchorage. The matrix assembled in alginate beads appears less dense and allows the detection of differences in proteoglycan retention. Matrilin-3 is smaller and can diffuse out of both types of matrix if anchorage to collagen IX and COMP is lost. The distribution of proteoglycans between pericellular and interterritorial matrix was not altered between genotypes and the total amount of collagen, mainly collagen II [272], did not differ significantly. However, the deposition of collagen was shifted from the cell-associated matrix towards the further removed matrix. Accordingly, the ratio between aggrecan and collagen decreases, particularly in the interterritorial matrix. These clear differences in matrix organization did not lead to detectable changes in mechanical properties.

Despite the compositional differences in cartilage matrix formed in the absence of collagen IX and COMP, electron microscopy revealed no obvious changes in the diameter of the collagen fibrils formed. This was surprising as an increased fibril diameter was found in mice lacking only collagen IX [268]. This increase was also observed in the matrix of collagen IX deficient chondrocytes cultured in alginate. Collagen IX is thought to limit lateral fibril growth by binding to the surface of the growing fibril [20]. On the other hand,

COMP has been shown to act as a catalyst of *in vitro* collagen fibrillogenesis [275], suggesting that COMP may also function to modulate fibril growth *in vivo* [276]. Fibrils formed in the absence of COMP show a more heterogenous diameter than in its presence [275]. Our studies show that, when both proteins are absent, fibrils of normal diameter are formed. These antagonistic effects of collagen IX and COMP in fibril assembly imply that the relative abundance of these proteins may regulate collagen fibril diameter *in vivo*.

Possibly, disturbances in secretion and/or function of collagen IX and COMP, as seen in human multiple epiphyseal dysplasia or pseudoachondroplasia, can affect collagen fibril assembly and through the resulting matrix reorganization promote the development of the osteoarthritis connected with these disorders. The synthesis, degradation and assembly of extracellular matrix components can be influenced by growth factors and cytokines that are known to be produced in osteoarthritic joints such as TGFβ [188, 277] Although collagen II has been most extensively studied in this regard, an increasing body of work now points to an effect of these cytokines on COMP production and degradation [256, 278, 279]. It is less clear how growth factors affect collagen IX expression. The observed changes in the deposition of matrix proteins caused by the loss of collagen IX and COMP point to that these in turn have regulatory influence on cartilage matrix assembly and may thereby influence the susceptibility for cartilage degeneration.

Appendix Chapter 7: comparing dko chondrocytes with COL9 single knock-out chondrocytes

Next to rib chondrocytes from double knock-out mice analyzed in chapter 7, rib chondrocytes from collagen type 9 (COL9) deficient mice were cultured and analyzed for biochemical parameters. In addition, chondrocytes from knee joints of newborn wild type mice, COL9 knock-out mice, and DKO mice were cultured and analyzed for GAG deposition and distribution, collagen deposition and distribution, and collagen cross-linking.

From these data we conclude that chondrocytes isolated from ribs and knees do not behave differently. Furthermore, missing both COMP and collagen type IX during matrix production does not seem to alter matrix production and distribution then when only collagen type IX is missing. The lack of collagen type IX alone even seems to have more effect then when both matrix molecules are missing, although this is not significant due to n=3.
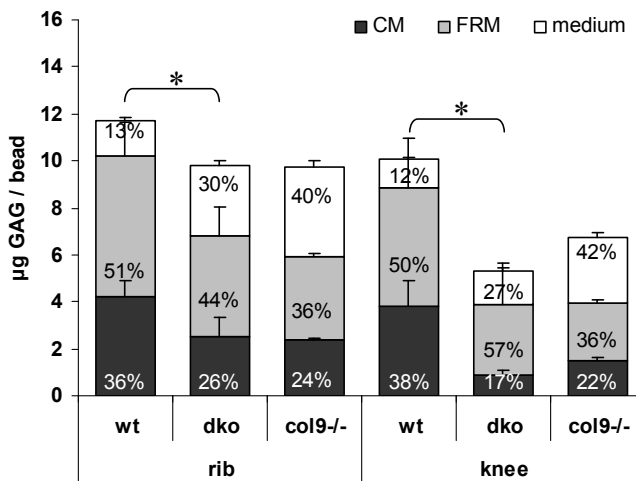
Figure 7.7. GAG distribution between cell-associated matrix (CM, black), further removed matrix (FRM, grey) and culture medium (white) in alginate cultures of wild type (wt) chondrocytes, chondrocytes deficient for collagen type IX (col9-/-) and chondrocytes deficient for collagen type IX and COMP (dko), all from mouse rib and knee origin. Distribution is shown as absolute production per alginate bead and as relative percentages. N=6 for wt and dko, N=3 for col9-/-. * indicates significant difference in absolute production and relative distribution.
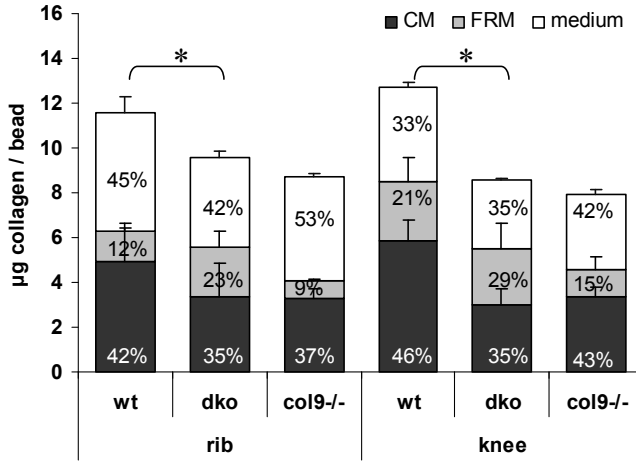
Figure 7.8. Collagen distribution between cell-associated matrix (CM, black), further removed matrix (FRM, grey) and culture medium (white) in alginate cultures of wild type (wt) chondrocytes, chondrocytes deficient for collagen type IX (col9-/-) and chondrocytes deficient for collagen type IX and COMP (dko), all from mouse rib and knee origin. Distribution is shown as absolute production per alginate bead and as relative percentages. N=6 for wt and dko, N=3 for col9-/-. * indicates significant difference in absolute production and relative distribution.
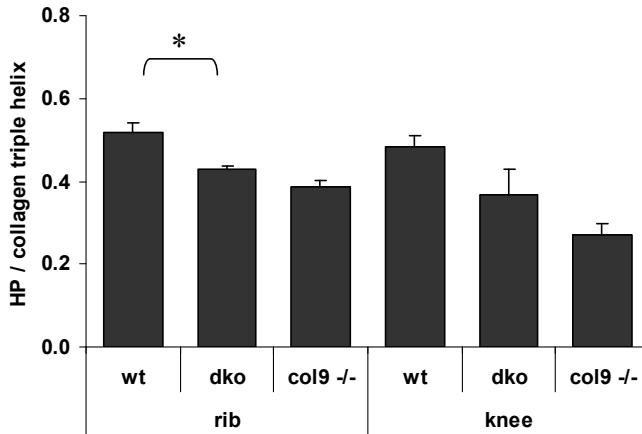
Figure 7.9 Collagen cross-linking in alginate cultures of wild type (wt) chondrocytes, chondrocytes deficient for collagen type IX (col9-/-) and chondrocytes deficient for collagen type IX and COMP (dko), all from mouse rib and knee origin. Cross-linking is shown as average number of HP cross-links per collagen triple helix ± sd. N=6 for wt and dko, N=3 for col9-/-. * indicates $p < 0.05$.

8

Proteoglycan production is required in initial stages of new cartilage matrix formation but inhibits integrative cartilage repair

Yvonne M. Bastiaansen-Jenniskens, Wendy Koevoet, Carola Feijt, P.Koen Bos, Jan A.N. Verhaar, Gerjo J.V.M. VanOsch, Jeroen DeGroot

Abstract

Objective

The optimal stimulus to repair or regenerate cartilage is not known. We therefore modulated collagen deposition, collagen cross-linking and GAG deposition simultaneously during cartilage matrix production and integrative repair, creating more insight in their role in cartilage repair processes.

Methods

Insuline-like Growth Factor 1 (IGF-1, increases proteoglycan and collagen synthesis), Beta-aminopropionitrile (BAPN, a reversible inhibitor of collagen cross-linking) and para-nitrophenyl-beta-d-xyloside (PNPX, interferes with proteoglycan production) were used. Bovine articular chondrocytes were cultured in alginate beads for three weeks with or without IGF-1, BAPN or PNPX alone and in all possible combinations followed by three weeks in control medium. DNA content, GAG and collagen deposition and collagen cross-links were determined. Cartilage constructs were cultured under same conditions and histologically analyzed for integration of two opposing cartilage matrices.

Results

In alginate cultures, inhibition of collagen cross-linking with BAPN in combination with promotion of matrix synthesis using IGF1 was most beneficial for matrix deposition. Addition of PNPX was always detrimental for matrix deposition. For integration of opposing cartilage constructs, the combination of BAPN, IGF1 and temporary prevention of proteoglycan formation with PNPX was most beneficial.

Discussion

When a new matrix is produced, proteoglycans are important to retain collagen in the matrix. When two already formed cartilage matrices have to integrate, temporary absence of proteoglycans and temporary inhibition of collagen cross-linking might be more beneficial in combination with stimulation of collagen production by for example IGF1. Therefore, the choice of soluble factors to promote cartilage regeneration depends on the type of therapy that will be used.

Introduction

Once damaged, adult articular cartilage has a poor repair capacity, which is probably due to the ineffective repair of the collagen network, since proteoglycan depletion is often reversible [142]. Although collagen turnover is increased in osteoarthritis (OA), this does not lead to the formation of a functional network [26, 68, 96, 143]. This suggests an activated repair mechanism in OA that appears however ineffective in repairing or maintaining the ECM homeostasis.

Several strategies are under investigation to promote matrix regeneration in cartilage repair. Growth factors are used to modulate matrix production, by directly adding them to chondrocytes in culture [123, 280] or by inducing over expression of the growth factor of interest [281, 282]. For example, we and others found that addition of IGF1 to chondrocytes in culture resulted in more proteoglycans and collagen than in the control condition without IGF1 [159, 168, 169, 188]

In addition to growth factors, modulation of collagen network formation is also employed to understand cartilage matrix formation and functionality, for example by the inhibition of collagen cross-linking by inhibition of lysyl oxidase (LOX) with β-aminopropionitrile (BAPN). It was found that collagen cross-links are important for the integrative repair and adhesive strength of cartilage [283] and that transient inhibition of the formation of these cross-links improved integrative repair and collagen cross-link maturation [284]. In cultures of chondrocytes in alginate, inhibition of collagen cross-link formation with BAPN resulted in an increase of collagen production [163, 208, 230]. In concordance with earlier explant studies [284], transient inhibition of LOX in alginate cultures resulted in accelerated cross-link maturation and improved functionality of the newly formed matrix [230].

The influence of glycosaminoglycans (GAGs) on cartilage growth and matrix production is also under investigation, but gets less attention than collagen. GAG depletion in cartilage explants resulted in less expansive growth and a more mature matrix with increased tensile integrity [141]. Preventing GAGs from binding to the proteoglycan core protein with para-nitrophenyl xyloside (PNPX) in the newly forming cartilage matrix of chondrocytes cultured in alginate resulted in less collagen deposition and a decrease in stiffness and ability to hold water [285]. The difference between these two studies is that in the explant study collagen was already deposited and cross-linked, whereas in the alginate cultures no collagen was yet deposited when GAG incorporation was inhibited. Both studies however indicate that modulating GAGs can influence the collagen network in an already existing cartilage matrix or during new cartilage matrix synthesis.

Most of the approaches mentioned above focused on modulating one matrix component (either collagen or proteoglycans). However, increasing collagen synthesis alone is not sufficient for cartilage repair. Because of the differential and potentially

complementary effects of these approaches, our hypothesis was that combining IGF1, BAPN and PNPX in one experiment has the potential to stimulate matrix production and integrative repair more than each single component. Chondrocytes cultured in alginate were used to examine the effect of the soluble factors on new matrix production, either alone or in all possible combinations. Cartilage explants were used to examine the effect of the factors mentioned above on integration of two existing cartilage matrices.

Materials and Methods

Cell culture

Articular cartilage was harvested from the metacarpophalangeal joints of calves aged 6–12 months. Chondrocytes were isolated, suspended in alginate in a concentration of 4 x 10^6 cells per ml of alginate, and alginate beads were made as described previously [188]. Beads were cultured in a six-well plate (BD Falcon, Bedford, MA, USA), with 75 µl/bead DMEM/F12 supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL). Chondrocytes were cultured for 21 days in the presence of 25 ng/ml IGF-1, 0.25 mM BAPN and/or 0.25 mM PNPX based on previous results [211, 230, 286] followed by 21 days in control medium. As known from these previous studies, BAPN inhibits collagen cross-linking and PNPX prevents incorporation of GAGs into the matrix. In the 21 days of additional culture without supplements, cross-links had the ability to form and GAGs to incorporate into the matrix. Culture medium was changed three times a week. Alginate beads were harvested after 42 days of culture.

Preparation of cartilage explants for integration study

Articular cartilage samples were harvested from the metacarpophalangeal joints of calves aged 6–12 months. Full-thickness cartilage explants of 8 mm diameter and with a thickness of 0.9–1.2 mm were prepared using a dermal biopsy punch and scalpel. From the centre of the explants, 3-mm cores were punched out. All samples (both outer ring and inner core) were incubated for 24 hours in 10 U/ml highly purified collagenase VII (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in DMEM-F12 with 10% fetal calf serum based on previous results [287-289]. From the study of Bos et al [287], it was concluded that there is some loss of matrix proteins by digesting for 24 hours with highly purified collagenase, but only on the locations where proteoglycans are lost, i.e. the area of the wound edge. Pre-treatment with collagenase is beneficial for integration because it increases the number of viable cells at the wound edge thereby enabling integration of the wound edges. All the cartilage explants were pre-treated with the collagenase irrespective of the condition. After incubation, the samples were washed

three times for 10 min in culture medium, and the 3-mm inner cores were reimplanted in their accompanying 8-mm outer rings. Constructs were cultured in parallel to the alginate beads for 21 days in the presence of 25 ng/ml IGF-1, 0.25 mM BAPN and/or 0.25 mM PNPX followed by 21 days in control medium. Constructs were cultured in 1.5 ml DMEM/F12 per construct supplemented with 10% fetal bovine serum (GibcoBRL), 50 µg/ml L-ascorbic acid 2-phosphate (Sigma), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (both GibcoBRL) in the presence of 25 ng/ml IGF-1, 0.25 mM BAPN and/or 0.25 mM PNPX during the first 21 days. After 42 days, constructs were harvested an immediately fixed in 4% phosphate buffered formalin.

Biochemical analysis of alginate beads

Alginate beads were digested overnight at 56°C in papain buffer (250 µg/ml papain in 50 mM EDTA and 5 mM L-cystein). Glycosaminoglycan (GAG) amount in the digest was quantified using dimethylmethylene blue (DMB) assay [160]. The metachromatic reaction of GAG with DMB was monitored with a spectrophotometer, and the ratio A530:A590 was used to determine the amount of GAG present, using chondroitin sulfate C (Sigma) as a standard. The amount of DNA in each papain-digested sample was determined using ethidium bromide with calf thymus DNA (Sigma) as a standard. High-performance liquid chromatography (HPLC) of amino acids (hydroxyproline, Hyp) and collagen cross-links (hydroxylysylpyridinoline, HP) was performed as described previously [162, 163]. The quantities of cross-links were expressed as the number of residues per collagen molecule, assuming 300 Hyp residues per collagen triple helix. Three samples were taken of 7 alginate beads per experimental condition for biochemical analyses.

Histochemical analysis of cartilage explants

Formalin fixed cartilage constructs were embedded in paraffin. To prevent any negative influence of processing the constructs for histological analysis, separate cartilage constructs were placed in little porous polymer bags directly after harvesting prior to fixation in formalin and processing. However, a certain risk of damage during histological procedure is unavoidable. Therefore we embedded all the samples at the same time and did the sectioning in a random order to exclude bias. Sections (6 µm) were cut using a standard microtome. Prior to the histological stainings, sections were deparaffinated in xylene and rehydrated through graded ethanol. For histological evaluation, serial sections were stained with Haematoxylin & Eosin (H&E). To evaluate integration, paraffin sections were stained with a thionine staining. For each sample we assessed the percentage of total interface length that had a matrix–matrix connection. A clear distinction could be made between parts with a matrix connection and parts of the cartilage touching each other but without a clearly connected matrix, which were scored as parts with a gap. Integration was determined at both integration sites within a paraffin

section. Interface integration percentages were obtained by two blinded observers from measurements of three to four different sections from each sample, resulting in an average value for each interface.

Statistical analysis

For the alginate bead cultures and the integration study, four pooled cartilage donors were used. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) software. All data are presented as mean ± standard deviation. Control groups and groups supplemented with PNPX, BAPN and/or IGF1 were compared with a ANOVA test followed by a post hoc Bonferroni test.

Results

Chondrocytes cultured in alginate

After 42 days, DNA content, GAG deposition, collagen deposition and collagen cross-linking were determined in the alginate beads. In the control condition, DNA content was 0.49 ± 0.03 µg DNA per alginate bead, which was unaffected by the addition of BAPN, IGF1 and or PNPX during the first 21 days of culture (Figure 8.1).

4.38 ± 0.39 µg GAG per bead was deposited in the control condition after 42 days of culture. As expected, this was significantly reduced in the condition with 0.25 mM PNPX during the first 21 days. Compared to the control condition, GAG deposition was also less when a combination of PNPX and IGF1 was present, when PNPX and BAPN were present together, and when all three components were present in the culture medium.

Figure 8.1. DNA content of the alginate beads after 42 days of culture. PNPX (P), BAPN (B) and/or IGF1 (I) were present in the first 21 days of culture, followed by additional culture for 21 days in control medium. Data are shown as mean DNA content per bead ± sd. N=3.
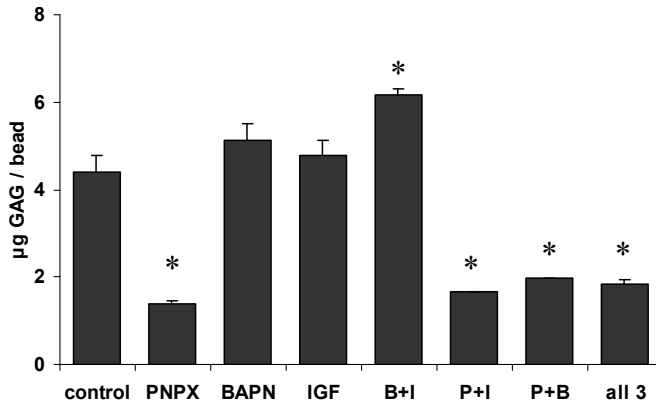
Figure 8.2. GAG content of the alginate beads after 42 days of culture. PNPX (P), BAPN (B) and/or IGF1 (I) were present in the first 21 days of culture, followed by additional culture for 21 days in control medium. Data are shown as mean GAG content per bead ± sd. N=3. * indicates a significant difference (p<0.05) from the control condition without additives.
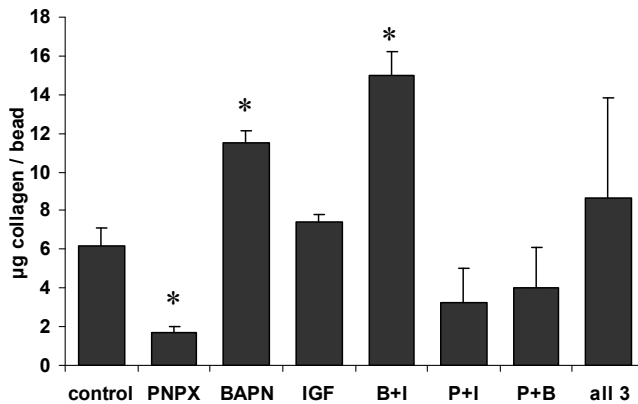
Figure 8.3. Collagen content of the alginate beads after 42 days of culture. PNPX (P), BAPN (B) and/or IGF1 (I) were present in the first 21 days of culture, followed by additional culture for 21 days in control medium. Data are shown as mean collagen content per bead ± sd. N=3. * indicates a significant difference (p<0.05) from the control condition without additives.

BAPN or IGF1 alone did not influence GAG deposition, rather their combination resulted in more GAG deposition (Figure 8.2).

Regarding the collagen deposition, 6.16 ± 0.91 µg was deposited in the control condition after 42 days. The presence of PNPX during the first 21 days resulted in less collagen deposition. Inhibition of collagen cross-link formation with BAPN on the other hand resulted in more collagen deposition, as expected. BAPN together with IGF1 increased the collagen deposition even more (Figure 8.3).

The presence of BAPN alone, BAPN with IGF1, BAPN with PNPX and all three factors during the first 21 days resulted in less cross-link formation, in line with the LOX
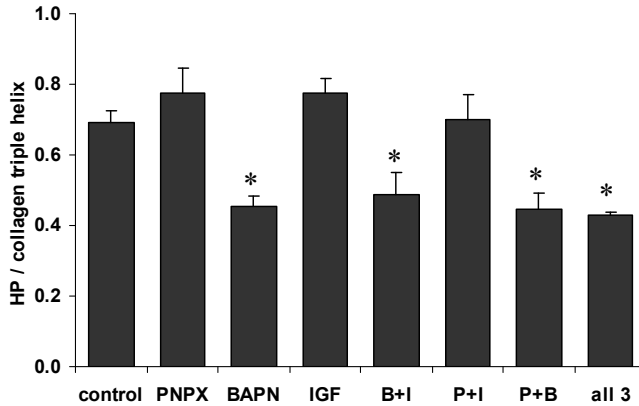
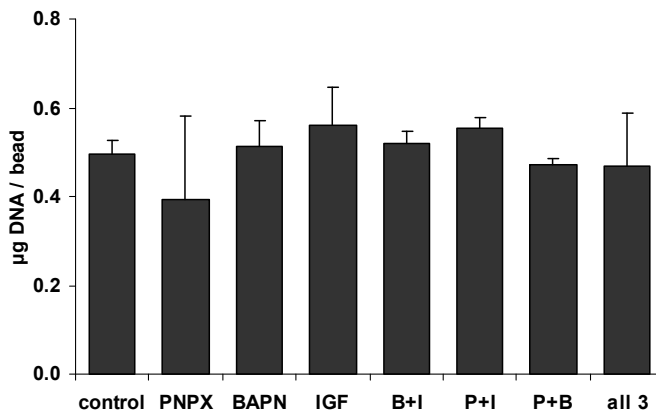**Figure 8.4.** Number of HP cross-links per collagen triple helix after 42 days of culture. PNPX (P), BAPN (B) and/or IGF1 (I) were present in the first 21 days of culture, followed by additional culture for 21 days in control medium. Data are shown as mean HP per collagen triple helix ± sd. N=3. * indicates a significant difference (p<0.05) from the control condition without additives.

inhibition by BAPN. Addition of IGF1 alone, PNPX alone or PNPX with IGF1 did not change the number of collagen cross-links from the control condition, which was 0.69 ± 0.03 HP per collagen triple helix (Figure 8.4).

Cartilage integration

In parallel to the alginate cultures, cartilage explants were cultured to investigate the integrative capacity in the presence of PNPX, BAPN and/or IGF1. As in the alginate cultures, soluble factors were only present during the first 21 days of culture followed by culture in control medium for an additional 21 days. Typical examples of integration after 42 days visualized with a thionine staining are shown in Figure 8.5. The average integration percentage in the control condition was less than 10%. Only when PNPX, BAPN and IGF1 were combined during the first 21 days, integration of the two opposing cartilage explants improved to 41.3 ± 31.4% (p < 0.05) (Figure 8.6). Although not reaching statistical significance, the presence BAPN and IGF1 alone or together also seems beneficial for integration.

Discussion

Many factors have been demonstrated to be able to influence proteoglycan production, collagen production or both. However, it is not fully known what the optimal balance of collagen and proteoglycan production is and how they influence each other to form a functional network.
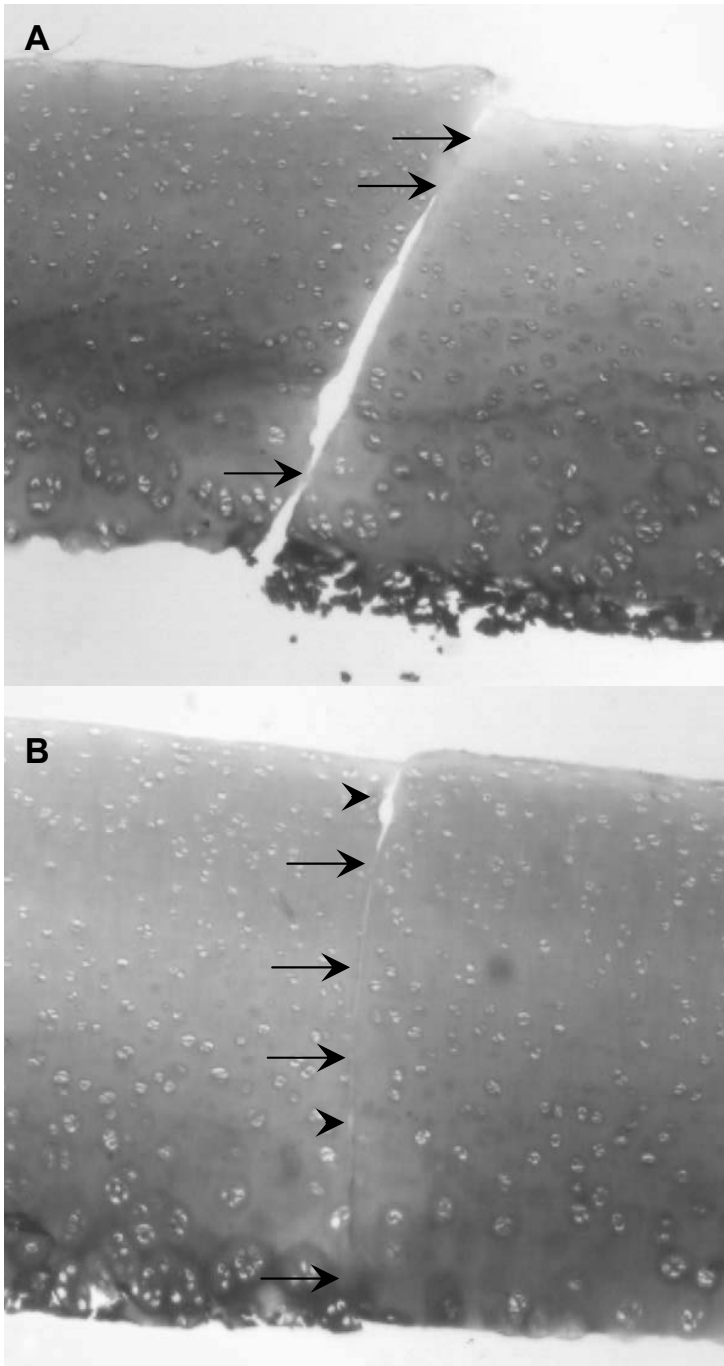
Figure 8.5. Examples of integration visualised with a thionine staining. A) 20% integration in a control sample, B) 70% integration in a sample where PNPX, BAPN and IGF1 were present for the first 21 days. Arrows indicate integration, arrowheads indicate no integration. Magnification is 40x.
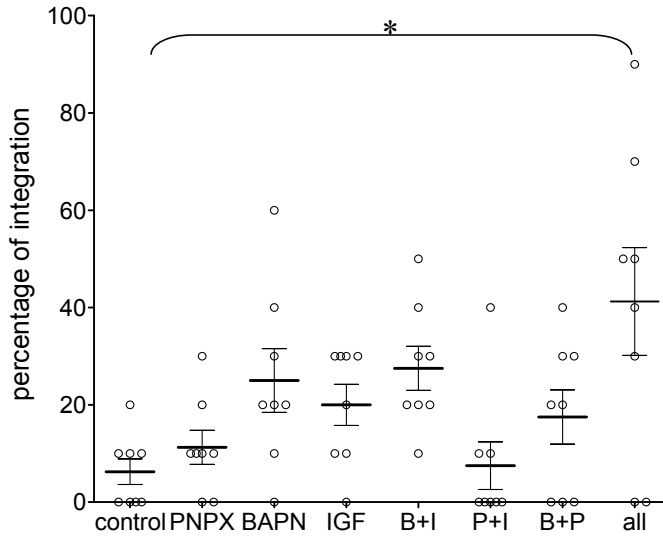
Figure 8.6. Percentage of integration of opposing cartilage tissue. PNPX (P), BAPN (B) and/or IGF1 (I) were present in the first 21 days of culture, followed by additional culture for 21 days in control medium. Data are shown as individual percentages with the mean percentage ± sd. N=8. * indicates a significant difference (p<0.05) from the control condition without additives.

Our goal was to examine the effect of modulating collagen deposition, collagen cross-linking and GAG deposition simultaneously during cartilage matrix production and cartilage integrative repair, thereby creating a wider insight in the role of proteoglycans and collagen in cartilage repair process.

In the alginate cultures, inhibition of collagen cross-linking with BAPN in combination with promotion of matrix synthesis using IGF1 was most beneficial for matrix deposition. Additional PNPX was always detrimental for matrix deposition. For integration, the combination of BAPN, IGF1 and temporary prevention of proteoglycan formation with PNPX was most beneficial.

Inhibition of GAGs from binding to the proteoglycan core protein not only resulted in less GAG deposition in the alginate bead, but also in less collagen deposition. This is in concordance to our previous study were the presence of PNPX during a culture period of 35 days resulted in less proteoglycan and collagen deposition in the alginate bead. In addition, excretion of both matrix proteins into the culture medium was higher due to the absence of an intact matrix network [285]. Because of the absence of this network and the loss of interaction with other matrix components, combining PNPX with BAPN and/ or IGF1 could not counteract the effects seen with PNPX alone. Earlier we found that the inhibition of collagen cross-linking in an alginate culture for 21 days and longer results in higher collagen deposition then when cross-links are formed. During this culture period, proteoglycan deposition was unaffected [230]. Even though cross-link inhibition

by BAPN stimulates collagen synthesis, the retention of collagen is very low because of the absence of a proteoglycan network. Previously, we and others showed stimulating effects of IGF1 on collagen and proteoglycan deposition [159, 168, 169, 188]. In the present setup, culture with IGF1 alone for 21 days did not significantly stimulate matrix deposition after 42 days, whereas IGF1 did previously already after 21 days [188]. However, the combination IGF1 and BAPN increased GAG deposition which is probably attributable to the presence of IGF1 since BAPN does not affect the GAG deposition. Even though IGF1 might have also stimulated GAG and collagen deposition when combined with PNPX or with PNPX and BAPN, GAGs were prevented from incorporation into the matrix. A possible positive effect of IGF1 on collagen synthesis was counteracted because of low collagen retention in the alginate bead. In every condition where the cross-link inhibitor BAPN was present for the first 21 days, less collagen cross-links were present even though the culture continued for 21 days after removal of BAPN. This might be explained by the fact that after removal of BAPN, it takes three weeks for mature HP cross-links to be formed [222]. In addition, BAPN binds irreversibly to lysyl oxidase (LOX) [212] and therefore extra time is also needed for LOX to be produced again. Based on our previous experiments, we expect that the number of collagen cross-links will be equal in all conditions when a longer culture period is used [230].

The combination of BAPN and IGF1 seems most beneficial for matrix synthesis when no matrix is formed yet, as in the alginate cultures. When aiming at the integration of two existing cartilage matrices, cross-link inhibition with BAPN and stimulation of matrix production with IGF1 is not sufficient to significantly improve integration. Transient inhibition of GAG incorporation in the matrix with PNPX in combination with BAPN and IGF1 did improve the integration. In these experiments, stimulation of matrix production alone with IGF1 had no effect. Transient inhibition of collagen cross-link formation could have resulted in more collagen synthesis, and in addition in better penetration of the newly formed collagen fibers in the opposing tissue that was partly depleted of GAGs following injury [290]. The latter is more likely since previous experiments with BAPN and cartilage explants did not result in more collagen synthesis [284]. The study by McGowan et al is also in concordance with the theory of better integration after BAPN treatment. The addition of PNPX and the resulting absence of GAG attachment to the proteoglycan core protein might have similar effects as seen with inhibition of cross-link formation. Without an intact proteoglycan network at the edges of the cartilage explants, newly formed collagen fibers might penetrate better into the opposing cartilage tissue. Because of the absence of a proteoglycan network, the collagen network might also have a better integrity [141]. This explains why the presence of IGF1 with PNPX or BAPN did not improve integration since the newly formed collagen stimulated by IGF1 could not penetrate into the cartilage. PNPX together with BAPN but without IGF1 was also not sufficient because of the absence of increased collagen production.

When cartilage transplantations are performed, the surgeon removes the damaged cartilage first. Removing the damaged cartilage will create a fresh wound area, similar to our experimental condition. We hypothesize that the integrative capacity of such an area is different from a long lasting wound area and we are therefore convinced that our explant model is close to the application in this respect.

The cartilage explants used for integration were cultured for 6 weeks in vitro. In an earlier study, we investigated the effect of improved integration on mechanical properties of the cartilage-cartilage interface [289]. There we observed a relation between the area of integration on histology and the mechanical properties of the interface. Since the previous study is performed after an in vivo culture period and our current study evaluates after in vitro culture, it is difficult to extrapolate these results. Earlier attempts in the lab have demonstrated us that after in vitro culture the bonding is not strong enough to measure reliably in our system. However, the improved histological integration in the present studies looks promising and suggests that when a construct is placed in vivo after modulation with our soluble factors (IGF1, PNPX and BAPN), integration might lead to functional cartilage.

Conclusions

Based on these results, combining IGF1 and cross-link inhibition with BAPN seems to be most promising in promoting the formation of a new cartilage matrix. When a new matrix is produced, proteoglycans are important to retain collagen in the matrix. When two already formed cartilage matrices have to integrate, the temporary absence of a proteoglycan network and the temporary absence of collagen cross-links might be more beneficial in combination with stimulation of collagen production by for example IGF1. Therefore, the choice of soluble factors to promote cartilage regeneration in OA depends on the type of therapy that will be used. In the case of cell therapy and the synthesis of a new matrix, stimulating both collagen and proteoglycan synthesis is important. The intra-articular administration of growth factors such as IGF1 to promote matrix production is the topic of ongoing research [291] and could be a part of future OA therapy. When two cartilage matrices have to integrate, temporary inhibition of proteoglycan network formation and collagen cross-linking together with stimulating collagen production results in better integration. In the case of autologous cartilage transplantation, the transplant can be pre-treated with hyaluronidase or chondroitinase to remove the proteoglycans and be subjected to temporary collagen cross-link inhibition. To generate a tissue engineered cartilage construct, culture in the presence of IGF1 and BAPN might be a promising approach. However, care must be taken with BAPN since it is mutagenic. Other approaches to inhibit collagen cross-linking might be the topic of future research.

9 Summary and General Discussion

Osteoarthritis (OA) is a complex disease of which the ethiopathology is not completely known. OA is a common, chronic, musculoskeletal disorder. Symptomatic osteoarthritis, particularly of the knee and hip, is the most common cause of musculoskeletal disability in elderly people. In the Western world it ranks fourth in health impact among women and eighth among men [292]. Given this high prevalence, therapeutic approaches for treatment are desired. However, treatment options recommended only consist of non-pharmacological and pharmacological interventions aiming for pain relief [128-130, 293]. Attempts are made to develop disease modifying osteoarthritic drugs (DMOADs). Glucosamine is claimed to have structure modifying effects on articular cartilage damaged in OA [294], although the effect is still under discussion [295, 296]. Anti-MMP, bisphosphonates and IL-1 receptor antagonist (IL-1ra) were proposed as possible DMOADs but had many side effects or had no or negative effects on OA development [297-299]. Since most currently available treatments are palliative, only effective in mild OA, and structure modifying compounds are still not proven to be effective, total joint replacement is still an often-performed procedure in end stage OA patients. Therapies to actually repair cartilage are thus still under investigation. The difficulty with cartilage repair and regeneration is the insufficient extracellular matrix production, especially collagen type II. The increase of collagen type II expression in osteoarthritic cartilage [95, 96] suggests an activated repair mechanism that is, however, ineffective in repairing or maintaining the ECM homeostasis. On a immunohistochemical level, it often appears that collagen type II and proteoglycans are present in high amounts because of a uniform and intense staining. However, biochemical quantification of tissue-engineered constructs reveals that both matrix constituents, but especially collagen type II, are present in very low amounts when compared to native articular cartilage [146]. This lead us to investigate the ability to modulate the formation of a functional collagen type II network that can ultimately contribute to innovation of cartilage repair in OA.

Influence of soluble factors on collagen deposition and functionality

By using growth factors known to play a role in cartilage matrix homeostasis and development, we found in chapter 2 that matrix production and especially collagen production can be modulated in vitro. Differential effects were shown of IGF1, FGF2 and TGFβ2. Our quantitative analysis shows beneficial effects of IGF on cartilage regeneration. Collagen cross-linking was not affected, mechanical properties improved, and MMP-1 gene expression downregulated.

The use of IGF1 was previously studied in animal models for cartilage repair. Histological analysis of these animal models showed that administration of IGF1 resulted in better cartilage morphology, including more collagen type II staining [259, 291, 300]. From

our biochemical data and the in vivo experiments, it can be concluded that IGF1 has disease-modifying potential in osteoarthritis.

FGF2 in the low concentration resulted in a small increase of matrix deposition, in contrast to the high concentration that resulted in no effect on proteoglycan deposition and less collagen deposition than in the control condition. Less collagen deposition resulted in a trend towards worsened functionality since the number of cross-links per collagen triple helix was comparable to the control condition. In animal models, FGF2 appears to have beneficial effects on cartilage repair [282, 301]. Even though cartilage morphology seems to improve when FGF2 is administered to cartilage lesions in vivo, our quantitative analysis shows the opposite. This might be explained by the positive effect FGF2 has on the phenotype of chondrocytes [121, 178] resulting in a different col2/col1 ratio, or the difference between histological analysis (in the in vivo studies) and biochemical analysis (in our studies) [146]. From our biochemical data, it can be concluded that FGF2 is less suitable for repair of the cartilage matrix.

Our experiments with TGFβ on primary calf chondrocytes resulted in slightly less matrix deposition. In general, intra-articular injection of TGFβ leads to an increase in proteoglycan content in articular cartilage although contrasting results also have been reported (see [302] for review). The positive effects seen in the in vivo studies might be explained by the counteracting effect of TGFβ on IL-1 induced matrix degradation [117, 303] and less by the stimulation of matrix production by TGFβ. Matrix incorporation into the existing cartilage matrix could be more effective because of less degradation in the presence of TGFβ. Earlier studies from our group showed that chondrocytes from immature cartilage were not sensitive to TGFβ, whereas addition of TGFβ to chondrocytes from more mature cartilage resulted in more proliferation and more GAG production [304]. The chondrocytes used in our studies were isolated from newborn calves. It is possible that these were less sensitive to TGFβ addition, which also explains the discrepancy between our results and some in vivo results. On the other hand, TGFβ stimulates proteoglycan synthesis more actively in OA than in healthy cartilage [305]. This is also an explanation for the difference between our results in healthy chondrocytes and the often seen stimulation of PG synthesis after TGFβ administration in other studies with in vivo OA models.

Next to the small effects on matrix deposition, TGFβ had striking effects on collagen cross-links; addition of TGFβ to chondrocytes in alginate resulted in less collagen cross-links. This was in contrast to our expectation since TGFβ is known to be a potent inducer of fibrosis in tissues such as skin and synovium [135, 193] which is characterized by an increase of HP cross-links [191, 220]. Therefore, we decided to study the effect of TGFβ in more detail in chapter 3. Several comparisons were made: chondrocytes versus fibroblasts, culture in alginate versus culture in monolayer on plastic, but also primary cells versus expanded cells since expanded chondrocytes display a fibroblast-like phenotype. Only expanded chondrocytes and fibroblasts deposited more collagen in reaction to

TGFβ addition, independent of the culture substrate and the type of collagen produced. Surprisingly, addition of TGFβ resulted in less HP cross-links in the alginate bead conditions and in more HP cross-links in the monolayer conditions, both independent of cell type or passage number. Contractile stress, as determined with a immunocytochemistry staining for α Smooth Muscle Actin, experienced by the cells in monolayer probably contributed to this effect. This lead us to conclude that the effect of TGFβ on collagen amount is dependent on cell type whereas the effect of TGFβ on cross-linking is independent of cell type but strongly dependent on the environment.

The difference in environment and how it affects the response to TGFβ might explain the positive effects seen on cartilage and the negative effects seen in synovium after intra-articular administration of TGFβ. Synoviocytes have a fibroblast-like shape and are stretched out on the collagen fibers of the synovial ECM. This is in contrast to the chondrocytes in the cartilage. Here, cells are round with a surrounding matrix of small collagens and proteoglycans. Therefore, cells in the synovium probably experience more contractile stress than cells in the cartilage, which results in more collagen cross-links in the synovium and less in the cartilage in response to TGFβ.

In addition, evidence exists that chondrocytes have an altered or dedifferentiated phenotype in OA. Based on our results, TGFβ could also induce collagen synthesis in OA, since only dedifferentiated/expanded cells deposited more collagen in response to TGFβ. This is in concordance with the study of Lafeber *et al* [305] where TGFβ stimulated OA chondrocytes more than healthy chondrocytes considering PG synthesis. Together with the beneficial properties of TGFβ in counteracting IL-1 mediated matrix degradation, this growth factor is also a promising target for modification of OA.

To examine the influence of reduced collagen cross-links in cartilage repair, the key enzyme involved in cross-linking (LOX) was temporarily inhibited in chondrocyte alginate cultures with the addition of BAPN in chapter 4. Inhibition of cross-link formation probably resulted in a higher collagen deposition, due to more collagen diffusion in the alginate bead and less chondrocyte-collagen interaction [163]. Initially, the increase in collagen deposition resulted in an improvement of matrix stiffness even without the presence of collagen cross-links. However, when the collagen deposition increased even more without cross-links present in the collagen, stiffness remained comparable to the control condition. A transient inhibition of collagen cross-linking resulted in more collagen that was cross-linked after removal of the cross-link inhibitor. This led to an improvement of matrix stiffness compared to the control condition and the condition cultured for the entire period with cross-link inhibition. Next to the amount of collagen, cross-links were required to improve matrix stiffness. The absence of cross-links also negatively influenced the susceptibility to MMP-1 degradation of the collagen matrix and the ability to hold water. These data confirm that collagen cross-links are needed for the functionality of cartilage. Not only to withstand mechanical loading but also

to withstand matrix degradation. This indicates that inducing collagen cross-linking in vivo has potential to prevent cartilage damage. Inducing collagen cross-linking might even stimulate cartilage repair in OA, since collagen synthesis is upregulated in OA but ineffective in maintaining matrix integrity.

Matrix constituents and the cartilage network

Collagen type II is the most abundant protein present in articular cartilage and interacts with a large variety of other molecules such as proteoglycans. Next to the importance of the collagen network in retaining proteoglycans in the cartilage matrix, the absence of a proteoglycan network also affects collagen network (chapter 5). By preventing the addition of glycosaminoglycan side chains to the proteoglycan core protein, collagen diffused further away from the chondrocyte and even into the culture medium, consequently worsening mechanical functioning of the matrix.

In OA, a large variety of matrix degrading enzymes are upregulated including aggrecanases [306]. Not only do they directly affect the proteoglycan content of the articular cartilage, but they possibly can also indirectly influence the collagen content as suggested by our data. This might explain the ineffective cartilage repair even though collagen synthesis is upregulated in early OA.

Next to proteoglycans, the pentameric protein COMP interacts with collagen type II in the cartilage matrix. The effect of growth factors on production of other matrix components next to collagen type II and proteoglycans is studied in chapter 6, where we focused on COMP deposition and distribution in reaction to the growth factors examined earlier. By adding TGFβ to chondrocytes in alginate, COMP gene and protein expression largely increased. FGF2 decreased COMP expression and possibly even resulted in COMP degradation. IGF1 had no effect on COMP expression. The distribution of COMP, collagens, and proteoglycans between the cell associated matrix and the further removed matrix indicates that COMP was not specifically associated with either the collagen or the proteoglycan component.

Because growth factors had many other effects next to influencing COMP deposition (as shown in chapter 2 and 3), COMP was also overexpressed in chondrocytes using a lentiviral overexpression system. This resulted in much more COMP production by the chondrocytes but only small effects on COMP incorporation in the cartilage matrix. This had no effect on matrix deposition, collagen cross-linking and mechanical properties. However, induction of COMP production with TGFβ or COMP lentivirus both resulted in collagen fibrils with a smaller diameter. Our results indicate that COMP is not a limiting factor in cartilage matrix production in these *in vitro* experiments. The decrease of collagen fibril diameter as a result of COMP induction however, might affect (mechanical) properties of the cartilage although not those parameters tested in our

study. The increase of COMP production often seen in osteoarthritic cartilage [67, 244] might therefore negatively affect the quality of the cartilage.

Next to our results on overexpressing COMP, it was found previously that mice deficient for COMP had a similar phenotype as the corresponding wild type mice [237]. To create a more severe phenotype, mice deficient for collagen type IX and COMP were developed. In these mice, a disorganized growth plate architecture and alterations in matrix deposition were observed both in newborn animals and at later stages of development [263]. The matrix produced by chondrocytes from these mice was examined in vivo and in vitro in chapter 7. As comparison, mice deficient only for collagen type IX (appendix chapter 7) or the corresponding wild type mouse strain was used. Mice deficient for COMP were not included in these studies since their phenotype is not different from the wild type [237]. When chondrocytes isolated from collagen type IX or collagen type IX/COMP deficient mice were cultured, less collagen and proteoglycans were produced in both knock-out conditions (although not statistically significant for collagen type IX knock-out because n=3). The absence of the matrix constituents also differentially influenced collagen and GAG distribution within the culture system. These effects seem to be mainly a result of collagen type IX deficiency since both collagen type IX knock-out and the double knock-out have similar effects. Having a COMP deficiency in addition to the collagen type IX deficiency does not change the effect seen with single collagen type IX knock-out regarding matrix deposition by knee and rib chondrocytes. This is in agreement with the effects seen with COMP overexpression were we also could not demonstrate an effect on matrix deposition and distribution. Collagen type IX seems more important in the production and formation of a functional cartilage matrix than COMP. However, matrix deposited by double knock-out chondrocytes had collagen fibrils with diameters similar to the matrix produced by wild type chondrocytes, whereas collagen fibrils of collagen type IX deficient mice were larger in diameter. Collagen type IX is known to limit lateral growth of the collagen fibrils [224]. Since inducing COMP production decreased the collagen fibril diameter (chapter 6), one would expect that the absence of COMP in addition to the absence of collagen type IX would increase the fibril diameter even more. Instead, fibril diameter normalized when both matrix molecules were absent. Possibly, the absence of COMP induced another matrix molecule to act as antagonist of collagen type IX.

COMP deficient mice lack an obvious phenotype [237], maybe due to a functional compensation by other matrix proteins. Mice deficient for collagen type IX display degenerative changes in articular cartilage, first detected at the age of 6 months [224, 261]. Combining the COMP and collagen type IX deficiency results in a disorganized growth plate architecture and alterations in matrix deposition both in newborn animals and at later stages of development [263]. However, only from mice deficient for collagen type IX it is known that they develop spontaneous OA [207, 261]. Double deficient mice need altered

mechanical loading of their joint such as running exercise to induce cartilage damage as seen in OA [307]. COMP single knock-out mice subjected to the same exercise only had flattening of their cartilage [307]. On the other hand, in an experimental arthritis model (collagen induces arthritis), mice deficient for COMP had an earlier onset and enhanced severity of the chronic arthritis [308]. Inducing OA by for example destabilization of the medial meniscus [309] would therefore probably lead to a quicker onset of OA when collagen type IX and COMP or COMP alone is absent then in the wild type mice.

Combining several approaches to modulate cartilage matrix deposition

As seen in our studies mentioned above, cartilage matrix deposition can be modulated with several factors such as addition of growth factors (chapter 2), by inhibition of collagen cross-link formation (with BAPN in chapter 4) or by inhibition of the formation of a functional proteoglycan network (with PNPX in chapter 5). The ability to modulate the formation of a new collagen network in a chondrocyte alginate culture by combining IGF1, BAPN and PNPX was examined in chapter 8. In parallel, the ability to modulate cartilage integrative repair, which also involves generation of a new collagen network, with these three factors was also examined. In both set-ups, factors were added temporarily to allow cross-link and proteoglycan formation after initial inhibition. The combination of IGF1 with transient collagen cross-link inhibition (with BAPN) was optimal for collagen deposition in the alginate bead. BAPN and/or IGF1 with PNPX, and therefore no intact proteoglycan network, resulted in less collagen deposition even though collagen deposition was stimulated in conditions without PNPX. Based on this study, we conclude that proteoglycans are necessary when new cartilage matrix is synthesized to retain the collagen matrix within the alginate bead. However, when two existing cartilage matrices have to integrate, the presence of proteoglycans seems to disturb the integration probably by preventing the collagen molecules to penetrate into the opposing matrix. Stimulating collagen production with IGF1, and simultaneously inhibiting the collagen from cross-linking with BAPN and preventing the formation of proteoglycans with PNPX was most beneficial for cartilage integration. Depending on the type of therapy in osteoarthritis, proteoglycans might be beneficial or disturbing in the repair. Cell therapy or matrix regeneration by chondrocytes would benefit from stimulating collagen and proteoglycan production in parallel. When transplanting cartilage into a defect, depletion of proteoglycans and inhibition of cross-links prior transplantation would probably be more beneficial.

Targeting collagen production: combining the knowledge

Taken together, the studies presented in this thesis show that several factors influence the collagen deposition by chondrocytes directly or indirectly via proteoglycan deposition. In

this section, the obtained knowledge will be combined. Of the growth factors used in this thesis, IGF1 had only positive effects on the parameters in our chondrocyte alginate cultures. It stimulated chondrocytes to deposit more collagen and proteoglycans. One can postulate on whether this was a direct stimulation of production of both matrix constituents or whether the deposition increased because of other factors. From literature for instance, it is known that IGF1 inhibits matrix degrading enzymes [310]. This could (also) have contributed to increased deposition, without induction of synthesis. However, increased gene expression of aggrecan core protein in the presence of IGF1 indicates that proteoglycan production is indeed increased resulting in more deposition. This increased proteoglycan deposition in turn could have resulted in more collagen deposition without increasing collagen production itself. This theory is supported by a lack of increased collagen type II gene expression in the presence of IGF1. From other studies it is known that IGF1 is even more potent in inducing matrix production when combined with OP1/BMP7[213]. FGF2 on the other hand had no positive effects in our culture system: It lowered the collagen deposition and did not affect the proteoglycan deposition. In addition, other studies showed that FGF2 inhibits the stimulatory effects of IGF1 [155] and that it stimulates the production of matrix degrading enzymes (chapter 2, and [311]). The effect TGFβ had on chondrocytes in culture was more complex and difficult to explain. Addition of TGFβ to chondrocytes in alginate had no large effect on proteoglycan and collagen deposition but did lower the number of cross-links per collagen molecule. In addition, it increased COMP deposition in our culture and has been shown to lower the production of matrix degrading enzymes by us and in other studies [117, 303]. Since induction of COMP by overexpression also resulted in collagen fibrils with a smaller diameter, the smaller collagen fibrils seen with TGFβ are most likely a result of the increased COMP production. This decrease of collagen fibril diameter could have also contributed to the decrease in collagen cross-links in the TGFβ cultures.

Collagen deposition could also be influenced by inhibition of LOX via covalent binding of BAPN. Here, the lack of collagen cross-links probably resulted in less collagen directly around the chondrocyte, resulting in less collagen-chondrocyte interaction. As a result, the chondrocyte produced more collagen (as shown by increased collagen type II gene expression) and thus also more collagen was deposited. This is not in line with the effects seen after TGFβ addition. Next to the effects known from ours and other studies, TGFβ probably has several other effects on chondrocytes, which makes it difficult to explain why less collagen cross-links in the TGFβ culture did not alter the collagen deposition. One possibility is the increase in COMP deposition and therefore the increase in network formation via other matrix molecules [63] that compensated for the lower number of cross-links present.

By inhibiting the formation of a proteoglycan network and therefore proteoglycan deposition, collagen deposition was also decreased. The absence of an intact

proteoglycan network lowered the collagen retention in our culture system. The same can be hypothesized for collagen type IX. Cultures of collagen type IX deficient chondrocytes also had altered collagen retention. Since collagen type IX forms a network together with collagen type II and other matrix molecules, the absence of this network probably also reduced collagen retention in the alginate bead. However, GAG distribution was also altered when collagen type IX was absent in the newly formed matrix. This could also have contributed to altered collagen retention. Interestingly, the absence of COMP in addition to collagen type IX deficiency did not alter matrix production and distribution. This leads to the conclusion that collagen type IX is more important in the matrix interaction than COMP.

Taken together, it seems that for the production of collagen in cartilage, focusing on the formation of an intact cartilage network is as important as stimulating collagen type II production. This network formation is important to retain the collagen type II in the newly formed matrix. From this thesis it can be concluded that at least proteoglycans and collagen type IX contribute to this.

Future perspectives

The goal of our study was to investigate the ability to modulate the formation of a functional collagen type II network in order to develop better treatments for OA in the future. The work presented in this thesis gave more insight in how matrix production and especially collagen type II production can be modulated and how these modulations contribute to the functionality of the cartilage matrix. For the repair of cartilage damage in OA, the modulations resulting in more and/or better collagen type II are considered most interesting. Removing a matrix constituent however, also gives insight in its importance. Several approaches for the repair of cartilage damage were examined in more detail, this might help future studies giving new starting points in the search for OA therapy.

Based on the positive effects of IGF1 in our experiments, IGF1 could be a possible disease modifier for OA. TGFβ is also extensively examined as possible DMOAD and our data gave more insight in the benefits of TGFβ considering the protection of matrix against degradation and stimulation of matrix production under specific conditions. As mentioned with TGFβ, care must also be taken here for the negative effects of intra-articular TGFβ administration (fibrosis, osteophyte formation). Preventing the TGFβ-side effects is the subject of ongoing research [114].

Other factors known to positively influence cartilage collagen type II production are BMP2 and platelet rich plasma (PRP). Addition of BMP2 resulted in higher gene expression and protein production of collagen type II [312]. Next to the effect of one or two growth factors, a pool of unknown growth factors in PRP also has the potency to increase collagen production [313].
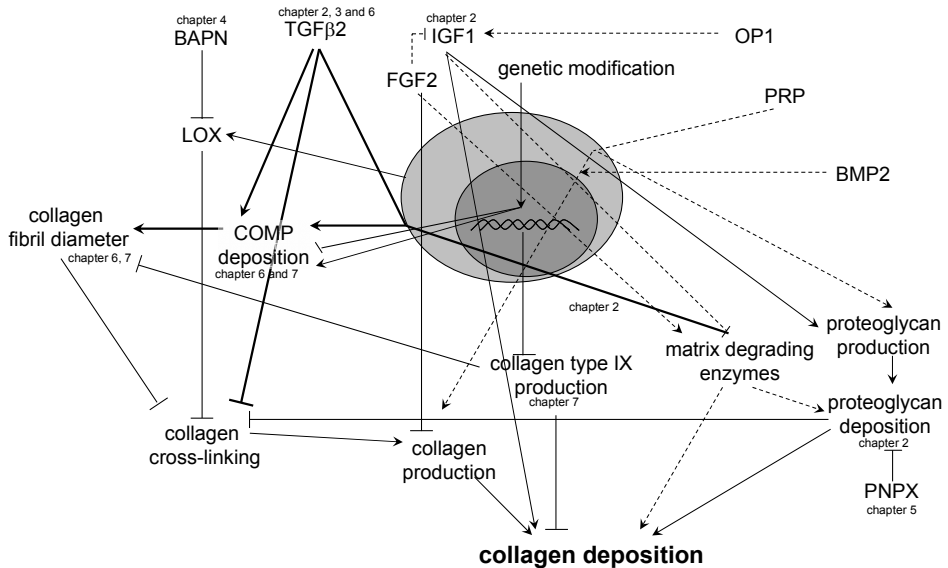
Figure 9.1. A selection of factors known to influence collagen type II expression, production and/or deposition in healthy chondrocyte alginate culture. The effect of factors used in this thesis are shown in solid arrows, effects of factors know from literature are shown in dashed arrows. Since TGFβ has a broad spectrum of effects and improve the clarity of the scheme, the effects of this growth factor are shown with a thick line. In addition, to illustrate the broad spectrum of effects, effects of TGFβ on chondrocytes in monolayer are shown too.

Next to promoting collagen type II production and collagen cross-linking, inducing expression of collagen type IX also might be an interesting approach to repair cartilage. More collagen type IX might result in more effective matrix repair than seen so far because of more efficient network formation. Overexpression by introduction of extra copies of the collagen type IX gene however is controversial. Gene therapy is under current investigation and might become available as future tool in modification of several diseases including OA. Alternatively, future research might include the identification of soluble factors that promote collagen type IX production. Care must be taken however that induction of collagen type IX might reduce the collagen fibril diameter leading the worsened mechanical properties [253]. This should also be investigated.

An other approach to repair cartilage damage is engineering cartilage in vitro and subsequent implantation of the engineered construct. For this approach, expansion of chondrocytes is sometimes needed. FGF2 might be beneficial here because this growth factor protects against dedifferentiation [121]. Based on our results, intra-articular injection of FGF2 might not be sufficient in repair of cartilage lesions because it had a negative effect on matrix deposition

Even though inhibition of collagen cross-linking resulted in an increase of collagen deposition in vitro, in vivo it has several side effects, especially when the compound

from our study is used, since BAPN is mutagenic/carcinogenic. Inhibiting collagen cross-linking with BAPN as a direct therapy for cartilage regeneration is therefore not advised. In addition, based on results in this thesis, stimulation of the formation of an extracellular matrix network instead of preventing network formation seems important for collagen deposition. Especially since collagen type II production is already increased in early OA [95, 96]. Using temporary cross-link inhibition to promote collagen deposition in cartilage tissue engineering however seems promising. Here, inhibition of collagen cross-link formation can be more controlled and systemic side effects in the patient are avoided. Temporary inhibition of collagen cross-linking also seems promising when the *in vitro* tissue engineered construct has to integrate in the existing matrix.

To conclude and based on the results presented in this thesis as well as the existing literature, collagen deposition by chondrocytes can improve when not only collagen type II production is stimulated but also when proteoglycan deposition and collagen type IX deposition is stimulated. On the other hand, temporarily preventing network formation might improve integration of two opposing cartilage matrices. This knowledge will help in the development of disease modifying osteoarthritic approaches.

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Artrose, ook wel gewrichtsslijtage genoemd, is een ziekte van de gewrichten waarbij onder andere het kraakbeen beschadigd is. Deze ziekte komt voor in alle gewrichten zoals de knie, heup, hand en de schouder. Bij gezonde personen bedekt het kraakbeen de uiteinden van het onderliggende bot waardoor gewrichten soepel bewegen en schokken worden opgevangen die tijdens het bewegen ontstaan. Door schade aan het kraakbeen bij artrose kunnen deze functies niet goed uitgevoerd worden wat pijn bij het bewegen en stijfheid van de gewrichten tot gevolg heeft. In ernstige gevallen van artrose kan de kraakbeenlaag zelfs helemaal verdwenen zijn. Momenteel is er geen goed herstel van het kraakbeen mogelijk en vindt vooral pijnbestrijding plaats. Wanneer pijnbestrijding niet meer voldoende is en het bewegen ernstig beperkt wordt, vindt een gewrichtsvervangende operatie plaats met een zogenaamd kunstgewricht van metaal en plastic.

Er zijn echter aanwijzingen dat kraakbeen de mogelijkheid heeft zich te herstellen. Eén van de meest voorkomende eiwitten in kraakbeen is collageen type 2. Van dit collageen is bekend dat dit meer wordt gemaakt wanneer artrose in een gewricht bestaat. Toch vindt er geen herstel van de schade plaats. Dit zou kunnen betekenen dat er wel een herstelmechanisme geactiveerd is, maar dat het niet effectief genoeg is om de schade daadwerkelijk te herstellen.

Dit leidde tot het onderzoek van dit proefschrift met de vraag of het mogelijk is de vorming van een collageen type 2 netwerk te beïnvloeden zodat dit kan bijdragen aan effectief kraakbeenherstel bij artrose.

In ons onderzoek hebben we allereerst in **hoofdstuk 2** gekeken naar vaak gebruikte groeifactoren en hoe deze het collageennetwerk in nieuwgevormd kraakbeen beïnvloeden. Groeifactoren zijn eiwitten die van nature in het lichaam voorkomen en daar allerlei processen regelen. Van de bestudeerde groeifactoren resulteerde de groeifactor IGF1 in de meest positieve effecten voor kraakbeenherstel: de productie van zowel proteoglycanen (andere belangrijke versuikerde eiwitten in kraakbeen) als collageen werd gestimuleerd en de hoeveelheid collageen cross-links bleef hierbij op peil. Deze cross-links zijn verbindingen tussen de collageenmoleculen en zijn belangrijk voor de sterkte van het collageennetwerk. De groeifactor FGF2 had weinig effect op de proteoglycaanproductie en remde in een hoge concentratie zelfs de collageenproductie.

Een andere groeifactor, TGFbeta2, had verrassende effecten op het collageen netwerk omdat dit de hoeveelheid collageen cross-links verminderde. Nader onderzoek in **hoofdstuk 3** toonde aan dat het effect van TGFbeta2 afhangt van de omgeving waarin de kraakbeencellen gekweekt worden. Wanneer TGFbeta2 toegevoegd werd aan kraakbeencellen die op een plat oppervlak gekweekt werden, nam de hoeveelheid collageen cross-links toe. TGFbeta2 bij kraakbeencellen in een 3-dimensionale omgeving

zorgde voor minder collageen cross-links. De invloed van collageen cross-links op het functioneren van het kraakbeeneiwitnetwerk (matrix) werd getest in **hoofdstuk 4** door tijdelijk de vorming van deze cross-links te remmen. Een bijkomstig voordeel van deze tijdelijke remming was de toename in de collageenproductie. Hieruit bleek dat bij lage hoeveelheden collageen alleen het aantal collageen moleculen belangrijk is voor de sterkte van de nieuwe kraakbeenmatrix. Naarmate er meer collageen gevormd wordt, neemt het belang van de cross-links toe. Dan zijn zowel de collageenhoeveelheid als structuur (cross-linking) nodig om de matrix sterker te maken. Daarnaast bleken collageen cross-links de kraakbeenmatrix te beschermen tegen afbraakenzymen

De invloed van andere kraakbeeneiwitten op het collageennetwerk is ook onderzocht. De productie van cartilage oligomeric matrix protein (COMP) werd in **hoofdstuk 6** beïnvloed door de eerder gebruikte groeifactoren: TGFbeta2 zorgde voor meer COMP, FGF2 juist voor minder. Door met een virus specifiek de COMP productie van kraakbeencellen te verhogen werd aangetoond dat een toename in COMP spiegels geen effect heeft op de collageenproductie, de collageencross-linking en het functioneren van de kraakbeenmatrix. Wel werden de gevormde collageenvezels dunner wanneer er meer COMP eiwit was. Verder vonden we in **hoofdstuk 7** dat kraakbeencellen die geen collageen type 9 of geen collageen type 9 en COMP konden maken, minder collageen type 2 en proteoglycanen produceerden. De matrixeiwitten die geproduceerd werden, waren ook anders verdeeld over het kweeksysteem en werden minder ingebouwd in de kraakbeenmatrix. Er was geen verschil te zien tussen cellen zonder collageen type 9 en cellen die zowel collageen type 9 als COMP niet konden maken wat betreft deze parameters. Opvallend was de collageenvezeldikte gemaakt door deze cellen. Kraakbeencellen die geen collageen type 9 konden maken, maakten collageenvezels die dikker waren dan normaal. Kraakbeencellen die zowel collageen type 9 als COMP niet konden maken, hadden vezels van normale dikte. Op basis hiervan lijken COMP en collageen type 9 tegenovergestelde effecten uit te oefenen op collageen.

Beïnvloeden van proteoglycaanvorming in **hoofdstuk 5** resulteerde ook in veranderingen in het collageennetwerk. De verdeling van het collageennetwerk in het kweeksysteem veranderde, er werd minder collageen ingebouwd, minder collageencross-links waren aanwezig en de functionaliteit was ook minder. Het collageennetwerk is dus niet alleen belangrijk om proteoglycanen vast te houden in het kraakbeen, maar bij de nieuwvorming van kraakbeen is een proteoglycaannetwerk belangrijk voor de inbouw van collageen. Zelfs wanneer IGF1 of de cross-linkremmer (beide resulterend in meer collageen) gecombineerd werden met de remmer van proteoglycaanvorming in **hoofdstuk 8**, zorgde dit voor verminderde collageen inbouw dan in de controle conditie zonder toevoegingen.

Wanneer echter twee stukken kraakbeen aan elkaar moeten groeien, lijkt een bestaand proteoglycaannetwerk storend te werken, evenals collageen cross-links. Beide

factoren werken mogelijk storend voor de collageenmoleculen om de tegenoverliggende matrix binnen te dringen.

Bovenstaande bevindingen kunnen gebruikt worden in het ontwikkelen van therapieën voor artrose. Verder onderzoek zal uitwijzen of het toedienen van bijvoorbeeld de groeifactor IGF1 in het kniegewricht herstellend werkt of dat de productie van collageen type 9 inderdaad de netwerkvorming stimuleert en het herstel zal bevorderen. Afhankelijk van de therapie kan gekozen worden voor juist het stimuleren van proteoglycanen en collageen cross-links of het tijdelijk remmen hiervan.

Dankwoord

Zo, het zit er op, mijn proefschrift is af. Omdat je promoveren niet alleen doet, wil ik graag een aantal mensen bedanken die mij hierbij hebben geholpen.

Prof.dr. J.A.N. Verhaar, mijn promotor, ik wil u hartelijk danken voor de mogelijkheid die u mij heeft geboden om op de afdeling Orthopaedie mijn promotieonderzoek te doen. Ik ervaar onze gesprekken altijd als erg stimulerend en stel uw kritische blik erg op prijs.

Dr. Gerjo van Osch en dr. Jeroen de Groot, mijn co-promotoren, jullie ook hartelijk dank voor jullie begeleiding tijdens mijn onderzoek. Ik had geen betere co-promotoren kunnen hebben. Gerjo, dat jouw deur altijd open staat is erg prettig, dank daarvoor. Als ik de draad even kwijt was, wist je mij te stimuleren en kon ik vol energie weer verder. Jeroen, onze besprekingen waren altijd erg motiverend ook al was het maar even bijpraten na een tijd in Rotterdam te hebben gezeten.

Naast mijn promotor en co-promotoren wil ik graag ook een aantal andere mensen danken voor de samenwerking: Harrie Weinans, dank je dat ik op het lab mijn onderzoek mocht doen en dank voor je hulp bij de mechanische onderdelen van mijn onderzoek. Ruud Bank, bedankt voor de prettige samenwerking, ik heb veel van je geleerd. Kaspar Jansen, Jacqueline van der Linden en Jos van Driel, ik wil jullie bedanken voor de mogelijkheid om mechanische testen in Delft uit te voeren en de hulp bij de interpretatie van de resultaten. Katrin Blumbach and Frank Zaucke, I really enjoyed working with you and hope we can continue our collaboration, herzlichen Dank. Piet van der Heul, bedankt voor alle tijd die je gestopt hebt in het creëren van mooie EM plaatjes. Arthur Westphal Taylor, even though you only stayed at the lab for a few months, your help with analyzing experiments was of great value. Koen Bos, bedankt voor de leuke en interessante discussies die we hadden en hebben tijdens jouw bezoeken aan het lab.

Met veel plezier heb ik aan mijn promotieonderzoek gewerkt, en niet in de laatste plaats door de hulp van collega's die altijd voor me klaar stonden en gezelligheid tussen het werk door, zowel in Rotterdam als in Leiden.

Elian, Jarno en Justus, de lunch of cake van de week was altijd hilarisch als jullie er bij waren. Nicole, lunch of cake van de week ìs altijd gezellig als jij er bij bent. Bedankt voor al je geduld en hulp. Judd, Luuk, Predrag en Olav bedankt voor de samenwerking. Ruud, Yvonne S, Sander en Eric, onze filmavondjes of avonden uit zorgden voor een welkome afwisseling. Erwin, Holger, Femke, Anna, Katja, Tom, Carola, Jennifer, Robert-Jan, Gerben, Michiel, Stefan, Esther en Sandra, fijn dat jullie mijn collega's zijn.

Anton, bedankt voor de fijne samenwerking en je grote bijdrage aan mijn onderzoek en dit boekje. Nico en Frits, jullie ook bedankt voor alle hulp en engelengeduld met het 'HPLC-en' en aanverwante zaken. Zonder jullie was dat nooit gelukt. Fritsie, hopelijk gaan wij ooit nog een keer een balletje slaan samen, moet ik wel eerst goed oefenen. Benno, Marjan, Joline, Annemarie, Anne-Marie, Jessica, Elly, Carla, Arianne, Geesje en Claudia, bedankt voor de goede en gezellige samenwerking bij TNO. Bea, bedankt voor jouw hartverwarmende interesse.

Marieke en Wendy, jullie staan naast mij als paranimfen op de spannende dag. Ik kan me geen betere paranimfen voorstellen.

Wendy, met jou heb ik een erg groot deel van het onderzoek samen gedaan. We vormden een goed team waarbij serieus onderzoek werd afgewisseld door veel gezelligheid. Ik hoop dat we nog veel en vaak gaan samenwerken.

Marieke, in onze tijd samen op het lab zijn we vrienden voor het leven geworden. Bedankt voor alle gezellige, hilarische en serieuze momenten. Echt stoep dat we straks allebei gepromoveerd zijn.

Niet alleen op het werk maar ook thuis kreeg ik veel steun van familie en vrienden.

The party of eight (nu nine): Alexandra & Charles, Wendy & Sjoerd, Tim & Marjolein, jullie zorgen voor welkome afwisseling tussen het werk door. Bedankt voor de vriendschap en de interesse in mijn werk.

Papa, mama, bedankt voor jullie onvoorwaardelijke steun en interesse. Het geeft een goed gevoel dat jullie er altijd voor me zijn. Pim, ik ben blij dat jij mijn broertje bent.

Anton-Pieter, lieve schat, jouw liefde geeft mij energie. Bedankt voor alle mooie momenten die we samen hebben beleefd en jouw troost in moeilijke tijden. Samen kunnen we de hele wereld aan.

Yvonne Bastiaansen-Jenniskens
Zevenbergen 2009

Curriculum vitae

Yvonne Maria Bastiaansen-Jenniskens werd geboren op 27 april 1980 te Breda. Van 1992 tot 1997 doorliep zij de HAVO op de Katholieke Scholengemeenschap het Newman College te Breda. Na het behalen van haar HAVO diploma ging zij Biologie en Medisch Laboratorium Onderzoek studeren aan de toenmalige Hogeschool West-Brabant (nu Avans Hogeschool). In het kader van haar specialisatie 'Histologie' heeft ze 9 maanden stage gelopen op de afdeling Hematologie van het Erasmus MC Rotterdam onder leiding van Dr. Ruud Delwel, Dr. Marieke Joosten en Yolanda Vankan. In 2001 studeerde zij met lof af waarna ze naar de Katholieke Universiteit Nijmegen (nu Radboud Universiteit Nijmegen) ging om Biomedische Wetenschappen te studeren. In het kader van het hoofdvak Pathobiologie liep zij vier maanden stage op het Reumatologie Research Laboratorium van het UMC St. Radboud te Nijmegen onder leiding van Dr. Leo Joosten en Birgitte Walgreen. In maart 2004 werd het doctoraal examen Biomedische Wetenschappen met goed gevolg afgelegd.

Van januari 2004 tot januari 2008 was Yvonne werkzaam als onderzoeker in opleiding op de afdeling Orthopaedie van het Erasmus MC Rotterdam. Zij deed haar onderzoek onder dagelijkse begeleiding van Dr. Gerjo van Osch (hoofd Connective Tissue, Cells and Repair groep, afdeling Orthopaedie Rotterdam) en Dr. Jeroen DeGroot (TNO Business Unit BioSciences, Leiden) waarvan dit proefschrift het resultaat is.

Yvonne Bastiaansen-Jenniskens is op 2 juni 2007 getrouwd met Anton-Pieter Bastiaansen. Momenteel is ze werkzaam als post-doc op de afdeling Orthopaedie van het Erasmus MC Rotterdam.

PhD Portfolio Summary

Name PhD student:	Yvonne Maria Bastiaansen-Jenniskens
Erasmus MC Department:	Orthopedic Research Laboratory
Research School:	MUSC
PhD period:	January 2004 – December 2007
Promotor:	Prof.dr. JAN Verhaar
Co-promotors:	dr. GJVM van Osch, dr. J. DeGroot

PhD training	**Year**
In-depth courses	
- Classical Methods for data-analysis (Nihes)	2004
- MUSC AIO Curus	2004
- Biomedical English Writing and Communication (EUR)	2005
- Cursus Basis Didactiek	2006
(Inter)national conferences, poster presentations	
- Targeting collagen synthesis in cartilage tissue engineering by using growth factors, European Tissue Engineering Society, Lausanne, Switzerland	2004
- Growth Factors to target collagen repair by chondrocytes, Orthopaedic Research Society (ORS), Washington DC, USA	2005
- β-Aminopropionitrile To Stimulate Collagen Matrix Repair, European League Against Rheumatism, Vienna, Austria	2005
- β-Aminopropionitrile To Stimulate Collagen Matrix Repair, 10th World Congress on Osteoarthritis, Boston, USA	2005
- Targeting collagen repair and biomechanical properties of cartilage by IGF1, TGFβ2 AND FGF2, 10th World Congress on Osteoarthritis, Boston, USA	2005
- TGF-beta Reduces the Formation of Collagen Crosslinks by Chondrocytes, effects of TGFbeta2 on collagen biochemistry in cartilage, 10th World Congress on Osteoarthritis, Boston, USA	2005
- TGF-beta reduced the formation of collagen crosslinks by chondrocytes, Dutch Society for Matrix Biology, Lunteren, The Netherlands	2006
- Modulation Of Collagen Network Formation To Improve Cartilage Regeneration, American College of Rheumatology, Washington DC, USA	2006
- Modulation of collagen network formation to improve functional properties in cartilage regeneration, Orthopaedic Research Society, San Diego CA, USA	2007
- TGF-beta affects collagen cross-linking independent of chondrocyte phenotype but strongly depending on physical environment, ORS, San Diego CA, USA	2007
- Collagen and its crosslinks determine functional properties of newly formed cartilage, Gordon Research Conference about Cartilage Biology & Pathology, Ventura CA, USA	2007
- Stimulating COMP production in cartilage tissue engineering, World Congress on Osteoarthritis, Ft Lauderdale FL, USA	2007
- Stimulating COMP production in cartilage tissue engineering, Dutch Society for Biomaterials and Tissue Engineering, Lunteren, The Netherlands.	2007
- Stimulating COMP production in cartilage tissue engineering, 54th Annual Meeting of the Orthopaedic Research Society, San Fransisco CA, USA	2008
- Collagen type IX and cartilage oligomeric matrix protein influence cartilage matrix assembly, the 2008 World Congress on Osteoarthritis, Rome, Italy	2008
- Stimulation of COMP Production in Cartilage Matrix Generation Decreases Collagen Fibril Diameter, 8th World Congress of the International Cartilage Repair Society, Miami FL, USA	2009

(Inter)national conferences, podium presentations

- Growth factors to target collagen repair by chondrocytes, Dutch Society for Biomaterials and Tissue Engineering, Lunteren, The Netherlands.	2004
- Impaired cartilage wound healing and how it affects tissue repair, Aegean conference on Tissue Engineering, Crete, Greece.	2005
- Growth factors to stimulate matrix repair targeting collagen production by chondrocytes, Dutch Society for Matrix Biology, Lunteren, The Netherlands	2005
- Effects of TGF-beta on collagen biochemistry in cartilage, European Tissue Engineering Society, Munich, Germany	2005
- β-Aminopropionitrile To Target Collagen Synthesis in Cartilage Tissue Engineering, European Tissue Engineering Society, Munich, Germany	2005
- β-Aminopropionitrile To Target Collagen Synthesis in Cartilage Tissue Engineering, Dutch Society for Matrix Biology, Lunteren, Nederland	2006
- Modulation of collagen network formation to improve cartilage tissue engineering, Tissue Engineering and Regenerative Medicine International Society Europe, Rotterdam, The Netherlands	2006
- Modulation of collagen network formation to improve functional properties in cartilage regeneration, OsteoArthritis Research Society International, Prague, Tsjech Republic	2006
- Stimulating COMP production in cartilage tissue engineering, Tissue Engineering and Regenerative Medicine International Society Europe, London, England	2007
- Does COMP influence cartilage regeneration? Dutch Society for Matrix Biology, Lunteren, The Netherlands	2008
- Inhibition of proteoglycan production influences cartilage collagen network formation, Tissue Engineering and Regenerative Medicine International Society Europe, Porto, Portugal.	2008
- Inhibition of proteoglycan production influences cartilage collagen network formation, the 2008 World Congress on Osteoarthritis, Rome, Italy	2008

Travel awards

- TERMIS travel award	2007
- TERMIS travel award	2008

Other

- Secretary of 'Tissue Engineering and Regenerative Medicine International Society – Students and Young Investigator Section Europe' (TERMIS-SYIS EU)	2006 – 2008
- Local organisor TERMIS-SYIS EU activities during the TERMIS meeting in Rotterdam	2006

Teaching activities	**Year**

Supervising practicals

- Practical 2nd year medical students, mechanical tests cartilage	2004
- Practical 2nd year medical students, mechanical tests cartilage	2005

Lecturing

- Lecture mechanical functioning cartilage, 2nd year medical students	2006
- Lecture mechanical functioning cartilage, 2nd year medical students	2007
- Practical mechanical functioning cartilage, Technical University Students Delft	2007
- Lecture mechanical functioning cartilage, 2nd year medical students	2008
- Lecture mechanical functioning cartilage, Technical University Students Delft	2008

Other

- Supervising short internship Technical University student	2006

List of publications

Bastiaansen-Jenniskens YM, Koevoet W, Feijt C, Bos PK, Verhaar JA, van Osch GJ, Degroot J. Proteoglycan production is required in initial stages of new cartilage matrix formation but inhibits integrative cartilage repair. Journal of Tissue Engineering and Regerative Medicine 2009 Feb;3(2):117-23.

Bastiaansen-Jenniskens YM, Koevoet W, Jansen KM, Verhaar JA, Degroot J, Vanosch GJ.Inhibition of glycosaminoglycan incorporation influences collagen network formation during cartilage matrix production. Biochem Biophys Res Commun. 2009 Feb 6;379(2):222-6

Bastiaansen-Jenniskens YM, Koevoet W, de Bart AC, Zuurmond AM, Bank RA. Verhaar JA, van Degroot J, Osch GJ. TGFbeta affects collagen cross-linking independent of chondrocyte phenotype but strongly depending on physical environment. Tissue Eng Part A. 2008 2008 Jun;14(6):1059-66

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Jenniskens YM, Koevoet W, de Bart AC, Weinans H, Jahr H, Verhaar JA, Degroot J, van Osch GJ. Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFbeta2 and FGF2. Osteoarthritis Cartilage. 2006 Nov;14(11):1136-46.

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Radstake TR, Roelofs MF, **Jenniskens YM**, Oppers-Walgreen B, van Riel PL, Barrera P, Joosten LA, van den Berg WB. Expression of toll-like receptors 2 and 4 in rheumatoid synovial tissue and regulation by proinflammatory cytokines interleukin-12 and interleukin-18 via interferon-gamma. Arthritis Rheum. 2004 Dec;50(12):3856-65.

Joosten M, Vankan-Berkhoudt Y, Tas M, Lunghi M, **Jenniskens Y**, Parganas E, Valk PJ, Lowenberg B, van den Akker E, Delwel R. Large-scale identification of novel potential disease loci in mouse leukemia applying an improved strategy for cloning common virus integration sites. Oncogene. 2002 Oct 17;21(47):7247-55.
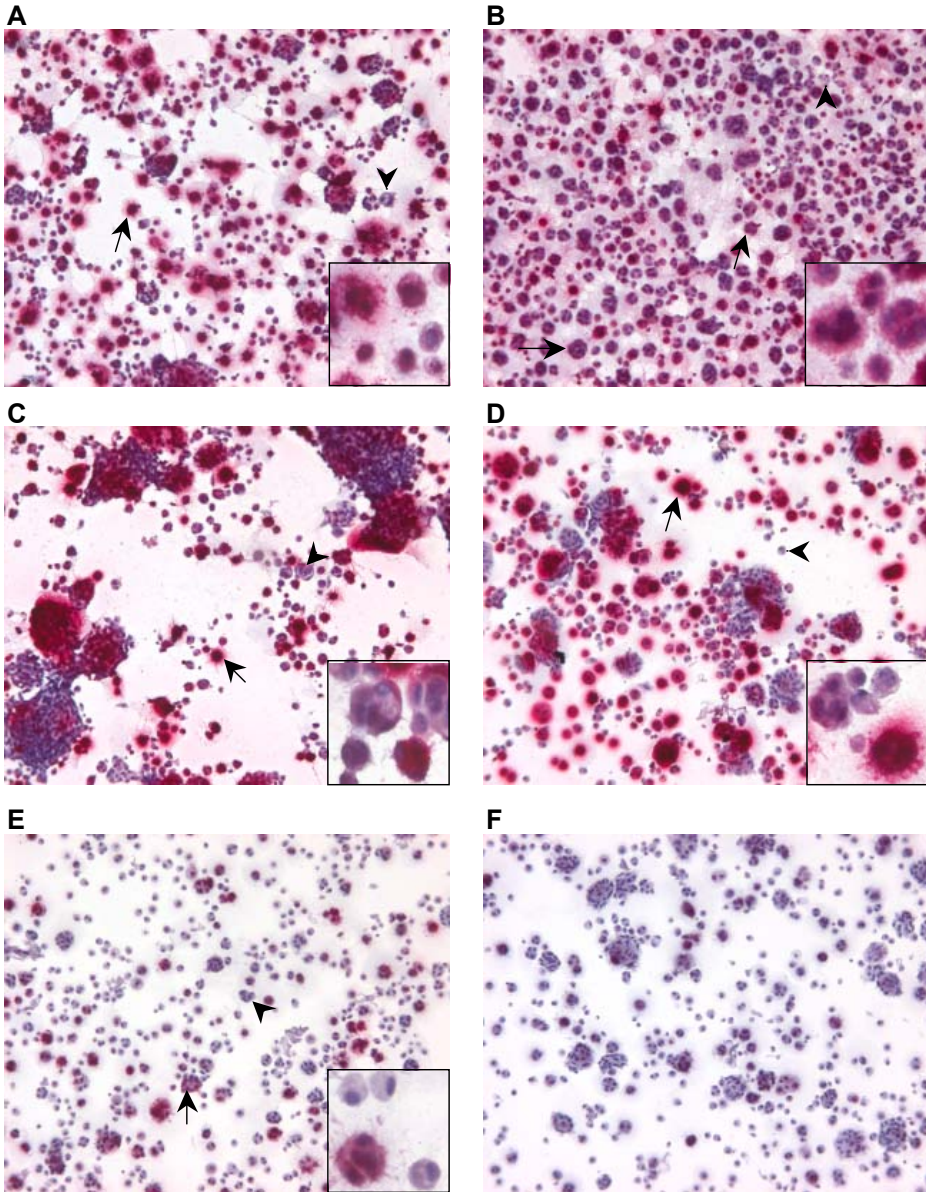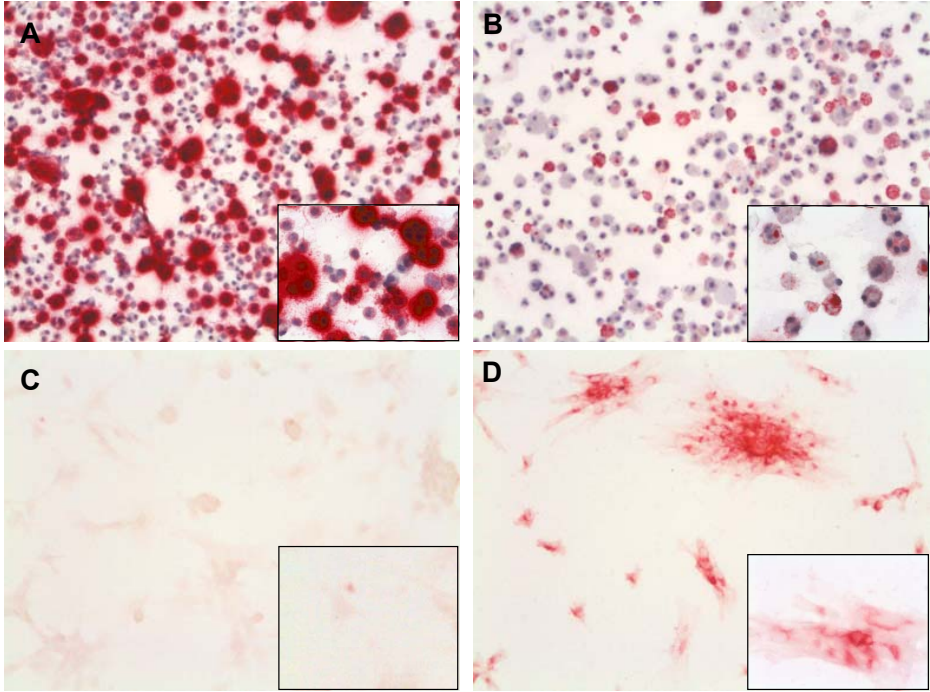
C Color figures

Figure 2.4

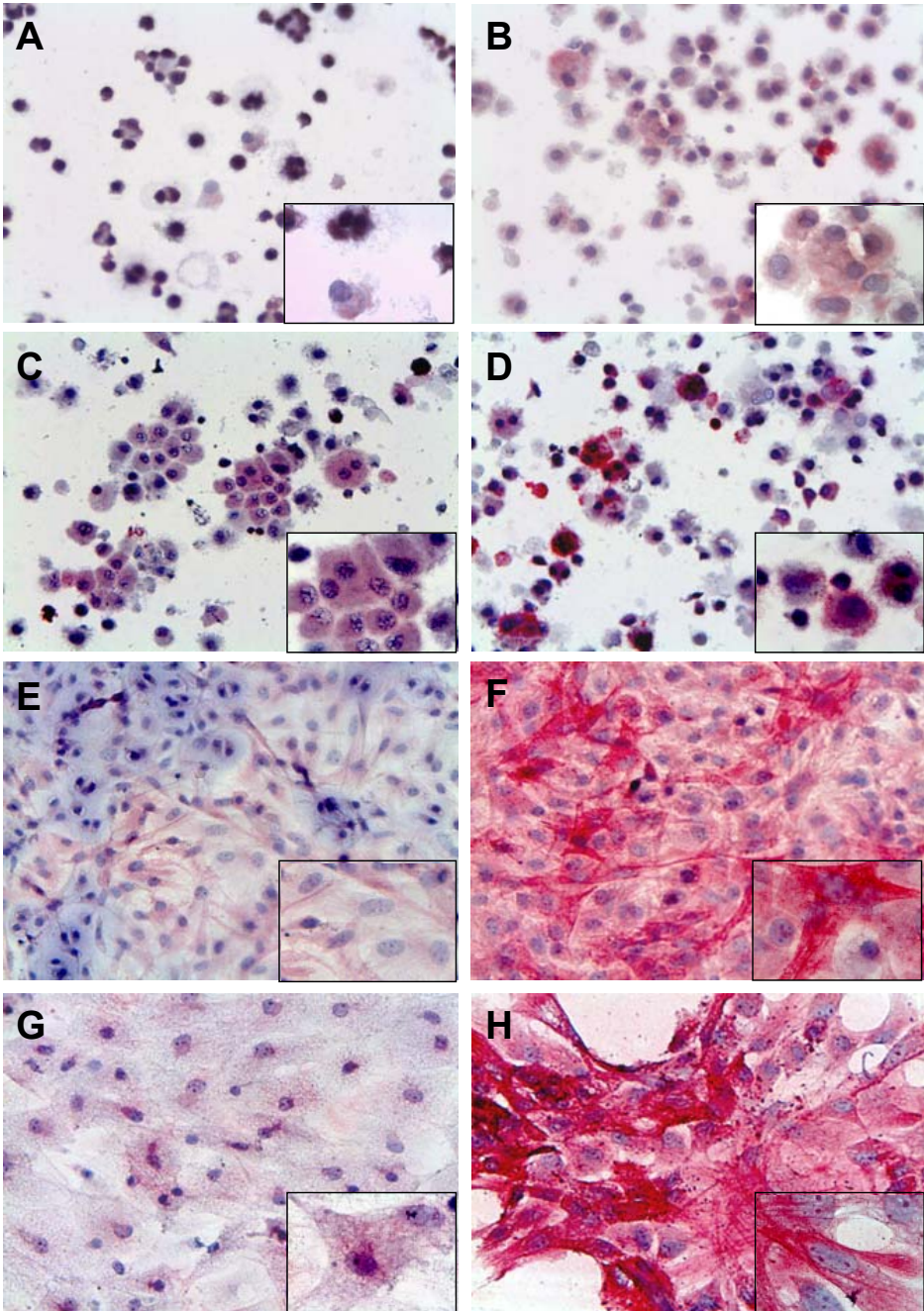**Figure 3.2**

Figure 3.4

Figure 4.6

A

B

Figure 6.3

wt   dko

collagen IX

COMP

collagen II

matrilin-3

HE/Alcian Blue
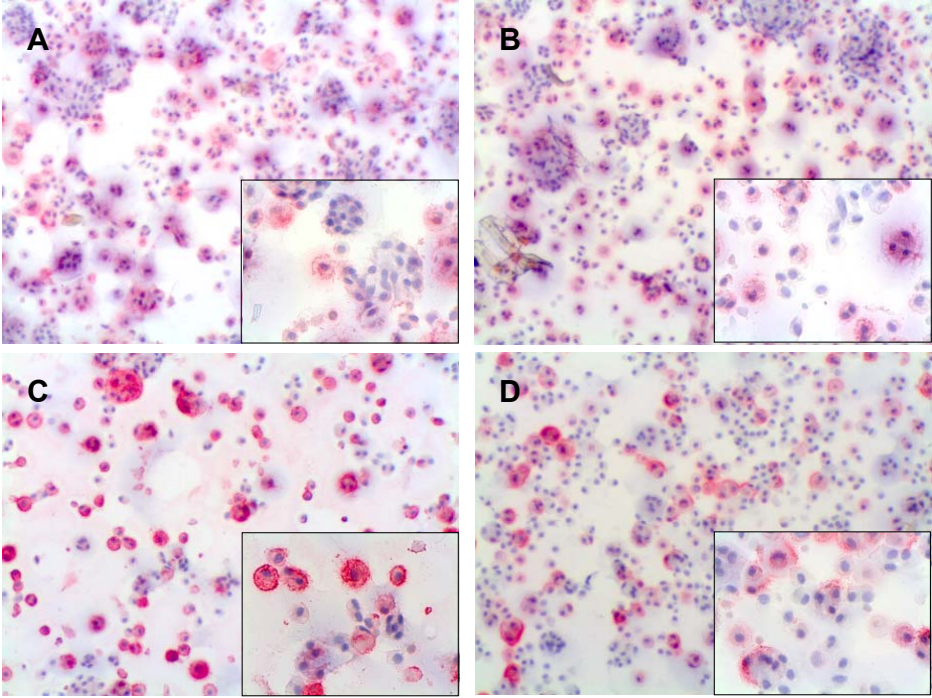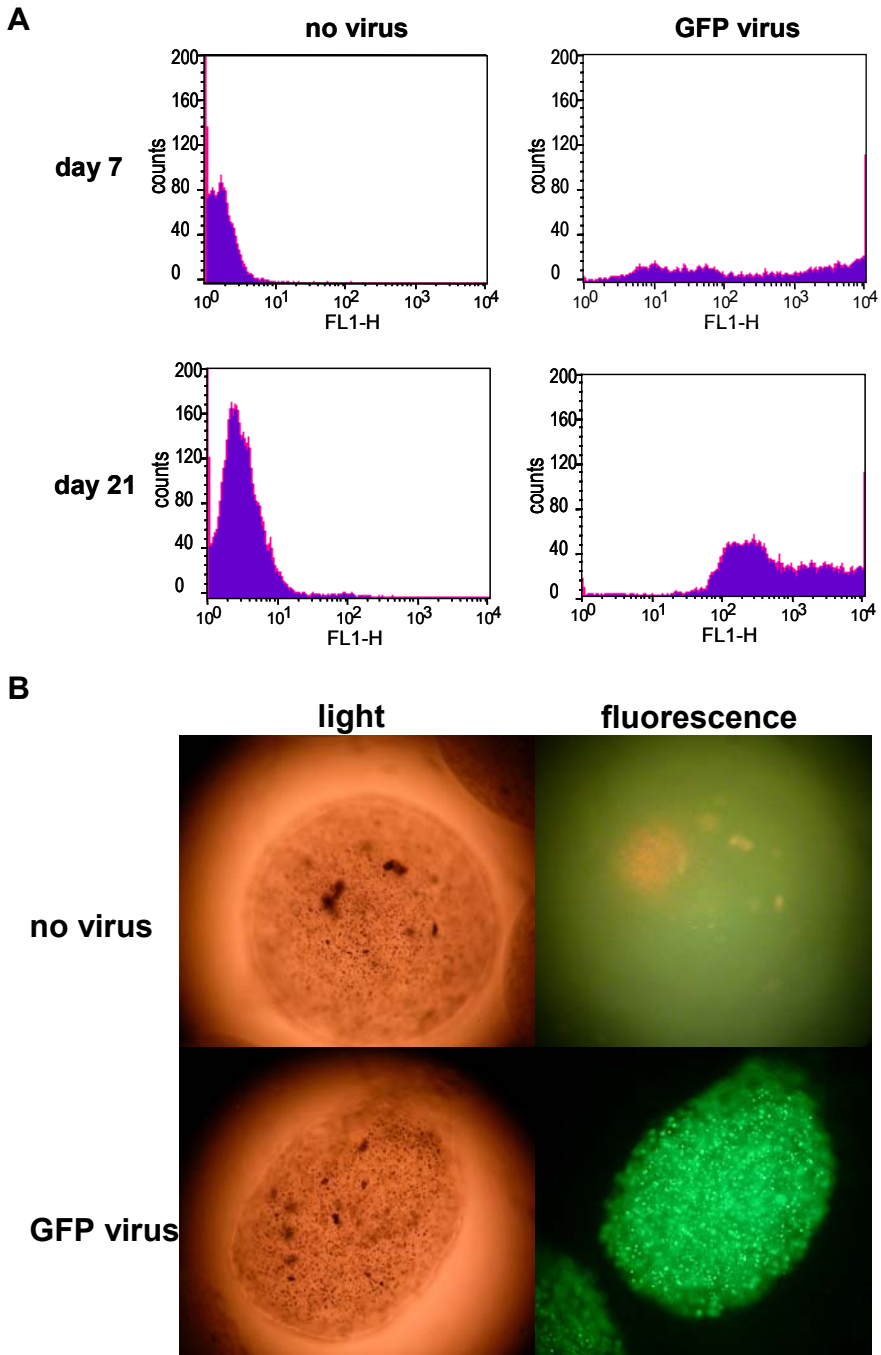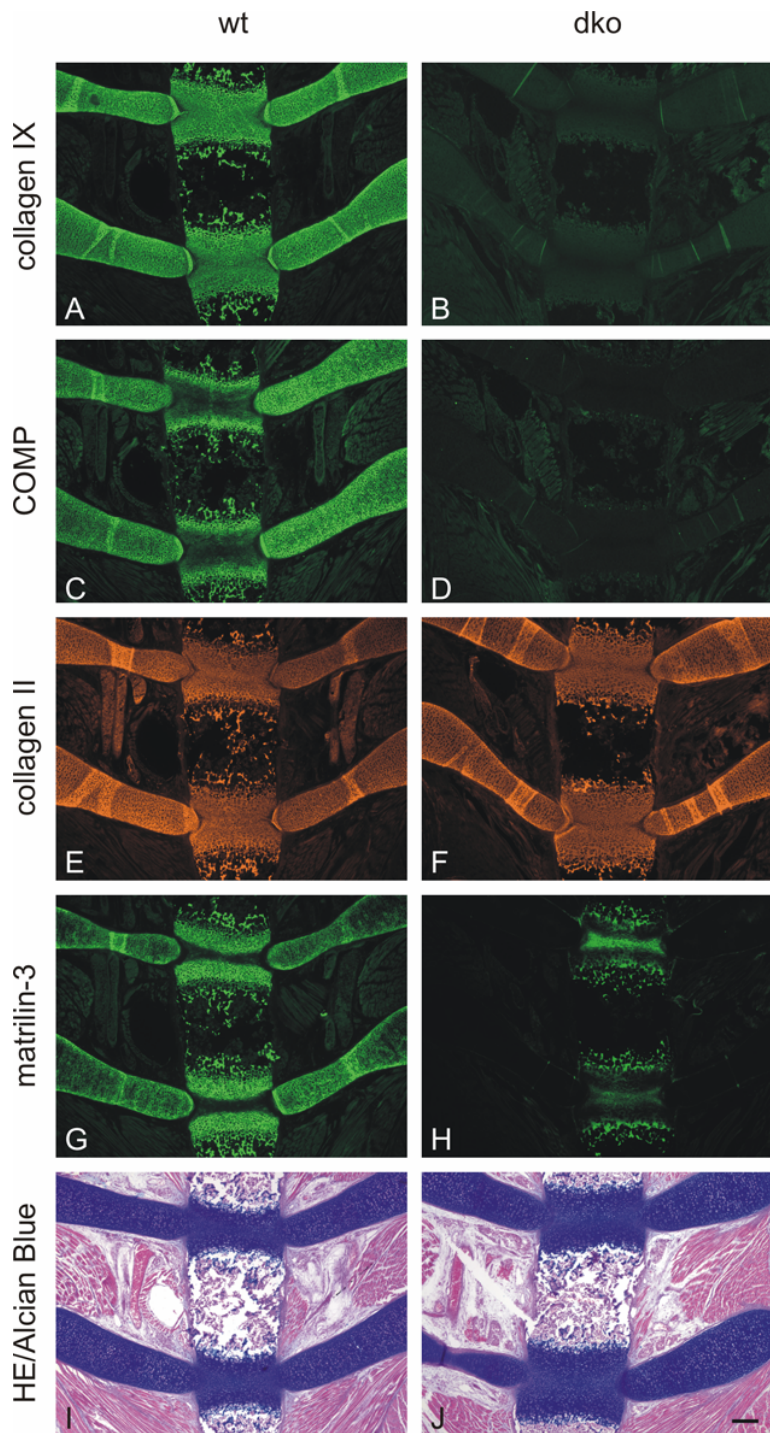
Figure 7.1

Figure 7.3

Figure 8.5