Lysyl hydroxylase 2b
the X factor in
OA-related synovial fibrosis

The cause of joint stiffness in OA?

Dennis F.G. Remst
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Chapter 1

General introduction: Unraveling OA-related synovial fibrosis: A step closer to solving joint stiffness

& Outline of this thesis

D.F.G. Remst\textsuperscript{1} · E.N. Blaney Davidson\textsuperscript{1} · P.M. van der Kraan\textsuperscript{1}

Abstract

Synovial fibrosis is often found in osteoarthritis (OA), contributing heavily to joint pain and joint stiffness, the main symptoms of OA. At this moment the underlying mechanism of OA-related synovial fibrosis is not known and there is no cure available.

In this review we discuss factors which have been reported to be involved in synovial fibrosis. This to get more insight in how these factors contribute to the fibrotic process and which would be the best targets for therapy in synovial fibrosis.

In this regard the following factors are discussed: Transforming growth factor beta (TGFβ), Connective Tissue Growth Factor (CTGF), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), Tissue inhibitor of metalloproteinase 1 (TIMP-1), urotensin-II (U-II), A disintegrin and metalloproteinase domain 12 (ADAM12), Prostaglandin F2α (PGF2α) and Hyaluronan.

Key messages
1) Synovial fibrosis, which cannot be cured yet, contributes to joint pain and stiffness in OA.
2) TGFβ signaling is on top of the fibrotic cascade in OA-related synovial fibrosis.
3) PLOD2 is an interesting target to block to interfere with synovial fibrosis in OA.
Introduction

Fibrosis is a non-physiological wound healing process characterized by excessive extracellular matrix deposition which is typically the result of inflammation or tissue damage. The accumulation of excess fibrous connective tissue leads to loss of tissue homeostasis and organ failure. Fibrosis is caused by a misbalance between extracellular matrix (ECM) synthesis and degradation and can occur in many tissues within the body including the synovium.

When synovial tissue is affected by fibrosis, which is often the case in OA, it becomes thicker and more rigid (1). Synovial fibrosis contributes to joint pain and stiffness which are the main symptoms of OA (2-4). The underlying mechanisms which cause OA are still not totally unraveled and, apart from joint replacement, no cure is available. This is an unmet need because OA is the most common joint disease and one of most important causes of disability in the elderly (5).

In the past, OA was considered a disease of the cartilage only. Nowadays, OA is recognized as a whole joint disease, not only involving the cartilage, but also the subchondral bone, ligaments, meniscus and the synovium. Understanding how synovial fibrosis contributes to OA pathology and symptoms might provide avenues for future OA therapies. In this review we focus on processes/factors shown to play a role in OA-related synovial fibrosis. This will aid in choosing the best targets to interfere with OA-related fibrosis in future studies.

Synovial fibrosis in OA

The synovium
The synovium can be distinguished in two different layers (which are not clearly separated by a membrane), the intima (synovial lining) which is 1-3 cell layers thick and subintima (sublining) which is up to 5 mm in thickness (in humans) (6). The intima forms an interface between the cavity containing synovial fluid and the subintimal layer. The subintima is composed of loose connective tissue and merges with the dense collagen-rich fibrous outer layer of the joint capsule.

Based on the structure of the subintimal layer, the synovium can be divided in three main types of synovium 1) areolar synovium, 2) fibrous synovium and 3) adipose synovium. Areolar synovium is composed of loose connective tissue and is often crimped in folds. It is highly cellular and has a rich vasculature. Fibrous synovium consist of a dense collagen matrix, that is poorly vascularized containing less synovial lining cells/synoviocytes compared to areolar synovium. Adipose synovium has a single layer of synovial lining cells and contains a high quantity of mature fat cells. (6)

The synovium produces synovial fluid, which is crucial for chondrocyte nutrition, and
protects the cartilage from wear and tear by lubrication. To maintain a healthy fluid, a healthy synovium is required and is therefore crucial for normal joint movement/function (6).

**Synovial abnormalities in OA**

Multiple studies have shown that the synovium of patients, suffering from early or advanced OA, have some form of pathology (7-10). Synovial pathology may impair joint functionality and contribute to disease progression by e.g. increased joint friction (7). Oehler et al. divided osteoarthritic synoviopathy in four different subtypes based on different characteristics of the synovium; hyperplastic, inflammatory, fibrotic and detritus-rich (9). Hyperplastic synoviopathy subtype is only present in early stage OA whereas the inflammatory synoviopathy is found in both early and late stage OA. Fibrotic and detritus-rich synoviopathy were only observed in late stage OA. Whereas no fibrosis was present in the hyperplastic subgroup and only to a minor extent in the inflammatory subgroup, fibrosis was abundantly present in both the fibrotic and detritus-rich synoviopathy. The main difference between the inflammatory, fibrotic and detritus-rich subgroup is that the inflammatory subgroup has less fibrosis but more lymphoplasmacellular infiltrate compared to the other two groups. Furthermore, the detritus-rich group differentiates itself by more fibrinous exudate and the presence of cartilage and bone debris. Instead of fibrotic and detritus-rich synoviopathy, we will use the more general term synovial fibrosis for both synoviopaties in the rest of the article. Although, dividing synoviopathy in different subtypes may help grouping OA-patients and/or disease progression, we have to keep in mind that the observation of synovial fibrosis, at different time points, is patient- and site-dependent. Moreover, in most cases inflammation and fibrosis can co-exist and are inter-dependent.

Kerna et al. reported enhanced level of inflammation, lining layer thickness, number of CD4+ T cells and macrophage infiltration in patients with very early OA compared to late stage OA (11). This confirms the observation by Oehler et al. that in early OA more inflammation was present where in late stage OA more fibrosis was observed (9). These outcomes also support the study of Haraoui et al. that reported that the amount of fibrosis is inversely proportional with the extent of cellular infiltrate in OA synovium, and the observation that fibrosis is mainly but not exclusively found in late stage OA. These results indicate a shift from inflammatory to the fibrotic subgroup which may suggest that the factors inducing fibrosis are upregulated in the inflammatory phase (8).

**Factors involved in synovial fibrosis**

A vast number of factors can contribute to fibrosis, many of which are cell type/disease specific. Therefore, we performed a search for “synovial fibrosis osteoarthritis” via pubmed limited for 2008 till 2015. This gave use 45 results and the following factors were reported to
be elevated in human with OA-related fibrosis (factors, only found to be induced on mRNA level are omitted from the list): Transforming growth factor beta (TGFβ), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) (also known as Lysyl hydroxylase 2b (LH2b)), Tissue inhibitor of metalloproteinase 1 (TIMP-1), A disintegrin and metalloproteinase domain 12 (ADAM12), Prostaglandin F2α (PGF2α), urotensin-II and Mammalian target of rapamycin (mTOR) (12-17). From this list PLOD2, TIMP-1 and mTOR are also shown to be elevated in experimental OA models (13, 17). Furthermore, lysophosphatidic acid is also found elevated in experimental OA (18).

Because we focus on the synovium, mTOR and lysophosphatidic acid were not described in more detail, as these factors were only found elevated in chondrocytes/cartilage and not in synovial fibroblasts or the synovium. Connective Tissue Growth Factor (CTGF) was added to this list because this is a well-known fibrotic factor that also is shown to induce synovial fibrosis.

In addition, we selected from our search results also the factors that are shown to be beneficial against fibrosis in an OA-like setting. Hyaluronan, polysulfated glycosaminoglycan (PS-GAG), parathyroid hormone PTH and Stanozolol were reported to be protective against OA-related fibrosis (Table 1) (19-23). We choose to describe hyaluronan in more detail because this factor is found effective against OA-related fibrosis by multiple groups in different species, were the other factors are only described by one group and for one species. For all the selected factors more additional and background information was acquired via PubMed.

Table 1. Factors found to be protective against OA-related fibrosis

<table>
<thead>
<tr>
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<th>Species</th>
<th>Proposed/Possible mechanism to reduce fibrosis</th>
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<td>Hyaluronan</td>
<td>Ovine, horse, mice</td>
<td>See chapter hyaluronan</td>
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<tr>
<td>Polysulfated glycosaminoglycan</td>
<td>Horse</td>
<td>Decrease in inflammatory mediators</td>
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<tr>
<td>parathyroid hormone</td>
<td>Rabbits</td>
<td>Inhibition of collagen, type 1, alpha 1</td>
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<tr>
<td>Stanozolol</td>
<td>Ovine</td>
<td>Reduced inflammatory phase</td>
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_Transforming growth factor beta (TGFβ) signaling: central in the fibrotic cascade_

TGFβ is the most well-known and best described fibrotic factor and a key player in many pro-fibrotic processes, including epithelial mesenchymal transition, enhancing expression of TIMPs and elevating ECM deposition (24, 25). To our knowledge, TGFβ has been found
involved/elevated in all fibrotic tissues researched so far, for example (but not exclusively): in fibrotic lesions of liver, lung, kidney, skin and heart tissue (26, 27). Furthermore, it has been shown in different fibrotic settings that inhibition of TGFβ signaling attenuates fibrosis whereas overexpression of TGFβ causes fibrosis (26, 28, 29). Recently, it was shown that blocking TGFβ can even diminish pre-existing fibrosis in a rat model of thioacetamide (TAA)-induced hepatic fibrosis (30). Because, the liver might have a higher repair capacity than the synovium it is uncertain whether established synovial fibrosis can be reversed. However, in other model systems, it is shown that pulmonary fibrosis, cardiac and renal fibrosis can be reversed, making it plausible also synovial fibrosis is reversible (31, 32). Furthermore, we showed in the past that CTGF-induced fibrosis is reversible overtime (33).

Ideally, to prevent fibrosis, one would like to block TGFβ, the top of the fibrotic cascade. However, TGFβ is a regulator of many crucial cellular processes. Blocking TGFβ would result in serious side-effects and thus cannot be considered the ultimate cure for fibrosis. Therefore, it is important to identify targets downstream of TGFβ that drive fibrosis to minimize unwanted side effects. To identify these downstream targets of TGFβ for fibrosis therapy one should first understand how TGFβ signals in fibrosis. It is now common knowledge that TGFβ, by binding the TGFβ type II receptor, can signal via two distinct type one receptors, namely ALK5 and ALK1, which in turn phosphorylate receptor-Smads, Smad2/3 and Smad1/5/8, respectively (34). The receptor-Smads can form complexes with the common Smad (Smad4) and translocate to the nucleus to induce gene transcription.

The role of ALK1 in fibrosis is not completely clear and literature on this seems to be inconsistent. For instance, in irradiation-induced kidney fibrosis, ALK1+/− mice developed less inflammation and fibrosis at 20 weeks after irradiation compared to wild type littermates (35). For scleroderma fibroblasts it was demonstrated that ALK5 dependent upregulation of collagen and connective tissue growth factor (CTGF), does not involve Smad2/3 activation but is mediated by ALK1/Smad1 and the TGFβ-induced non-Smad-dependent ERK1/2 pathways (36, 37). These observations indicate a pro-fibrotic role for ALK1. However, ALK1+/− mice with ureteral unilateral obstruction (UUO)-induced kidney fibrosis showed after 15 days significantly higher expression of type I collagen compared to wild-type mice (38). Furthermore, cultured renal fibroblasts from ALK1+/− mice expressed more collagen type I and fibronectin than fibroblasts derived from wild-type mice. These results indicate a more anti-fibrotic role for ALK1, which is in contrast to the studies mentioned above. Apparently within one organ, like kidney, the use of a different model system can result in a different outcome. This suggests that the role of ALK1 is not only cell type and tissue dependent, but also may influenced by the ailment of the tissue (39).

ALK5-mediated signaling is known to induce most of TGFβ’s pro-fibrotic effects and inhibition of ALK5 have been shown to repress fibrosis in several fibrotic diseases (40-42). Because ALK5 signals via both Smad2 and Smad3, which can potentially have different
effects, their individual roles in fibrosis have been investigated, most frequently in epithelial cells. In these cells Smad3 acts pro-fibrotic whereas Smad2 protects against Smad3-mediated fibrosis (43-45). Because the specific roles of either smad2 or smad3 can be tissue-dependent the individual functions of Smad2 and Smad3 in the synovium have yet to be determined.

Besides the TGFβ-Smad pathways which are well described in general and specifically regarding fibrosis, there are also Smad-independent TGFβ signaling pathways. The Smad-independent TAK-1 pathway is shown to have pro-fibrotic effects by regulating the expression of extracellular matrix proteins, including collagens and fibronectin (46). Furthermore, in a TGFβ-driven murine model of dermal fibrosis, inhibition of TGFβ-dependent ERK phosphorylation showed strong and dose-dependent anti-fibrotic effects on skin thickening (47). This indicates that not only the TGFβ-Smad pathways, but also the Smad-independent TGFβ signaling pathways have pro-fibrotic properties. Unfortunately, not much is known about these Smad-independent TGFβ signaling pathways concerning synovial fibrosis and their functions in the synovium, which puts limitations on selecting the optimal target to interfere with synovial fibrosis. These non-Smad signaling factors are central mediators in multiple pathways, which makes their mechanism of action very elaborate and therefore they are potentially less suitable as targets to interfere with synovial fibrosis.

Connective Tissue Growth Factor (CTGF), TGFβ’s right hand in the fibrotic cascade

CTGF is also known as CCN family protein 2 (CCN2). A primary function of CTGF is to modulate and coordinate signaling responses involving cell surface proteoglycans, key components of the extracellular matrix, and growth factors (48). During adulthood CTGF is expressed in endothelia and neurons in the cerebral cortex, where it promotes angiogenesis and tissue integrity, and in the female reproductive tract were it regulates both follicle development and ovulation (49-51). In addition CTGF is expressed in wound healing, vascular diseases and fibrosis (52-54).

CTGF is like TGFβ found to be elevated in many fibrotic diseases. There is no unique receptor known for CTGF to which it binds with high affinity, and therefore CTGF is considered a matricellular protein that modulates the interaction of cells with the matrix which modifies the cellular phenotype (55). It is suggested by Leask et al. that CTGF mediates its effects through integrin- and heparin sulfate proteoglycan dependent mechanisms and that the ability of CTGF to bind cell surface heparin sulfate proteoglycans, which are highly present in the joint, is essential for CTGF activity (56).

Because, no data is available about the interaction between TGFβ and CTGF in the synovium, we will discuss “in our opinion” the best alternative, cellular signaling responses in fibroblasts in other tissues. As CTGF is a potent enhancer of fibroblast proliferation, chemotaxis, and extracellular matrix (ECM) deposition, CTGF is thought to mediate some of TGFβ’s fibrogenic effects after being upregulated by TGFβ (48, 57). Furthermore, CTGF decreases Smad7, an
inhibitory Smad which can inhibit TGFβ-signaling on multiple levels, and via this mechanism promoting TGFβ signaling (58). The mechanism by which CTGF regulates Smad7 is not yet fully unraveled. However, one proposed mechanism in what way CTGF inhibits Smad7 is by induction of TIEG-1, which is upregulated via the TrkA signaling receptors for CTGF (59, 60). Depletion of CTGF in foreskin fibroblasts with the use of adenoviral CTGF siRNA almost completely abrogated TGFβ-induced upregulation of collagen synthesis, indicating CTGF not only enhances some of the pro-fibrotic effects of TGFβ but is also obligatory for certain pro-fibrotic effects (61).

Where others have demonstrated that only the combination of TGFβ and CTGF leads to persistent fibrosis, we have previously published that overexpression of TGFβ alone causes persistent synovial fibrosis whereas CTGF alone in murine knee joint only causes transient synovial fibrosis (28, 29, 33, 62). Because TGFβ is a potent inducer of CTGF we cannot rule out the possibility that it is essential for the induction of persistent synovial fibrosis. It is suggested by Wang et al. that the threshold level of CTGF necessary to induce persistent fibrosis may not be always reached by injecting TGFβ alone (29). Therefore, a possible explanation for this discrepancy might be that overexpressing Ad-TGFβ results in higher levels of TGFβ and subsequently higher CTGF levels compared to protein injection of TGFβ and therefore might reach the CTGF threshold required to induce persistent synovial fibrosis. In addition, both the synovial fibroblasts as well as the chondrocytes in the cartilage strongly induce CTGF expression upon TGFβ stimulation (15, 63). Our finding that CTGF can cause transient fibrosis is in line with the observations that CTGF by itself can promote collagen synthesis. However, one or more additional factors, elevated by TGFβ, seem to be required to induce persistent fibrosis (57, 64).

To validate CTGF as a potential anti-fibrotic target, it is important to determine whether CTGF is necessary for the persistence of TGFβ-induced synovial fibrosis. Especially because a CTGF blocking antibody (FG-3019) is available. This antibody attenuated the fibrotic response in three independent models of fibrosis, A model of multiorgan fibrosis induced by repeated intraperitoneal injections of CTGF and TGFβ, an unilateral ureteral obstruction (UUO) renal fibrosis model; and an intratracheal bleomycin instillation model of pulmonary fibrosis (29).

**procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2)**

PLOD2 is a collagen cross-linking enzyme, which activity induces the formation of pyridinoline cross-links (65). Increased expression of Plod2 mRNA is found in different fibrotic fibroblasts (66). Also the pyridinoline cross-links, which make collagen fibrils less susceptible to enzymatic degradation and more rigid, are found to be elevated in different fibrotic tissues (66). Diminished collagen degradation as a result of more pyridinoline cross-links per collagen triple helix, results in collagen accumulation, which is one of the hallmarks
of fibrosis. One of the most potent inducers of PLOD2 is TGFβ (15, 67). However, for skin fibroblasts it was shown that also IL-4, BMP-2, activin A and TNF-α can enhance PLOD2 expression (67). For synovial fibroblasts it was shown that besides TGFβ also prostaglandin f2α induces PLOD2 expression (14).

We observed in OA-induced fibrosis, that PLOD2 expression as well as the number of pyridinoline cross-links per collagen triple helix in the synovium were elevated (28). Most importantly, we also found elevated levels of PLOD2 in human end-stage OA synovium (13). Because the presence of fibrosis in these OA patients was unknown, the average PLOD2 level might be even higher in the subpopulation of OA patients with fibrosis. This elevation suggests that PLOD2 may be crucial in OA-related synovial fibrosis. At this moment, to our knowledge no blocking or overexpression studies of PLOD2 exist, to determine its direct function in the fibrotic process. However, based on the function of PLOD2 and the fact that it is highly induced in OA synovium, PLOD2 is an appealing target to interfere with synovial fibrosis.

**Tissue inhibitor of metalloproteinase 1 TIMP-1**

TIMP-1 is an inhibitor of the matrix metalloproteinases (MMPs), peptidases involved in extracellular matrix degradation, and is found to be elevated in different fibrotic diseases e.g. pulmonary, liver and kidney fibrosis (68-70). We found that TIMP-1 is elevated in both the synovium of human end-stage OA patients and mice with experimental OA (13). TIMP1 is induced by TGFβ and is typically proposed as an enhancer of fibrosis development but does not induce fibrosis itself (69, 70). Inhibition of TIMP-1 is expected to result in higher MMP activity and therefore more ECM breakdown, which might be beneficial to diminish fibrosis. However, in unilateral urethral obstruction-induced fibrosis there was no difference in the degree of interstitial fibrosis between wild-type and TIMP1-deficient mice (71). Most likely the role of TIMP-1 may vary between different types of fibrosis and its role in synovial fibrosis has yet to be discovered.

**Urotensin II (U-II)**

Urotensin II is a potent vasoconstrictor which is involved in cardiac remodeling and may influence cardiovascular homeostasis and pathology (72, 73). Furthermore, it may also influence the central nervous system and endocrine function in man (73). In different fibrotic diseases, for example hepatic-, pulmonary- and cardiac fibrosis, urotensin II levels are elevated (74-77). Moreover the authors of these articles suggest that urotensin II is involved in the development of fibrosis. Most fascinating, urotensin II levels were also reported to be significantly higher in the synovial fluids of the osteoarthritis patients compared to control and may be associated with synovial fibrosis in osteoarthritis (16). It is reported that urotensin II may stimulate collagen synthesis via the ERK1/2 and TGFβ/Smad2/3 signaling
pathway and in this way contribute to fibrosis (72, 78). The exact signaling mechanism of urotensin II is however, largely unknown. Therefore more knowledge is needed about the interplay between urotensin II and TGFβ-signaling in synovial fibroblast and its potential role in synovial fibrosis.

**A disintegrin and metalloproteinase domain 12 (ADAM12)**

ADAM12 is primarily involved in cell adhesion and fusion, extracellular matrix restructuring, and cell signaling. There are two different splice variants, a shorter secreted form (ADAM12-S) and a longer membrane-bound form (ADAM12-L) (79). Elevated serum levels of ADAM12-S are associated with elevated serum inflammatory markers, severity of skin fibrosis, and increased activity of interstitial lung disease in diffuse cutaneous systemic sclerosis, suggesting a pro-fibrotic role for ADAM12 (80). Furthermore ADAM12-L was found to be elevated in the cartilage of OA patients (81). Most interestingly both ADAM12-S and ADAM12-L were upregulated in synovial tissue of patients with OA and positively correlated with the grade of synovial fibrosis, suggesting a role for ADAM12 in OA-related synovial fibrosis (11, 81).

ADAM12 is potently induced by TGFβ on both mRNA and protein level in different cell types including fibroblasts, enhancing epithelial to mesenchymal transition (EMT), a-SMA expression and ECM production (82-84). The proposed mechanism by which ADAM12 induces its pro-fibrotic effects, is by positively regulating TGFβ signaling, due to the stabilization of the TβRII protein (85). This stabilization might be accomplished by suppressing the association of TβRII with Smad7, which prevents the receptor complex degradation by Smad7 (85, 86). In line with these data, it was shown in hepatic stellate cells that adding ADAM12 stimulates TGFβ-induced-phosphorylation of Smad2/3, whereas treatment of cells with antisense to ADAM12 diminishes the TGFβ–dependent induction of TGFβ-induced Smad2P (Smad3P was not measured in this study) as well as COL1A2 mRNA expression (86, 87). ADAM12 can therefore be an important modulator of TGFβ-induced fibrosis.

**Prostaglandin F2α (PGF2α)**

PGF2α normally regulates a number of important physiological functions, like uterine contraction and bronchoconstriction. However, elevated plasma concentrations of PGF2α metabolites found in idiopathic pulmonary fibrosis are significant associated with both disease severity and prognosis (88). Oga et al. have shown that both PGF2α and TGFβ increased the promoter activity of COL1A2, and simultaneous addition of both factors synergistically increased the COL1A2 promoter activity (89). Furthermore, prostaglandin F2α deficiency and inhibition of TGFβ signaling additively decrease fibrosis in mice with idiopathic pulmonary fibrosis, suggesting that TGFβ and PGF2α recruit different signaling molecules to induce collagen production (89). These results indicate that PGF2α has pro-fibrotic effects that works independently of TGFβ.
The PGF2α isoforms 8-iso-PGF2α and 15-keto-dihydro-PGF2α were found significantly increased in the synovium fluid of patients with OA (90). Also relative high levels of PGF2α were measured in infrapatellar fat pad (from OA patients) conditioned medium (FCM) (14). Collagen production by fibroblast-like synoviocytes was positively associated with PGF2α levels in this FCM. In addition, gene expression of the collagen crosslinking gene, Plod2 was increased in fibroblast-like synoviocytes in the presence of this FCM. Inhibition of PGF2α levels reduced the extent of FCM-induced collagen production and Plod2 expression whereas inhibition of the TGFβ-ALK5 pathway with SB505124 did not alter the FCM-induced effects on fibroblast-like synoviocytes. These results indicate that elevated levels of PGF2α and its isoforms are present in an OA joint and that PGF2α has pro-fibrotic effects on the synovium that might differ from those induced by TGFβ.

Hyaluronan

Hyaluronan, a glycosaminoglycan that binds the CD44 receptor. Injection of hyaluronan 24 h after TGFβ injection in the TGFβ prior to treadmill running model of OA inhibited the cascade of OA-like joint changes including gait changes and synovial fibrosis. Furthermore, hyaluronan injection post-surgery in the meniscectomy-induced OA model in sheep reduced synovial fibrosis (23). These results show that hyaluronan protects against OA-related fibrosis in both “intra-articular injection of TGFβ prior to treadmill running” model of OA and the meniscectomy-induced OA model in sheep (22, 23). This is in consensus with the observation that exogenously provided hyaluronan antagonized TGFβ1-dependent myofibroblast differentiation (91). However, the exact mechanism how hyaluronan interferes with synovial fibrosis is unknown. One suggested mechanism by Plaas et al., is that hyaluronan may act as anti-fibrotic by blocking ADAMTS5-mediated activation of profibrotic pathways in periarticular cells (92). This, because hyaluronan can form a complex with Adamts5 and ablation of Adamts5 is shown to prevent both cartilage erosion and fibrotic remodeling in challenged joints (93). Another possible explanation is that interaction of hyaluronan with its receptor results in an increase in the association of the TGFβ receptor with Smad7, leading to TGFβ receptor degradation (85). This degradation leads to a decrease in TGFβ-signaling and therefore in less fibrosis. That hyaluronan may be beneficial to reduce fibrosis by attenuating TGFβ-signaling, again suggests the major role of TGFβ signaling in fibrosis.

Targets to block synovial fibrosis in OA

The main cause of synovial fibrosis seems to be TGFβ-ALK5 signaling. Unfortunately, blocking ALK5 may not be without any consequences for the cartilage. This because ALK5 is reported to promote type II collagen and aggregcan expression in chondrocytes (94, 95). Additionally, TGFβ-Smad3 signaling, which is mediated via ALK5, suppresses MMP13 expression, and is
reported in multiple papers to repress chondrocyte hypertrophic differentiation and therefore OA (96-98). In contrast, there are also papers that propose that transition from an ALK5-mediated fibrogenic signaling to ALK1-mediated signaling in joint cells represents a transition from a nonreparative to a reparative chondrogenic cell phenotype (99). Taking all these results together indicate that inhibition of ALK5 comes with a certain risk for the cartilage and therefore better not can be inhibited in an OA-joint. Unless it is specifically blocked in the synovium to prevent fibrosis, which is unfortunately not yet possible.

Whether inhibition of ALK1 in an OA joint has pro- or anti-fibrotic effects remains to be elucidated. This may be worth investigating because besides the potential anti-fibrotic effects, inhibition of ALK1 is expected to reduce MMP13 expression in chondrocytes and therefore MMP mediated cartilage damage: a potential win-win situation (94, 95). However, to minimize potential side effects, inhibition of a gene with as single or limited function has the preference over blocking genes with multiple function or that are at the top of an extensive signaling pathway, such as TGFβ or PGF2α. In this regard the two most attractive options of the discussed factors are TIMP-1 and PLOD2 (Table 2). A major drawback of targeting TIMP1 in an OA joint is that elevated MMP activity will contribute to cartilage damage (100). Therefore inhibition of TIMP-1 in an OA-joint is not a preferred option to interfere with OA-related synovial fibrosis. PLOD2 on the other hand is a potential target to block in synovial fibrosis. Noteworthy, cartilage containing high levels of pyridinoline collagen cross-links, which are increased due to PLOD2 activity, seems to fail mechanically in long term loading whereas areas containing low pyridinoline levels are less prone to degeneration (101). This suggests that inhibition of PLOD2, besides the potential anti-fibrotic effects may also favor cartilage repair in an OA joint. Unfortunately, there are no small molecular inhibitors available yet to block PLOD2. Therefore alternative methods to target this intracellular enzyme in vivo, perhaps siRNA, have to be used. We look forward to an experiment were PLOD2 is blocked in an OA-model accompanied by fibrosis to determine whether this approach indeed prevent synovial fibrosis.
Table 2. Pros and cons of inhibiting CTGF, PLOD2 or TIMP1 for the synovium and cartilage.

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Expected effect on the synovium</th>
<th>Expected effect on the cartilage</th>
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<tbody>
<tr>
<td><strong>CTGF inhibition</strong></td>
<td>Pro</td>
<td>Less collagen synthesis. might attenuate the inflammatory cascade. (44, 52, 88)</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>unknown</td>
</tr>
<tr>
<td><strong>PLOD2 inhibition</strong></td>
<td>Pro</td>
<td>Less pyridinoline cross-links in the collagens triple-helixes. Prevents that the collagen becomes harder to degrade and therefore prevents long lasting collagen accumulation. (16, 54, 55, 90)</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>unknown</td>
</tr>
<tr>
<td><strong>TIMP1 inhibition</strong></td>
<td>Pro</td>
<td>More collagen degradation, as a result of more MMP activity may reverse the fibrosis. (91)</td>
</tr>
<tr>
<td></td>
<td>Con</td>
<td>unknown</td>
</tr>
</tbody>
</table>
Discussion

Because it is estimated that over half of all OA patients suffer from synovial fibrosis it is important that this pathological process receives more attention. Especially because fibrosis is one of the main causes of joint stiffness (2-4, 102). At present, there are no options to interfere with synovial fibrosis, however, preventing or reversing fibrosis in OA might result in major symptom relieve. The goal of this review was to provide an overview of the known factors that play a role in initiating and sustaining synovial fibrosis to facilitate selection of targets for anti-fibrotic therapies.

Several factors can contribute to an excessive deposition of the ECM, which will result in synovial fibrosis by either increasing ECM synthesis or decreasing its degradation. The majority of these pro-fibrotic factors are downstream of TGFβ or modulate TGFβ signaling. There are other pathways that may contribute to synovial fibrosis independently of TGFβ, for instance PGF2α. It is hard to predict the relative contribution of those factors to the fibrotic process. Blocking studies will have to elucidate whether inhibition of one of these factors will break the fibrotic cascade in synovial fibrosis.
Outline of this thesis.

Besides cartilage damage and osteophyte formation, synovial fibrosis is one of the characteristics of osteoarthritis (OA). More than 50% of the patients with end stage OA suffer from synovial fibrosis (chapter 1). This fibrosis greatly contributes to joint stiffness, one of the main symptoms of OA.

In previous experiments, we found that overexpression of Transforming Growth Factor beta (TGFβ), a growth factor involved in a multitude of cellular functions, causes persistent synovial fibrosis in murine knee joints, whereas Connective Tissue Growth Factor (CTGF) only induces transient fibrosis. Furthermore, inhibition of TGFβ prevents OA-related fibrosis in murine experimental OA models. Because TGFβ-signaling has an important role in cartilage maintenance and repair, simply blocking TGFβ in an OA joint may have adverse effects and is for that reason not suitable to treat OA-related fibrosis. Therefore we searched for targets downstream of TGFβ to prevent OA-related fibrosis without interfering with joint homeostasis (chapter 2). The two genes, LH2b and TIMP1, which were the highest up-regulated in different OA-related fibrotic conditions, were also examined on protein level on histology of murine knee joints with collagenase-induced OA.

Because we were very intrigued by our earlier observation that showed that TGFβ caused persistent fibrosis whereas CTGF only induced transient fibrosis, we wanted to elucidate the origin of this difference (chapter 3). Therefore, we compared the expression of a number of genes thought to be relevant in fibrosis in synovium obtained from murine knee joints exposed to either TGFβ or CTGF. Various extracellular matrix (ECM) components, ECM degraders, growth factors and collagen cross-linking-related genes were compared. The most pronounced differences between persistent (TGFβ) and transient (CTGF) fibrosis was the high LH2b expression in the TGFβ-induced fibrosis group. LH2b activity results in the formation of pyrodinoline cross-links, which make collagen harder to degrade and more rigid, and is reported to be elevated in different fibrotic tissues. For that reason we investigated whether these pyridinoline cross-links were elevated in an experimental OA model accompanied by fibrosis and therefore, may influence the persistence of OA-related fibrosis.

To test our hypothesis that these pyridinoline cross-links are responsible for the persistence of synovial fibrosis we overexpressed CTGF combined with LH2b to examine whether this will make the CTGF-induced transient fibrosis persistent (chapter 4). Because LH2b appeared to be a highly attractive target to interfere with the fibrotic process, we wanted to elucidate via which signaling routes TGFβ activate LH2b (Chapter 5). Finally, we summarized the findings of this thesis and gave our final considerations (chapter 6).

The general aim of this study was to elucidate the involvement of LH2b in OA-related synovial fibrosis.
References


96 Chen CG, Thuillier D, Chin EN, Alliston T. Chondrocyte-intrinsic Smad3 represses Runx2-inducible matrix metalloproteinase 13 expression to maintain articular cartilage and prevent osteoarthritis. Arthritis Rheum 2012;64(10):3278-89.


Chapter 2

Gene expression analysis of murine and human osteoarthritis synovium reveals elevation of TGFβ–responsive genes in osteoarthritis-related fibrosis

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Abstract

Objective. Synovial fibrosis is a major contributor to joint stiffness in osteoarthritis (OA). Transforming growth factor beta (TGFβ), which is elevated in OA, plays a key role in the onset and persistence of synovial fibrosis. However, blocking of TGFβ in OA as a therapeutic intervention for fibrosis is not an option since TGFβ is crucial for cartilage maintenance and repair. Therefore, we undertook the present study to seek targets downstream of TGFβ for preventing OA-related fibrosis without interfering with joint homeostasis.

Methods. Experiments were performed to determine whether genes involved in extracellular matrix turnover were responsive to TGFβ and were elevated in OA-related fibrosis. We analyzed gene expression in TGFβ-stimulated human OA synovial fibroblasts and in the synovium of mice with TGFβ-induced fibrosis, mice with experimental OA, and humans with end-stage OA. Gene expression was determined by microarray, low-density array, or quantitative polymerase chain reaction analysis.

Results. We observed an increase in expression of procollagen genes and genes encoding collagen cross-linking enzymes under all of the OA-related fibrotic conditions investigated. Comparison of gene expression in TGFβ-stimulated human OA synovial fibroblasts, synovium from mice with experimental OA, and synovium from humans with end-stage OA revealed that the genes PLOD2, LOX, COL1A1, COL5A1, and TIMP1 were upregulated in all of these conditions. Additionally, we confirmed that these genes were upregulated by TGFβ in vivo in mice with TGFβ-induced synovial fibrosis.

Conclusion. Most of the upregulated genes identified in this study would be poor targets for therapy development, due to their crucial functions in the joint. However, the highly upregulated gene PLOD2, responsible for the formation of collagen crosslinks that make collagen less susceptible to enzymatic degradation, is an attractive and promising target for interference in OA-related synovial fibrosis.
**Introduction**

Synovial fibrosis, characterized by fibroblast proliferation and an imbalance between collagen synthesis and collagen degradation, is a hallmark of osteoarthritis (OA) (1). This imbalance results in excessive deposition of collagen into the extracellular matrix (ECM) leading to thickening and stiffening of the synovial membrane, which is believed to be a major contributor to both joint pain and joint stiffness (2, 3). A key event in the onset of fibrosis is the response of fibroblasts to transforming growth factor beta (TGFβ) (4). TGFβ levels have been found to be elevated in the synovial fluid of OA patients (5–7); since large amounts of TGFβ are stored in cartilage, its elevated concentrations in the synovial fluid could be the result of OA-related cartilage damage (8). We have previously demonstrated that intraarticular injection of adenoviral TGFβ into murine knee joints results in persistent synovial fibrosis and, more importantly, that blocking of TGFβ in experimental OA strongly reduces synovial fibrosis (9–11). These findings implicate TGFβ as the driving force in OA-related fibrosis.

However, inhibition of TGFβ locally in the joints to prevent fibrosis in OA is not a feasible treatment option because TGFβ has an essential role in cartilage homeostasis (12): TGFβ and Smad2/3 signaling gives rise to the main cartilage components aggrecan and type II collagen and suppresses matrix metalloproteinase 13 (MMP-13) (13). To overcome this problem we sought to identify genes downstream of TGFβ that are elevated in OA-related synovial fibrosis and therefore could be potential targets for antifibrotic therapy in OA. We selected genes that encode for collagens, collagen-modifying enzymes, proteinases, and collagen receptors (Table 1). The proteins/enzymes encoded by these genes affect collagen synthesis and degradation at different levels and could therefore influence the onset and progression of fibrosis. For example, collagen crosslinking enzymes, which are necessary for formation of stable fibrils, affect the susceptibility of collagen molecules to MMP activity. A collagen network with a high amount of pyridinoline crosslinks is less susceptible to degradation by MMPs, which may lead to collagen accumulation and eventually fibrosis (14). In addition, reduced MMP activity or increased expression of tissue inhibitor of metalloproteinases (TIMP) could be an alternative cause of collagen accumulation.

In the present study, complementary DNA (cDNA) obtained from a variety of sources was investigated in order to elucidate which of the above-described genes were upregulated by TGFβ and/or elevated in OA. Specifically, our studies were performed using cDNA from TGFβ-stimulated human OA synovial fibroblasts, synovium from mice with experimental OA or TGFβ-induced fibrosis, and most importantly, synovium from the affected joints of humans with end-stage OA.
Table 1. All extracellular matrix turnover–related genes measured in transforming growth factor beta–stimulated fibroblasts from patients with osteoarthritis (OA), synovium from patients with end-stage OA, and synovium from mice with collagenase-induced OA.

<table>
<thead>
<tr>
<th>Gene category, protein or enzyme encoded</th>
<th>Gene name</th>
<th>Gene category, protein or enzyme encoded</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-modifying enzymes</td>
<td></td>
<td>Collagens</td>
<td></td>
</tr>
<tr>
<td>Lysyl hydroxylase 1</td>
<td>PLOD1</td>
<td>Collagen type I 1 chain</td>
<td>COL1A1</td>
</tr>
<tr>
<td>Lysyl hydroxylase 2</td>
<td>PLOD2</td>
<td>Collagen type I 2 chain</td>
<td>COL1A2</td>
</tr>
<tr>
<td>Lysyl hydroxylase 3</td>
<td>PLOD3</td>
<td>Collagen type III 1 chain</td>
<td>COL3A1</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase 1</td>
<td>P4HA1</td>
<td>Collagen type IV 1 chain</td>
<td>COL4A1</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase 2</td>
<td>P4HA2</td>
<td>Collagen type V 1 chain</td>
<td>COL5A1</td>
</tr>
<tr>
<td>Prolyl 4-hydroxylase 3</td>
<td>P4HA3</td>
<td>Proteinases</td>
<td></td>
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<td>Protein disulfide isomerase</td>
<td>P4HB</td>
<td>Collagenase 1</td>
<td>MMP1</td>
</tr>
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<td>Prolyl 3-hydroxylase 1</td>
<td>LEPRE1</td>
<td>Collagenase 2</td>
<td>MMP8</td>
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<td>LEPREL1</td>
<td>Collagenase 3</td>
<td>MMP13</td>
</tr>
<tr>
<td>Prolyl 3-hydroxylase 3</td>
<td>LEPREL2</td>
<td>Membrane type 1 matrix metalloproteinase</td>
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<td>Lysyl oxidase</td>
<td>LOX</td>
<td>Tissue inhibitor of metalloproteinases 1</td>
<td>TIMP1</td>
</tr>
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<td>Lysyl oxidase–like 1</td>
<td>LOXL1</td>
<td>Cathepsin K</td>
<td>CTSK</td>
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<td>Lysyl oxidase–like 2</td>
<td>LOXL2</td>
<td>ADAMTS-1, 2</td>
<td>ADAMTS2</td>
</tr>
<tr>
<td>Lysyl oxidase–like 3</td>
<td>LOXL3</td>
<td>ADAMTS-1, 3</td>
<td>ADAMTS3</td>
</tr>
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<td>Lysyl oxidase–like 4</td>
<td>LOXL4</td>
<td>ADAMTS-1, 14</td>
<td>ADAMTS14</td>
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<td>Heat-shock protein 47</td>
<td>SERPINH1</td>
<td>Bone morphogenetic protein 1</td>
<td>BMP1</td>
</tr>
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<td>Procollagen C-endopeptidase enhancer</td>
<td>PCOLCE</td>
<td>Collagen receptors</td>
<td></td>
</tr>
<tr>
<td>Procollagen C-endopeptidase enhancer 2</td>
<td>PCOLCE2</td>
<td>Discoidin domain receptor family, member 1</td>
<td>DDR1</td>
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<td>MEP1A</td>
<td>Discoidin domain receptor family, member 2</td>
<td>DDR2</td>
</tr>
<tr>
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<td>MEP1B</td>
<td>Endocytic receptor 180</td>
<td>MRC2</td>
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<td>Glycosyltransferase 25 domain-containing 1</td>
<td>GLT25D1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycosyltransferase 25 domain-containing 2</td>
<td>GLT25D2</td>
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<td></td>
</tr>
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<td>FKBP10</td>
<td></td>
<td></td>
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<tr>
<td>Solute carrier family 39 member 13</td>
<td>SLC39A13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cartilage-associated protein</td>
<td>CRTAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptidylprolyl isomerase B</td>
<td>PPIB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Materials and methods

Human OA synovial fibroblasts.
To examine which ECM turnover–related genes were responsive to TGFβ, we isolated human OA fibroblasts. Human synovium was obtained, with written informed consent, from 8 OA patients at the time of knee arthroplasty. The synovium was cut into samples of 3 mm³, and the samples were placed in a 6-well plate (3 samples/well). Fibroblasts were allowed to grow out of the tissue and to proliferate for 4 weeks, after which they were transferred to a culture flask and cultured for a maximum of 10 passages in monolayer in BME medium (Gibco Invitrogen) enriched with 10% fetal calf serum (Thermo Scientific HyClone), 50 g/ml gentamicin (Centrafarm), and 1 mM sodium pyruvate (Gibco Invitrogen) (15). The fibroblasts were then cultured in a 24-well plate (Greiner Bio-One, CellStar) in serum-free medium for 24 hours and subsequently stimulated with 10 ng TGFβ 1 (BioLegend) in serum-free medium for a further 24 hours. At the end of the experiment the cells were harvested in 0.5 ml Tri Reagent (Sigma). Gene expression levels in the TGFβ-stimulated fibroblasts were compared to those in unstimulated controls.

Animals.
Twelve-week-old male C57BL/6J mice were used for animal studies. The mice were kept in filtertop cages with wood chip bedding. They were fed a standard diet with tap water adlibitum. All animal experiments were approved by the Radboud University Nijmegen Medical Centre Committee on Animal Research and Ethics.

Collagenase-induced OA.
Experimental OA was induced in mice by intra articular injection, on day 0 and day 2, of 1 unit of bacterial collagenase (Sigma) in a total volume of 6 μl 0.9% NaCl. The injection of bacterial collagenase leads to joint laxity and subsequent OA lesions (16). This model represents an equivalent of human secondary OA resulting from joint instability. Mice were killed on day 7, 21, or 42 (n = 8 per group) and synovial samples were obtained for RNA isolation. Synovium obtained from the contralateral uninjected knee joints was used as a control.

Isolation of messenger RNA (mRNA) from human OA synovial fibroblasts.
Human OA synovial fibroblasts harvested in Tri Reagent as described above were incubated with 100 μl chloroform. After 3 minutes of incubation the suspensions were centrifuged at 11,000 revolutions per minute for 15 minutes at 4°C. Supernatants (240 μl) were obtained and mixed with 240 μl isopropanol. This mixture was incubated for 10 minutes at room temperature and subsequently centrifuged at 11,000 rpm for 10 minutes at 4°C. The RNA
pellets obtained were washed twice with 75% ethanol, dried, and dissolved in 8 μl RNase-free water (Fresenius Kabi). To remove chromosomal DNA contamination in the isolated mRNA samples, the samples were treated with DNase I (Invitrogen).

**Isolation of mRNA from murine and human synovial tissue.**
RNA isolation from murine or human synovium was performed with an RNeasy Mini kit (Qiagen). Synovial tissue samples were placed in RLT buffer (provided in the kit) in MagNA Lyser Green Beads tubes (Roche) and disrupted in the MagNA Lyser for 4 cycles of 20 seconds at 6,500 rpm with 1 minute of cooling on ice between cycles. The remainder of the isolation procedure was performed according to the protocol recommended by the manufacturer.

**Reverse transcription–polymerase chain reaction (RT-PCR).**
Isolated mRNA was transcribed into cDNA before being used for quantitative PCR (qPCR) or low-density array analysis. Eleven microliters (1 μg) of mRNA was mixed with 9 μl RT-PCR mix (1.9 μl distilled water, 2.4 μl 10× DNase buffer, 2.0 μl 0.1M dithiothreitol, 0.8 μl dNTPs [each at 12.5M], 0.4 μl oligo[dT] primer [0.5 μg/μl], 1.0 μl reverse transcriptase [200 μg/μl; Invitrogen], and 0.5 μl RNasin [Promega]). The RT-PCR protocol was as follows: 5 minutes at 25°C, followed by 60 minutes at 39°C and a final step of 5 minutes at 94°C. After the RT-PCR procedure, the cDNA was diluted 20 times for further use in low-density array or qPCR analysis.

**Low-density array analysis.**
For each sample studied by low-density array analysis, 300 ng cDNA was loaded onto a custom-made 384-well TaqMan plate and run on a ViaA7 Real-time PCR system (Applied Biosystems). Expression levels of the genes of interest were corrected for GAPDH levels and for levels in control samples, and relative mRNA levels ($2^{ΔΔCt}$) were calculated.

**Microarray analysis of murine synovium.**
Synovial samples obtained from the mice with collagenase-induced OA were analyzed using Affymetrix GeneChip Genome 430 2.0 arrays. Three arrays of pooled synovia from 2 mice were used at each time point. For microarray analysis of both human and murine synovial tissue, the quality of RNA was assessed using an Agilent Bioanalyzer. RNA (1 μg) was labeled and hybridization was performed according to the Affymetrix Expression Analysis Technical Manual for 1-cycle amplification. Array data were analyzed using GeneChip operating software (version 1.4) and Partek Genomics Suite software.
Microarray analysis of human OA synovium.
To examine which of the selected ECM-related turnover genes were elevated in human OA, synovial tissue samples were obtained, with written informed consent, from 7 OA patients at the time of arthroplasty. Synovial biopsy specimens obtained during an arthroscopic procedure in 5 patients with suspected acute trauma of the knee joint were used as control samples. Human samples were analyzed using Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays.

Quantitative PCR.
For qPCR, a primer mix consisting of 1 μl (2.5 μM) forward and reverse primer (see Supplementary Table 1, on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38266/abstract) was added to 5 μl SYBR Green Master Mix (Applied Biosystems). Next cDNA (3 μl) was added and the qPCR was run on a StepOnePlus Real-Time PCR System according to the instructions of the manufacturer (Applied Biosystems). Relative mRNA levels (2^ΔΔCt) were calculated by correcting the genes of interest for GAPDH levels and levels in control samples.

Induction of fibrosis by TGFβ overexpression.
To induce fibrosis, the right knees were injected intraarticularly with 1 × 10^7 plaque-forming units of an adenovirus overexpressing active porcine TGFβ 1 (Ad-TGFβ 223/225) in 6 μl 0.9% NaCl (Fresenius Kabi). Ad-luciferase was used as a viral control. The mice were killed on day 7 after injection for histologic analysis or on day 21 after injection for gene expression analysis (n = 5 per group). Synovial biopsy specimens from individual mice were obtained for RNA isolation.

Histologic and immunohistochemistry analyses.
Mouse knee joints were processed and immunohistochemically stained as previously described (17). Specific primary antibodies against lysyl hydroxylase 2 (LH-2) (1:150; Proteintech) or TIMP-1 (R&D Systems) were incubated overnight at 4°C. Incubation with secondary antibody (biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG [both from Vector]) was performed for 1 hour. A biotin–streptavidin detection system was used according to the instructions of the manufacturer (Vector). Sections were counterstained with hematoxylin and mounted with Permount.
Statistical analysis.
Statistical analysis of the low-density array and qPCR data was performed with SPSS 18.0. All low-density array and qPCR data were first checked for normality, by Shapiro-Wilk test. Student’s paired t-test was used to assess the significance of differences between groups in the human OA synovial fibroblast experiments. To assess the significance of differences between groups in the TGFβ-induced fibrosis experiments, an independentsample t-test was performed. The significance of differences between non-normally distributed groups was determined by Wilcoxon’s signed rank test. Analysis of variance with step-up false discovery rate correction for multiple testing (Benjamini and Hochberg method) was used to determine the significance of microarray results. P values less than 0.05 were considered significant.
Results

Induction by TGFβ of genes encoding for collagen and crosslinking enzymes in human OA synovial fibroblasts.

Expression levels of ECM turnover–related genes were measured in TGFβ-stimulated human OA synovial fibroblasts on a low-density array, to determine which of the selected genes were downstream of TGFβ. We found increases in expression of the collagen genes COL1A1 (4.6-fold), COL3A1 (2.5-fold), COL4A1 (4.8-fold), and COL5A1 (3.7-fold) (Table 2), indicative of increased collagen synthesis, which is characteristic of fibrosis. Moreover, expression of several genes encoding for crosslinking enzymes was elevated after TGFβ stimulation, with PLOD2 exhibiting the highest increase (25.2-fold). The other genes encoding for crosslinking enzymes that were upregulated by TGFβ were LOX (4.5-fold), LOXL2 (3.7-fold), LOXL3 (10.7-fold), and LOXL4 (3.9-fold). Furthermore, levels of the collagen-modifying enzymes PLOD3, P4HB, and LEPRE1 were elevated (4.6-fold, 2.2-fold, and 2.6-fold, respectively). These results show that genes encoding for collagens and crosslinking enzymes are highly responsive to TGFβ in OA synovial fibroblasts, with the latter regulated at the highest amplitude.

Table 2. Results of low-density array studies of human OA fibroblasts stimulated with TGFβ*

<table>
<thead>
<tr>
<th>ECM turnover-related gene</th>
<th>Relative expression (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLOD2</td>
<td>25.2 (8.5–74.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>LOXL3</td>
<td>10.7 (3.2–35.6)</td>
<td>0.013</td>
</tr>
<tr>
<td>COL4A1</td>
<td>4.8 (1.0–22.1)</td>
<td>0.046</td>
</tr>
<tr>
<td>PLOD3</td>
<td>4.6 (1.4–9.5)</td>
<td>0.016</td>
</tr>
<tr>
<td>COL1A1</td>
<td>4.6 (2.9–7.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LOX</td>
<td>4.5 (3.6–5.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LOXL4</td>
<td>3.9 (1.7–8.9)</td>
<td>0.007</td>
</tr>
<tr>
<td>LOXL2</td>
<td>3.7 (2.2–6.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>COL5A1</td>
<td>3.7 (1.1–12.0)</td>
<td>0.036</td>
</tr>
<tr>
<td>P4HA1</td>
<td>3.0 (0.9–10.1)</td>
<td>0.069</td>
</tr>
<tr>
<td>PLOD1</td>
<td>2.9 (1.3–6.7)</td>
<td>0.068</td>
</tr>
<tr>
<td>LEPRE1</td>
<td>2.6 (1.3–5.3)</td>
<td>0.019</td>
</tr>
<tr>
<td>COL3A1</td>
<td>2.5 (2.1–3.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP14</td>
<td>2.4 (0.5–11.4)</td>
<td>0.184</td>
</tr>
<tr>
<td>P4HB</td>
<td>2.2 (1.1–4.2)</td>
<td>0.017</td>
</tr>
<tr>
<td>TIMP1</td>
<td>2.2 (1.5–3.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>PPIB</td>
<td>2.0 (1.5–2.5)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Extracellular matrix turnover–related genes whose expression was increased >2-fold after stimulation with transforming growth factor beta (TGFβ) are shown. OA = osteoarthritis; CI = confidence interval.
Elevated levels of TGFβ-inducible ECM turnover–related genes in the synovium of mice with collagenase-induced OA.

To investigate whether the TGFβ-responsive ECM genes identified in the experiments using fibroblasts were also upregulated in OA synovium, we performed microarray analysis on cDNA from the synovium of mice with collagenase-induced OA. Performing the experiments at several different time points enabled us to study the kinetics of the selected ECM genes during the progression of fibrosis.

At each time point assessed (day 7, day 21, and day 42), expression levels of Col1a1, Col1a2, Col3a1, Col4a1, and Col5a1 were all increased in the synovium of mice with experimental OA compared to the levels in synovium obtained from contralateral joints at the same time (Table 3). In parallel with the findings in fibroblasts, Plod2 was the most highly upregulated crosslinking gene in the synovium of mice with collagenase-induced OA. Moreover, the crosslinking genes Lox, Loxl1, Loxl2, and Loxl3 were upregulated at all 3 time points, indicating elevation of many crosslinking enzymes. The results did not differ significantly between time points. Expression levels of most genes, with the exception of Timp1, Plod2, P4ha3, and Loxl3, were highest on day 7, with levels of the latter 4 genes peaking at later time points. Overall, upregulation of genes encoding for collagen and crosslinking enzymes in this OA model was consistent with the results observed in TGFβ-stimulated OA fibroblasts, indicating comparable TGFβ signaling regulatory mechanisms.

Table 3. Results of microarray studies of synovium from mice with collagenase-induced OA*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day 7 Fold change</th>
<th>P</th>
<th>Day 21 Fold change</th>
<th>P</th>
<th>Day 42 Fold change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timp1 ‡</td>
<td>15.3 ± 1.3</td>
<td>0.003</td>
<td>13.5 ± 1.3</td>
<td>0.004</td>
<td>19.0 ± 0.4</td>
<td>0.002</td>
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<tr>
<td>Plod2 ‡</td>
<td>6.2 ± 1.2</td>
<td>0.014</td>
<td>8.3 ± 1.1</td>
<td>0.006</td>
<td>6.9 ± 0.2</td>
<td>0.011</td>
</tr>
<tr>
<td>Ctsk</td>
<td>9.2 ± 0.7</td>
<td>0.0003</td>
<td>6.5 ± 0.8</td>
<td>0.001</td>
<td>5.0 ± 0.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Col3a1 ‡</td>
<td>8.1 ± 0.7</td>
<td>0.0007</td>
<td>5.2 ± 0.6</td>
<td>0.004</td>
<td>4.4 ± 0.1</td>
<td>0.008</td>
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<tr>
<td>Col1a1 ‡</td>
<td>6.7 ± 0.6</td>
<td>0.0004</td>
<td>4.6 ± 0.7</td>
<td>0.002</td>
<td>4.3 ± 0.1</td>
<td>0.003</td>
</tr>
<tr>
<td>Mmp14 ‡</td>
<td>8.3 ± 0.7</td>
<td>0.0005</td>
<td>5.0 ± 0.7</td>
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<td>4.3 ± 0.2</td>
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<td>3.0 ± 0.1</td>
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<tr>
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<td>5.7 ± 0.6</td>
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</tr>
<tr>
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<td>Crtap</td>
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<td>2.5 ± 0.3</td>
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<td>Col1a2</td>
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<td>3.0 ± 0.4</td>
<td>0.001</td>
<td>2.5 ± 0.2</td>
<td>0.004</td>
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<td>Day 21 Fold change</td>
<td>Day 21 P</td>
<td>Day 42 Fold change</td>
<td>Day 42 P</td>
</tr>
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<td>Serphin1</td>
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<td>0.005</td>
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<tr>
<td>Loxl3 ‡</td>
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<td>2.5 ± 0.2</td>
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<td>Adamts2</td>
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<td>0.0012</td>
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<td>2.4 ± 0.2</td>
<td>0.023</td>
</tr>
<tr>
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</tr>
<tr>
<td>Loxl2 ‡</td>
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<td>0.0003</td>
<td>4.3 ± 0.9</td>
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<td>2.1 ± 0.1</td>
<td>0.045</td>
</tr>
<tr>
<td>Ppib ‡</td>
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<td>0.0013</td>
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<td>1.9 ± 0.1</td>
<td>0.035</td>
</tr>
<tr>
<td>Ddr2</td>
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<td>0.0013</td>
<td>2.0 ± 0.3</td>
<td>0.006</td>
<td>1.5 ± 0.2</td>
<td>0.090</td>
</tr>
<tr>
<td>Col4a1 ‡</td>
<td>2.7 ± 0.3</td>
<td>0.00002</td>
<td>2.1 ± 0.2</td>
<td>0.0003</td>
<td>1.4 ± 0.2</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* ECM turnover–related genes whose expression was increased >2-fold on day 7 are shown. Values are the mean ± SEM. See Table 2 for definitions.
‡ Also upregulated in TGFB-stimulated human OA fibroblasts.

**Elevated levels of genes encoding for both collagen and crosslinking enzymes in human end-stage OA.**

To investigate whether the upregulated expression of genes identified in the above-described experiments reflects expression in human end-stage OA, we performed microarray analysis of ECM-related genes in human end-stage OA synovium. Expression levels of both COL1A1 and COL5A1 were elevated in synovium from patients with end-stage OA (6.7-fold and 4.0-fold, respectively) (Table 4), suggesting that procollagen synthesis in synovium remains elevated even at this phase of the disease. An even more noteworthy finding was that expression levels of P4HA2, P4HA3, P4HB, LOX, LOXL1, LEPRE1, LEPREL1, PLOD1, and PLOD2 were increased (2.0–3.8-fold) in end-stage OA synovium. We thus demonstrated upregulation of genes for both collagen and collagen-modifying enzymes (including those involved in crosslinking), indicating that fibrosis is maintained and/or still progressing in human end-stage OA.
Table 4. Results of microarray studies of synovium from patients with end-stage OA*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>P</th>
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<tr>
<td>COL1A1</td>
<td>6.7 ± 2.3</td>
<td>0.036</td>
</tr>
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<td>PCOLCE</td>
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<tr>
<td>COL5A1</td>
<td>4.0 ± 1.0</td>
<td>0.012</td>
</tr>
<tr>
<td>P4HA3</td>
<td>3.8 ± 1.8</td>
<td>0.142</td>
</tr>
<tr>
<td>TIMP1</td>
<td>3.3 ± 0.7</td>
<td>0.006</td>
</tr>
<tr>
<td>LOX</td>
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</tr>
<tr>
<td>MRC2</td>
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<td>GLT25D1</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>SLC39A13</td>
<td>2.5 ± 0.4</td>
<td>0.001</td>
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<tr>
<td>LEPRE1</td>
<td>2.4 ± 0.3</td>
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<td>LEPREL1</td>
<td>2.4 ± 0.7</td>
<td>0.067</td>
</tr>
<tr>
<td>LOXL1</td>
<td>2.4 ± 0.6</td>
<td>0.039</td>
</tr>
<tr>
<td>FKBBP10</td>
<td>2.4 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
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<td>MMP1</td>
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<td>0.4652</td>
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<td>CRTAP</td>
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<td>P4HA2</td>
<td>2.2 ± 0.4</td>
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<tr>
<td>ADAMTS2</td>
<td>2.2 ± 0.5</td>
<td>0.027</td>
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<tr>
<td>PLOD1</td>
<td>2.0 ± 0.4</td>
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<tr>
<td>PLOD2</td>
<td>2.0 ± 0.4</td>
<td>0.035</td>
</tr>
<tr>
<td>P4HB</td>
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<td>0.008</td>
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</table>

* ECM turnover–related genes whose expression was increased >2-fold are shown. Values are the mean ± SEM. See Table 2 for definitions.

Elevated levels of PLOD2, LOX, COL1A1, COL5A1, and TIMP1 in all 3 OA-related fibrotic conditions.

By combining the data from the above-described experiments, we were able to determine which TGFβ-inducible genes were elevated in both experimental and human OA. We found that PLOD2, LOX, COL1A1, COL5A1, and TIMP1 were upregulated by 2-fold in all of the OA-related fibrotic conditions tested (Figure 1) and therefore are most likely both downstream of TGFβ and elevated in OA. To validate the microarray data, expression of PLOD2, LOX, COL1A1, COL5A1, and TIMP1 in both the human and the murine samples was measured by qPCR. These results confirmed that each of the genes was upregulated in the OA-related fibrotic conditions compared to controls (for PLOD2, 17-fold and 7-fold in humans and mice, respectively; for LOX, 15-fold and 2-fold, respectively; for COL1A1, 24-fold and 7-fold, respectively; for COL5A1, 17-fold and 5-fold, respectively; and for TIMP1, 19-fold and 23-fold, respectively).
To further validate our findings, TGFβ was over-expressed with an adenovirus in murine knee joints to determine whether gene expression of PLOD2, LOX, COL1A1, COL5A1, and TIMP1 is actually regulated by this growth factor in vivo. Analysis by qPCR revealed that after TGFβ overexpression, expression levels of these genes in mouse knee joints were indeed significantly elevated in the synovium (P < 0.05), with upregulation of 18-fold, 13-fold, 10-fold, 9-fold, and 8-fold for Col1a1, Plod2, Timp1, Lox, and Col5a1, respectively. Furthermore, immunohistochemistry analysis of Timp1 and Plod2 confirmed that their protein levels were also elevated in mouse knees with TGFβ-induced fibrosis (Supplementary Figure 1, on the Arthritis & Rheumatology web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38266/abstract). These results, taken together with the upregulation of the genes in experimental and human end-stage OA and the fact that TGFβ is a major factor in fibrosis, strongly suggest that one or a combination of these genes plays a crucial role in OA-related fibrosis.

Figure 1. Venn diagram showing extracellular matrix turnover–related genes that were upregulated by >2-fold in one or more of the following: transforming growth factor beta (TGFβ)–stimulated human osteoarthritis (OA) synovial fibroblasts, synovium from mice with collagenase-induced OA, and synovium from humans with end-stage OA.
Discussion

Elevated TGFβ levels are the main cause of synovial fibrosis in OA (9). Recently it was shown that inhibition of TGFβ activity in the subchondral bone attenuated the degeneration of articular cartilage (18); however, blocking of TGFβ in an OA-affected joint to interfere with fibrosis would have adverse effects on cartilage homeostasis and is therefore not a feasible therapeutic option. In this study we sought ECM turnover–related genes that are downstream of TGFβ and regulated in OA-related synovial fibrosis, that could potentially be used for anti-fibrotic therapy in OA. We found that PLOD2, LOX, COL1A1, COL5A1, and TIMP1 were upregulated both in OA fibroblasts stimulated with TGFβ and in mice with TGFβ-induced fibrosis, which indicates that these genes are downstream of TGFβ and potentially crucial for fibrosis. The same genes were also elevated in collagenase-induced OA as well as in human end-stage OA. Since these ECM-related genes are downstream of TGFβ and elevated in both experimental and human OA as well as in TGFβ-induced fibrosis, they might be interesting treatment targets for interference with OA-related synovial fibrosis (Figure 2).

TIMP-1, which inhibits MMP activity, has been found to be elevated in various fibrotic diseases such as pulmonary, liver, and kidney fibrosis (19–21), and was also enhanced in the OA-like fibrotic conditions investigated in the present study. In rats with immune–mediated fibrosis serum TIMP-1 levels were shown to reflect the severity of liver fibrosis (22), whereas in the collagenase-induced OA model the gene expression levels of TIMP1 did not reflect the severity of the fibrosis, with fibrosis being much more severe on day 21 compared to day 7, while TIMP1 gene expression was higher on day 7 (11, 16). This is comparable to observations in rats with CCl4-induced fibrosis, in which TIMP1 levels also did not correlate with fibrosis severity (22). It is also possible that TIMP1 gene expression levels do not reflect TIMP-1 protein levels. TIMP-1 is typically considered to be an enhancer of fibrosis development that does not induce fibrosis by itself (19,20). However, in unilateral urethral obstruction–induced fibrosis there was no difference in the degree of interstitial fibrosis between wild-type and TIMP1-deficient mice (23). This observation was explained by the possibility that, due to other proteinase inhibitors, the function of TIMP1 was compensated for or that inhibition of the intrinsic MMP activity does not contribute to a profibrogenic event in the kidney (23). Increased MMP activity due to diminished TIMP1 activity might be beneficial for reducing fibrosis.

A possible consequence of targeting TIMP1 in an OA joint is that this will lead to elevated MMP activity, which contributes to cartilage damage (24). Interference with collagen synthesis might therefore be a better option for interfering with fibrosis.

The upregulation of COL1A1 we observed in the OA-related fibrotic conditions met our expectations since type I collagen is the main collagen found in fibrotic diseases (25, 26).
In contrast to COL1A1, the increase in COL5A1 gene expression was not anticipated since this is not a general phenomenon in fibrotic diseases (27), although, consistent with the upregulation of COL5A1 observed in our models, elevation of type V collagen has been demonstrated in rats with CCl4-induced liver cirrhosis (28). It has been suggested that in CCl4-induced liver cirrhosis, type V collagen not only functions to connect type IV collagen with type I collagen fibrils, but also protects the parenchyma against excess type I collagen deposition. Furthermore, it has been reported that type V collagen is important in the determination of fibril structure and matrix organization (29). Thus inhibition of type V collagen may lead to a dysfunctional collagen network. This, and the potential role of type V collagen in protecting against excess type I collagen deposition, makes COL5A1 a less attractive target than COL1A1. Furthermore, in all of the conditions we studied, induction of COL1A1 was higher than that of COL5A1, supporting the notion that COL1A1 is the major collagen in fibrosis.

There are currently no available data on the effect of blocking type 1A1 or type 5A1 collagen in synovial tissue. Neither type I collagen nor type V collagen has a crucial role in cartilage (30), and therefore it can be anticipated that these genes can be safely blocked locally in an OA joint without compromising cartilage integrity.

Tendons and ligaments are composed primarily of type I collagen, but it is unlikely that inhibition of type I collagen synthesis locally would affect the tendons and ligaments in the joint since collagen turnover is very low in these tissues (31, 32). Although blocking of the synthesis of these collagens might have little effect in healthy tendons and ligaments, it can, however, be expected that healing of damaged tendons and ligaments would be impaired.

The genes for both lysyl oxidase and LH2b (LOX and PLOD2, respectively) were upregulated in all OA-like fibrotic conditions tested in our study. It is known that the degree and type of crosslinks influences the susceptibility of collagen to enzymatic degradation (14, 33). Two crosslink routes are responsible for the formation of collagen crosslinks: the allysine and the hydroxyallysine routes (34, 35). In the allysine route LOX converts lysine residues within the telopeptide into the aldehyde allysine (36). This is a critical step in crosslinking of collagen fibrils. In the hydroxyallysine route a hydroxylysine residue within the telopeptide is converted by LOX into the aldehyde hydroxyallysine. LH2b converts the lysine in the telopeptides into hydroxylysine, thereby inducing the hydroxyallysine route. This route lead to an elevation in pyridinoline crosslinks that results in greater resistance of collagen to enzymatic degradation (14, 34, 37). Interestingly, both LH2b and pyridinolines are elevated in various fibrotic tissues and organs (34,38). In the present study, the combined elevation of LOX and PLOD2 in OA-related fibrotic conditions indicates elevated crosslinking, favoring the hydroxyallysine route (38, 39).

To our knowledge there are no published reports describing the effect of blocking of PLOD2 on fibrosis. However, PLOD2 might be an interesting target to block in the OA joint.
because this would reduce pyridinoline crosslink formation in the synovium. This would, in turn, result in type I collagen that is more prone to degradation by MMPs, and therefore synovial fibrosis might be prevented/diminished. Furthermore, cartilage with collagen containing low hydroxylsine and pyridinoline levels might be less prone to mechanically induced degeneration since collagen with high levels of lysyl hydroxylation and pyridinoline crosslinking seems to fail mechanically under conditions of long-term loading (40). Therefore, inhibition of PLOD2 will most likely favor cartilage repair attempts. In addition, blocking of PLOD2 would be a safer alternative than blocking of type I collagen synthesis with regard to tendon and ligament repair, as the former does not affect collagen synthesis and “normal” crosslinking by LOX is not altered. The only potential drawback could be that missing/reduced pyridinoline crosslinks might reduce the strength of repaired tendons or ligaments.

Others have shown that inhibition of LOX with β-aminopropionitrile significantly improves the bone marrow fibrotic phenotype in a mouse model of myelofibrosis (GATA-1–low mice) (36). Moreover, in Yorkshire pigs, inhibition of LOX with β-aminopropionitrile reduced chamber and myocardial stiffness by inhibiting collagen crosslinking compared to that in normal untreated pigs (41). Therefore, blocking of LOX may be considered a potential therapeutic target for interference with OA-related fibrosis.

Of the genes identified in the present study, we propose that PLOD2 is the most suitable as a target for interfering with OA-related fibrosis without negatively affecting joint homeostasis. Blocking of TIMP1 or COL5A1 in the OA joint is not advised due to the disadvantages described above. With inhibition of PLOD2, as opposed to LOX, only the hydroxyallysine route is blocked, and normal collagen maturation is still possible since the allysine crosslink route remains intact (39). Furthermore, inhibition of LOX may lead to decreased crosslinking of type II collagen, resulting in less mature and weaker collagen, which will hinder cartilage repair (42). With regard to COL1A1, we assume that complete blocking would be difficult to achieve, and a possible disadvantage of partial blocking of COL1A1 synthesis would be that an even greater imbalance between procollagen synthesis and crosslinking enzymes could result. When this is the case the collagen helixes produced will be highly crosslinked, resulting in collagen that is even more resistant to degradation, which eventually also can lead to collagen accumulation.

We mainly measured gene expression levels, which is a limitation of this study given that gene expression does not always reflect levels of protein. Therefore, we additionally performed immunohistochemistry analysis for TIMP-1 and LH2b to confirm that the elevated mRNA levels actually translated into highly elevated protein expression. Another limitation of this study is that the human control synovium was obtained from patients who, though not diagnosed as having OA or rheumatoid arthritis, had suspected acute trauma requiring arthroscopy. These patients are likely not fully representative of the healthy population.
Furthermore, the OA patients were not subdivided into groups with and without fibrosis, which might have resulted in an underestimation of the actual fibrotic genotype. Despite this potential underestimation, however, we still identified genes that were significantly induced during OA compared to controls. We used 4 different conditions to reflect “OA fibrotic” and compared these conditions in order to investigate which genes were elevated in all conditions. The genes identified are thus highly likely to be of importance in OA-related fibrosis. Given the current findings, one of our future goals is to create an inducible PLOD2-knockout mouse, in which PLOD2 could be knocked out before and during OA progression in order to study its effect on fibrosis. We postulate that blocking of PLOD2/LH2 during OA will prevent the formation of pyridinoline crosslinks and will therefore reduce long-term fibrosis.

In conclusion, in the present study we identified 5 potential target genes for therapeutic interference with synovial fibrosis. Of these 5 genes—COL1A1, COL5A1, PLOD2, LOX, and TIMP1—we postulate that PLOD2 is a promising target. Previously we demonstrated that elevated PLOD2 expression levels result in more pyridinoline crosslinks per triple helix in experimental OA (11). Now we have shown that PLOD2 is upregulated not only in mouse models of OA-related fibrosis, but also in human end-stage OA.

**Figure 2.**
Schematic diagram showing the hypothetical mechanism of onset and maintenance of osteoarthritis (OA)–related fibrosis. TGFβ transforming growth factor; TIMP-1 tissue inhibitor of metalloproteinases 1; MMP matrix metalloproteinase.
Acknowledgments

The authors would like to thank Eug`ene Verwiel (Radboud University Nijmegen Medical Center) for analyzing the murine microarray data, Nozomi Takahashi (Ghent University, Ghent, Belgium) for analyzing the human microarray data, Saskia de Rond (University Medical Center Groningen, Groningen, The Netherlands) for performing the low-density arrays, and Dr. C. D. Richards (University of Newcastle, Newcastle-upon-Tyne, UK) for providing the Ad-TGFβ virus.

References


Chapter 3

Osteoarthritis-related fibrosis is associated with both elevated pyridinoline cross-link formation and lysyl hydroxylase 2b expression

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Abstract

Objective. Fibrosis is a major contributor to joint stiffness in osteoarthritis (OA). We investigated several factors associated with the persistence of transforming growth factor beta (TGFβ)-induced fibrosis and whether these factors also play a role in OA-related fibrosis.

Design. Mice were injected intra-articularly (i.a.) with an adenovirus encoding either TGFβ or connective tissue growth factor (CTGF). In addition, we induced OA by i.a. injection of bacterial collagenase into the right knee joint of C57BL/6 mice. mRNA was isolated from the synovium for Q-PCR analysis of the gene expression of various extracellular matrix (ECM) components, ECM degraders, growth factors and collagen cross-linking-related enzymes. Sections of murine knee joints injected with Ad-TGFβ or Ad-CTGF or from experimental OA were stained for lysyl hydroxylase 2 (LH2). The number of pyridinoline cross-links per triple helix collagen in synovium biopsies was determined with high-performance liquid chromatography (HPLC).

Results. Expression of collagen alpha-1(I) chain precursor (Col1a1), tissue inhibitor of metalloproteinases 1 (TIMP1) and especially procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2b (Plod2b) were highly upregulated by TGFβ but not by CTGF. Elevated expression of Plod2b mRNA was associated with high lysyl hydroxylase 2 (LH2) protein staining after TGFβ overexpression and in experimental OA. Furthermore, in experimental OA the number of hydroxy pyridinoline cross-links was significant increased compared to control knee joints.

Conclusions. Our data show that elevated LH2b expression is associated with the persistent nature of TGFβ-induced fibrosis. Also in experimental OA, LH2b expression as well as the number of hydroxy pyridinoline cross-link were significantly upregulated. We propose that LH2b, and the subsequent increase in pyridinoline cross-links, is responsible for the persistent fibrosis in experimental OA.
Introduction

Important hallmarks of osteoarthritis (OA) are cartilage degeneration, osteophyte formation and fibrosis, resulting in both joint pain and stiffness. Fibrosis is characterized by excess connective tissue accumulation, which can occur in many organs and can lead to organ failure (1,2). In OA, fibrosis is a major contributor to joint stiffening and is involved in joint pain (3). Two main players in fibrotic diseases which are also elevated in OA are transforming growth factor beta (TGFβ) and connective tissue growth factor (CTGF) (4,5).

TGFβ controls cell proliferation, differentiation and fulfills different roles in immunity and wound healing but also plays a role in fibrosis in different organs (6-12). We have previously shown that TGFβ plays an important role in synovial fibrosis in experimental OA, as blocking TGFβ could prevent synovial fibrosis (13). However, blocking TGFβ in OA is not an option as it is crucial for cartilage maintenance and repair (14,15). Besides TGFβ, CTGF has been proposed as a major player in fibrotic diseases. CTGF can be induced by TGFβ through a TGFβ response element, however it can also function independent of TGFβ (16-18). During adulthood, CTGF is expressed in endothelia and neurons in the cerebral cortex where it promotes angiogenesis and tissue integrity (19-22). When it is expressed in other tissues it is mostly associated with wound healing, vascular diseases and fibrosis (22).

By injecting an adenovirus overexpressing CTGF in murine knee joint we previously showed that CTGF can induce synovial fibrosis (23). However, in contrast to TGFβ-induced synovial fibrosis, which is persistent, CTGF overexpression resulted in fibrosis that was resorbed by day 28. Others have also found that adenoviral expression of TGFβ induced pronouncedly prolonged fibrosis, while adenoviral expression of CTGF induced only transient fibrosis (10,24). This raised the question: What causes this difference between TGFβ and CTGF-induced fibrosis, and specifically which factor(s) are responsible for the persistent nature of TGFβ-induced fibrosis?

Fibrosis results from an imbalance between matrix synthesis and matrix degradation. Therefore, we assessed the effect of either TGFβ or CTGF overexpression on mRNA expression levels of various matrix turnover related genes and whether these give an indication of the observed differences. Thereafter we examined whether these factor(s) play a role in OA-related fibrosis.

Here we report that lysyl hydroxylase 2b is strongly induced in TGFβ-induced persistent fibrosis and not in CTGF-induced transient fibrosis. Lysyl hydroxylases are collagen modifying and crosslinking enzymes that convert lysine into hydroxylysine, thereby leading to cross-links that make collagen harder to degrade (25-29). Especially LH2b, which hydroxylases the telopeptides and so induces the formation of pyridinoline collagen cross-links, makes collagen harder to degrade. LH2b and the pyridinoline cross-links it induces were both significantly elevated in OA-related fibrosis.
Materials and methods

Animals.
Male C57Bl/6 mice aged 12 weeks were used. Animals were kept in filtertop cages with woodchip bedding. They were fed a standard diet with tap water ad libitum. The local committee on animal research and ethics has approved this study.

CTGF and TGFβ overexpression.
We injected murine knee joints intra-articularly (i.a.) with $1 \times 10^7$ pfu virus in the right knee joint, thereby transfecting the synovial lining with an adenovirus overexpressing active porcine TGFβ1 (Ad-TGFβ223/225) (gift from Dr. C.D. Richards) or human CTGF (Ad-CTGF) (FibroGen, Inc., South San Francisco, CA, USA). As a viral control Ad-luc was used. The mice were sacrificed on day 3, 7 and 21. Synovial biopsies from at least five individual mice per group, were taken from the right knee joint for RNA isolation and subsequently Q-PCR. The right knee joints of mice injected with Ad-luc were used as controls for TGFβ1 and CTGF injected joints. This experiment was repeated and whole knee joints were isolated for histology ($n = 6$ per group). Since the adenoviruses overexpress either porcine TGFβ or human CTGF we could distinguish these from endogenous murine TGFβ and CTGF with specific primer sets.

Collagenase-induced OA.
To induce OA, five units of bacterial collagenase in a total volume of 6 ml were injected i.a. into the right knee joint as previously described (30). Mice were sacrificed on day 7, 21, and 42 and synovial biopsies were taken for RNA isolation ($n = 4$ per group*). This experiment was repeated for pyridinoline cross-link measurement and synovial biopsies were taken at day 2, 7, 21, and 42 ($n = 12$ per group*). Whole knee joints were isolated for histology ($n = 10$ per group*) (day 7, 28 and 42). The left non-injected knee joints served as controls. The injection of bacterial collagenase leads to joint laxity and subsequent OA lesions resembling those occurring naturally in old mice. This model represents an equivalent to human secondary OA resulting from joint instability. *Each number represents an individual mouse.

Quantitative PCR (Q-PCR).
RNA was isolated from the synovial biopsies with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) after which an reverse transcriptase PCR (RT-PCR) was performed. Q-PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) according to manufacturers protocol. The used primer sets are displayed in Table 1. Ct values of the genes of interest were corrected for glyceraldehyde-3-phosphate
dehydrogenase (Gapdh) and their corresponding controls (delta delta Ct (ΔΔCt)). Each biological sample was measured once.

**Histology and immunohistochemistry.**
The murine knee joints were processed and immunohistochemically stained as previously described (14). Specific primary Abs against LH2 (1/100) (Proteintech Group Inc., Chicago, IL, USA) were incubated overnight at 4 °C. The second antibody biotinylated goat anti-rabbit IgG (Vector Laboratories) was incubated for 1 h. A biotin–streptavidin detection system was used according to the manufacturer’s protocol (Vector Laboratories). Sections were counterstained with hematoxylin and mounted with Permount.

The presence of fibrosis was determined by the synovial thickening, due to the accumulation of extracellular matrix and increase in fibroblasts (histology), and at the increase in collagen alpha-1(I) chain precursor (Col1a1) gene expression (Q-PCR).

**Cross-link measurement.**
Synovial tissue samples (n = 12) were hydrolyzed in an oven with 6 M HCl at 110 °C for 20 h. After drying (Speed Vac) samples were dissolved in 100 ml internal standard. The amount of hydroxyproline (Hyp) and the cross-links lysyl pyridinoline (LP) and hydroxylysyl pyridinoline (HP) in these samples were determined by reversed-phase high-performance liquid chromatography (HPLC) as described by Bank et al. (31). To calculate the amount of HP per triple helix the amount of HP (pmol) in the sample was divided by the total amount of collagen (=Hyp/300). Values are expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

**Statistical analysis.**
First all data were checked for normality with the Shapiroe-Wilk test. To determine significant (P < 0.05) differences between groups that were normally distributed a One-Way ANOVA with Gamese Howell post hoc test for multiple comparison was performed. Significant (P < 0.05) differences between groups that were not normally distributed were determined with the Wilcoxon Signed Ranks Test. The statistical analyses were performed with the statistical software package SPSS 18.0 (SPSS, Chicago, IL, USA).
Table 1. Primers used for Q-PCR. Primers were accepted if the deviation from slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. Efficiencies (E) for all primer sets were determined using a standard curve of five serial cDNA dilutions in water in duplicate.

<table>
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<tr>
<th>Gene</th>
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<th>E</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5'→ 3')</th>
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<td>Col1a1</td>
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Table 2. Ct values of human CTGF and porcine TGFβ. Mice were injected i.a. with an adenovirus encoding either TGFβ or CTGF or a control virus. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. Primers detecting only viral CTGF (human, not murine) and only viral TGFβ (porcine, not murine) were used to evaluate whether expression was sustained over a period of time. Ct values were corrected for Gapdh. Higher Ct values thus represent a lower expression. Nd = not detectable. Clearly, viral CTGF was only found in synovial biopsy punches of mice injected with Ad-CTGF and viral TGFβ was only detected in synovial biopsy punches of mice injected with Ad-TGFβ. The expression levels declined in time, but expression was still clearly detectable at day 21.
Results

To address the question which factors could be responsible for difference in TGFβ-induced persistent and CTGF-induced transient fibrosis, we evaluated the expression of several extracellular matrix components, matrix proteases, as well as growth factor expression and modulators of collagen cross-linking. First we checked whether the adenoviruses, which were intra-articular injected in the right murine knee joint, induced expression of human CTGF and porcine TGFβ. This showed clear expression of both factors until at least day 21 (Table 2).

**Ad-TGFβ induces both endogenous TGFβ and CTGF mRNA expression.**

We examined the effect of adenoviral overexpression of CTGF or TGFβ on the endogenous expression of CTGF and TGFβ. Adenoviral overexpression of TGFβ induced relatively high levels of endogenous (murine) TGFβ1 and CTGF, with maximal induction levels of 3.5 ΔΔCt (P < 0.0001) for TGFβ1 at day 7 and 3.8 ΔΔCt (P < 0.0001) for CTGF on day 3 (Figure 1a). Adenoviral overexpression of CTGF only resulted in a small increase (1.3 ΔΔCt) of TGFβ on day 7. Further there were hardly any changes induced by Ad-CTGF with regard to the endogenous expression levels of CTGF and TGFβ (Figure 1b).

![Figure 1](image).

**Figure 1.** mRNA expression (ΔΔCt) of endogenous TGFβ and CTGF in murine synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGFβ1 or CTGF. Three, 7 and 21 days after injection of the adenovirus, synovial biopsy punches were taken of which RNA was isolated. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and their corresponding controls. [a] TGFβ induced elevated levels of TGFβ1 RNA. In addition, Ad-TGFβ overexpression resulted in elevation of CTGF RNA expression. [b] Ad-CTGF only resulted in a significant (P = 0.02) increase of TGFβ on day 7. The endogenous levels of CTGF were not affected by Ad-CTGF.
Elevated gene expression of Mmp3/9/13, Adamts4 and Timp1 by Ad-TGFβ

TGFβ induced the expression of most of the studied matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) and tissue inhibitor of metalloproteinases 1 (Timps). Expression of Mmp13 and Adamts4 were strongly elevated on all measured days (Figure 2a). Timp1 expression was highly elevated by TGFβ overexpression with a maximum at day 7 of 5.8 ΔΔCt (P < 0.0004) compared to normal expression. Compared to TGFβ, CTGF only significantly induced Adamts4 (1.9 ΔΔCt; P = 0.0168), further it did not significantly change any of the measured expression levels more than 1.5 ΔΔCt (Figure 2b).

Figure 2. mRNA expression (ΔΔCt) of various enzymes in the synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGFβ or CTGF. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and for control samples. [a] Ad-TGFβ induced the highest changes on day 7, with most marked changes in expression of MMP3, MMP13, ADAMTS4 and TIMP1. [b] Overexpression of Ad-CTGF resulted in a significant induction of MMP3 (P = 0.008) and ADAMTS4 (P = 0.017) at day 7. MMP9 was downregulated by Ad-CTGF at day 7 (P = 0.003) and 21 (P = 0.011).
Ad-TGFβ, but not Ad-CTGF, induces procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2b (Plod2b) mRNA expression in murine synovium. Since, collagen cross-linking can have a major impact on the degradation of collagen we also evaluated the levels of mRNA expression of the lysyl hydroxylases (enzymes involved in collagen cross-linking). The family of lysyl hydroxylases consists of: LH1, LH2 and LH3 which are coded by Plod1, Plod2, and Plod3 respectively. LH2 has two alternative splice-variants: LH2a and LH2b. TGFβ induced all Plods with the highest expression on day 7 (Figure 3a). Strikingly, TGFβ overexpression resulted in very high Plod2b expression levels, with an induction of approximately 4 ΔΔCt (P < 0.003) on all measured days. Adenoviral overexpression of TGFβ also resulted in elevated expression levels of Col1a1 mRNA in synovium on all measured days, with a maximum increase (4.7 ΔΔCt; P < 0.0001) on day 7 compared to controls (Figure 3a). CTGF had no effect on Plod gene expression levels on day 3 and day 7, at day 21 Plod2(b) and Plod3 were somewhat downregulated but this was not significant (Figure 3b). In contrast to TGFβ, and rather unexpected, CTGF did not induce the expression of Col1a1.

**Figure 3.** mRNA expression (ΔΔCt) of various lysyl hydroxylases and Col1a1 in the synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGFβ or CTGF. At day 3, 7 and 21 after injection of the adenovirus synovial biopsy punches were taken for RNA isolation. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and for control samples. [a, b] In contrast to CTGF, TGFβ induced expression levels of all measured Plods. The most marked change was observed in Plod2(b) expression, which was strongly induced on all measured days. Col1a1 expression was also induced after Ad-TGFβ overexpression but not after Ad-CTGF overexpression.
Ad-TGFβ-induces persistent fibrosis which is accompanied by elevated LH2 protein expression.

Having found a particularly high Plod2(b) gene expression in the synovium, we investigated if this translates into LH2 protein expression. To this end, we stained paraffin sections of the murine knee joint transduced with either CTGF or TGFβ for LH2. First we investigated if the synovium was thickened. After TGFβ overexpression there was a strong increase in the thickness of the synovium over time, which was still present on day 21 (Figure 4c). CTGF overexpression resulted in synovial fibrosis with a peak on day 7, which was completely resolved on day 28 (Figure 4b). These results support our earlier published observations that overexpressing TGFβ leads to persistent fibrosis whereas CTGF induces transient fibrosis. Similar to LH2b mRNA expression after TGFβ overexpression, LH2 protein expression was the highest on day 21 (Figure 4c). However, on day 3 and 7 there was also a clear increase in LH2 protein expression compared to the control knee joints. As expected, CTGF did not influence LH2 protein expression (Figure 4b).

Figure 4. [a] Normal murine knee joint, with the area that is displayed in the histology figures. [b, c] Immunohistochemically staining for LH2 protein expression in the synovial tissue of murine knee joints after i.a. of CTGF or TGFβ. TGFβ overexpression resulted in a strong increase over time in the thickness of the synovial membrane starting at day 3. CTGF-induced fibrosis was most abundant at day 7 and was completely resorbed at day 28. In contrast to CTGF, overexpressing of TGFβ strongly induced LH2 expression in the synovium, especially at day 21 (Original magnification × 100).
Plod2b mRNA is elevated in collagenase-induced OA

Because Plod2b was strongly upregulated in TGFβ-induced persistent fibrosis we investigated if Plod2b was also elevated in OA-related synovial fibrosis in collagenase-induced OA. We found that in collagenase-induced OA there was a significant and longlasting increase of Plod2b gene expression. The highest induction of 6.3 ΔΔCt (P< 0.0001) was measured on day 7, however Plod2b was still significant increased on day 21 and 42 (Figure 5a). Thus, comparable to TGFβ-induced fibrosis, Plod2b gene expression is also elevated in experimental OA-related fibrosis.

LH2 protein expression is strongly induced in OA-related fibrosis.

Histological sections of murine knee joints were stained for LH2, to assess if LH2 protein expression was induced in OA-related fibrosis. A clear increase in LH2 protein expression was observed in the synovium at all measured time points (Figure 5b). Especially on day 7 there was a very strong increase in LH2 expression, while on both day 21 and 42 LH2 protein expression was still elevated but less intense than on day 7. The thickness of the synovium of the murine knee joints with collagenase-induced OA was mildly increased at day 7, while a large increase was seen on day 28 and 42. LH2 protein expression being strongly elevated in collagenase-induced OA, indicating LH2(b) may play a key role in OA-related fibrosis.

Elevated number of HP cross-links in synovium in experimental OA.

To determine whether a higher LH2b expression level results in an increase of pyridinoline cross-links, the number of LP and HP per triple helix was measured in the synovium of mice with collagenase-induced OA. The amount of LP was under the detection limit at all measured time points. However, there was a significant increase in the number of HP cross-links per triple helix on both day 21 and 42, with a 1.8-fold increase on day 21 and a 2.6-fold increase on day 42 compared to unaffected left control joints (Figure 5c). This indicates that elevated levels of LH2b expression during experimental OA indeed result in more pyridinoline cross-links per triple helix. Suggesting the formation of more degradation-resistant collagen (27).

Figure 5.

[a] Plod2b mRNA expression (ΔΔCt) in the synovial tissue of murine knee joints with collagenase-induced OA. Plod2b mRNA expression was significantly increased on all measured days in the synovium of OA-affected joint compared to the healthy control joints. This increase was with an induction of 6.3 ΔΔCt the strongest on day 7, but was still 1.6 ΔΔCt increased at
Figure 5. [b] Immunohistochemically staining for LH2 protein on murine knee joints with collagenase-induced OA (Original magnification × 100). The picture is focused at the synovial tissue lateral of the growth plate (see Figure 3a). At day 7 a mild increase in the thickness of the synovial membrane was seen whereas on day 28 and 42 a strong increase in synovial thickening was observed. LH2 expression was strongly induced in the synovium at day 7, on day 28 and 42 LH2 was still elevated but less intense compared to day 7. [c] HPLC cross-link measurement in synovial punches, obtained from mice with collagenase-induced OA (n = 12 per group). The number of pyridinoline crosslinks per triple helix was significantly elevated on day 21 and 42 in the synovium of murine knee joints with collagenase-induced OA compared to unaffected left control joints.
**Discussion**

Synovial fibrosis is thought to contribute significantly to joint stiffness (3). Previously we have shown that TGFβ induces persistent fibrosis whereas CTGF induces reversible fibrosis (23,32,33). In this study we evaluated several factors to get an indication why TGFβ induces persistent fibrosis and CTGF induces only transient synovial fibrosis and whether the factors are involved in the persistent nature of OA-related fibrosis. The most pronounced and abundant induction we detected was the induction of LH2b by TGFβ. In addition, we found that LH2b gene expression and LH2 protein expression as well as the number of pyridinoline cross-links per triple helix were strongly induced in experimental OA.

First we explored if the endogenous levels of TGFβ1, and CTGF were altered by overexpressing either TGFβ or CTGF. Overexpression of TGFβ resulted in the induction of endogenous TGFβ and CTGF, whereas overexpressing CTGF had no noteworthy effects on endogenous CTGF and TGFβ. TGFβ autoinduction could contribute to the potentiation of TGFβ induced effects. However, TGFβ-induced fibrosis persists for months whereas TGFβ autoinduction strongly diminished in time. Therefore it is unlikely that TGFβ autoinduction is the cause of the persistence of TGFβ induced fibrosis.

As expected, endogenous CTGF was induced by overexpressing TGFβ. In contrast to our finding, several other groups have established that fibrosis induced by TGFβ alone was not persistent and that only simultaneous application of CTGF and TGFβ resulted in long-term fibrotic tissue formation in skin of mice (34-36). In our system, TGFβ was sufficient to maintain fibrosis maybe supported by the induced CTGF. Our results show that Ad-CTGF overexpression, which induces high CTGF expression, gives mild and transient fibrosis. Since CTGF alone only induces transient fibrosis, CTGF is most likely not the factor responsible for the persistence of TGFβ-induced fibrosis.

Expression of Col1a1 mRNA levels was highly upregulated by TGFβ when compared to CTGF or controls. We expected Col1a1 upregulation by both TGFβ and CTGF, but we found almost no upregulation after Ad-CTGF exposure. This was unexpected as type 1 collagen is the major component of fibrosis and has frequently been found upregulated by CTGF (17, 24, 37, 38). However, Bonniaud et al. found a 3-fold upregulation of Col1a1 mRNA at day 14 after Ad-CTGF exposure but they did find only a small increase (approximately 1.5-fold) in Col1a1 mRNA expression on day 3 and 7 and approximately 0.75 at day 21 (24). Thus, we might have missed the window in which this increased expression could have been present.

High type 1 collagen production maybe crucial in the building up process of fibrosis. However type 1 collagen, without an elevation in the number of pyridinoline cross-links can be degraded by different enzymes and may therefore have a limited role in the persistence of fibrosis. We expected that the reversibility observed in CTGF-induced fibrosis might
be due to a higher degree of ECM degradation, but in fact TGFβ led to elevated levels of MMP expression, whereas CTGF hardly influenced the expression of the proteases explored. TGFβ also induced elevated Timp1 expression, proteins that are able to inhibit MMPs and might prevent or reduce MMP-mediated matrix degradation. The outcome of the effect of Timp1 on TGFβ-induced synovial fibrosis is probably determined by the balance between the TIMPs and MMPs. The increase at day 21 in Mmp13 mRNA expression was higher than Timp1 mRNA expression, suggestive for Timp1 not being responsible for the persistence of TGFβ-induced fibrosis. The net effect of this balance on matrix turnover however is hard to predict.

Since the previous described genes did not clarify the persistent nature of TGFβ-induced fibrosis, we also examined differences in collagen cross-linking enzymes. A prominent observation was that TGFβ induced high expression of Plod2 in the synovium, especially Plod2b, whereas CTGF did not induce any significant changes in Plod mRNA expression levels. That TGFβ is able to induce Plod2/LH2b was earlier shown by van der Slot et al. in skin fibroblasts (25). They also showed that fibroblasts of several fibrotic disorders had increased levels of Plod2/LH2b mRNA expression. Induced LH2b results in overhydroxylation of lysine residues within collagen telopeptides which leads to increased formation of pyridinoline cross-links, making collagen harder to degrade by proteases (27, 39, 40). This could explain why the elevated Mmp13 and Adamts4 mRNA levels do not interfere with the persistence of TGFβ-induced fibrosis. This also might explain why CTGF-induced fibrosis is transient, whereas TGFβ-induced fibrosis lingers at least for months.

We investigated if Plod2 is also involved in OA-related synovial fibrosis in collagenase-induced OA. In this OA model that is accompanied by fibrosis both Plod2b mRNA and LH2 protein expression levels were strongly elevated. We postulated that the strong induction of LH2b in the beginning of collagenase-induced OA lead to overhydroxylation of lysyl groups in the telopeptides of procollagen molecules. During the maturation process these overhydroxylated procollagen molecules will form more pyridinoline cross-links per triple helix compared to not overhydroxylated procollagen. We measured the amount of pyridinoline cross-links per triple helix and as hypothesized these were significantly elevated on day 28 and 42. This finding was not unexpected because collagen cross-link maturation may take several weeks (41). This increase in pyridinoline cross-links will result in collagen that is harder to degrade, and lead to persistent fibrosis after TGFβ exposure and in experimental OA.

We have shown that LH2b gene expression and LH2 protein were induced in both TGFβ-induced fibrosis and collagenase-induced OA-related fibrosis. For different fibrotic diseases an increase in pyridinoline cross-links is reported, for instance in systemic sclerosis, alcoholic cirrhosis, and glomerulosclerosis (26). Since these pyridinoline cross-links are increased due to the elevated LH2b expression levels, we propose that LH2b is responsible for the persistence of fibrosis in OA. Most likely TGFβ, that is elevated in OA, is the driving
force of enhanced LH2b expression in the OA process. We and other groups have shown that blocking TGFβ prevents fibrosis (13,42). However, complete blockage of TGFβ in OA is not an option as it is crucial for cartilage maintenance and repair. Selective blocking of LH2b in an early stage of OA may prevent the formation of pyridinoline cross-links, and therefore persistent fibrosis. This makes LH2b an interesting target for the treatment of OA-related synovial fibrosis.
Acknowledgments

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References


Chapter 4

The role of Plod2b in the onset and maintenance of synovial fibrosis.

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Abstract

Introduction: Synovial fibrosis is often present in osteoarthritis (OA) patients and may greatly contribute to joint stiffness. Previously we found that procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (Plod2b), a collagen cross-linking enzyme was highly upregulated in different OA-related fibrotic conditions in the synovium. Based on its function, Plod2 might be responsible for the persistence of synovial fibrosis. Therefore we want to investigate whether Plod2b is responsible for the persistence of synovial fibrosis.

Methods: We intra-articular injected Ad-CTGF, Ad-Plod2b or a combination of both in the right knee joint of mice. At day 5, 13 and 27 we isolated synovial biopsies for gene expression and cross-link measurement and whole knee joints for histology.

Results: Exposing the synovium to CTGF or a combination of CTGF + Plod2b increased the thickness of the synovium compared to controls. Col5a1 was significantly upregulated by overexpressing Ad-hPlod2b, Ad-CTGF or the combination of both. Moreover, Col5a1 mRNA expression was significantly correlated with both Ad-Plod2b and Ad-CTGF expression. All our selected OA-related fibrotic genes (Plod2b, Col1a1, Col5a1, Lox and Timp1) significantly correlated with each other. In none of the tested conditions the amount of pyridinoline cross-links was altered in comparison with the control group, which suggests that the hPlod2b was not overexpressed high enough or did not function properly.

Conclusion: To our knowledge, we show for the first time that both CTGF and Plod2b are able to induce Col5a1, one of our other selected OA-related fibrotic genes, which may provide new insight in OA-related synovial fibrosis. However, valid conclusions about the role of Plod2b in the persistence of synovial fibrosis can only be drawn when PLOD2b can specifically and functionally overexpressed or inhibited and lead to more or less pyridinoline cross-links respectively.
Introduction

Synovial fibrosis is a common problem amongst osteoarthritis (OA) patients. The excessive deposition of the extracellular matrix caused by the fibrotic process makes the synovial tissue thicker and more rigid (1). Therefore, synovial fibrosis may greatly contribute to joint stiffness, one of the main symptoms of OA (2, 3). The key triggers for fibrosis are inflammation and tissue damage, which are both present in OA and might initiate the synovial fibrosis (4). TGFβ, which is enhanced and activated during these processes is the main inducer of the fibrotic cascade, but cannot be blocked in an OA joint without undesirable side effects due to its necessity for maintenance of cartilage (5).

In our previous studies we searched for targets downstream of TGFβ for preventing OA-related fibrosis without interfering with joint homeostasis. We found that the collagen crosslinking enzyme PLOD2b, which activity leads to an elevation in pyridinoline crosslink formation, was highly elevated in fibrotic synovial tissue. We confirmed that these pyridinoline cross-links as well as Plod2b expression were elevated in the synovium of mice with collagenase-induced OA (6). The degree of pyridiniline cross-links influences the susceptibility of collagen to enzymatic degradation and therefore may cause the persistent fibrosis (7, 8). Because Plod2 based on its function does not negatively affecting joint homeostasis, we concluded from these studies that Lysyl hydroxylase 2b (Plod2b) is the most suitable target for interfering with OA-related fibrosis (6, 9). However, we do not have direct evidence that Plod2b is indeed involved in the onset and maintenance of synovial fibrosis.

Plod2b is responsible for crosslinking of developing collagen, but does not induce collagen by itself. Therefore, in order to investigate its role in making collagen persistent to degradation, we require an additional factor in our experiments that induces collagen production without elevated crosslinking capacity. TGFβ is a potent inducer of Plod2b and therefore not suitable for our experiment. CTGF, which causes transient synovial fibrosis, does not increase Plod2b and is thus a suitable candidate to co-injected with Plod2b (6, 10).

Therefore we intra-articularly (i.a.) injected an adenovirus (Ad) overexpressing CTGF (Ad-CTGF), Plod2b (Ad-Plod2b) or a combination of both in the right knee joint of mice. By performing this experiment we can investigate whether Plod2b increases the amount of pyridinoline cross-links and if this enables the transition from transient fibrosis, which is normally induced by CTGF, into persistent fibrosis, due to the additional presence of Plod2b. Furthermore, we can elucidate whether it affects and or correlates with, the gene expression levels of other OA-related fibrotic genes (Col1a1, Col5a1, Lox and Timp1), so we can determine whether Plod2 influences fibrosis by more than one mechanism (6, 9).
Materials and Methods

Animals
Twelve-week-old male C57BL/6J mice were used for animal studies. The mice were kept in filtertop cages with wood chip bedding. They were fed a standard diet with tap water ad libitum. All animal experiments were approved by the Radboud University Nijmegen Medical Centre Committee on Animal Research and Ethics.

CTGF and Plod2b overexpression
To investigate if Plod2b can enhance pyrodinoline cross linking and thereby cause persistent fibrosis and whether it effects other OA-related fibrotic genes, right knee joints were intraarticular injected with adenoviruses Ad-hPlod2b and/or Ad-hCTGF and/or Ad-Luc as a control. Mice were injected with $0.5 \times 10^7$ plaque-forming units (pfu) Ad-hPlod2b + $0.5 \times 10^7$ pfu Ad-Luc in 6 μl 0.9% NaCl (Fresenius Kabi), $0.5 \times 10^7$ pfu Ad-hCTGF + $0.5 \times 10^7$ pfu Ad-Luc, $0.5 \times 10^7$ pfu Ad-hPlod2b + $0.5 \times 10^7$ pfu Ad-hCTGF or $1 \times 10^7$ pfu Ad-Luc alone as control. In the remainder of the article the Ad-hPlod2b + Ad-Luc and Ad-CTGF + Ad-Luc group are referred to as the Ad-Plod2b and Ad-CTGF. The mice were sacrificed on day 5, 13 and 27 after injection. Two synovial biopsies were isolated per mouse from the right knee joint, which were used for cross-link measurement and for mRNA isolation (n = 6 per group). Whole knee joints were isolated for histology (n = 6 per group). Since the adenoviruses overexpressed the human equivalent of Plod2b and CTGF we could distinguish these from endogenous murine Plod2b and Ctgf with specific primer sets (Table 1).

Isolation of mRNA from murine synovial tissue
For mRNA isolation, murine synovial tissue samples were placed in 1 ml Tri Reagent (Sigma) in MagNA Lyser Green Beads tubes (Roche) and disrupted in the MagNA Lyser for 4 cycles of 20 seconds at 6,500 rpm with 1 minute of cooling on ice between cycles. The Tri Reagent was transferred to a 1.5 ml eppendorf tube and 200 μl chloroform was added. After 3 minutes of incubation the suspensions were centriped at 11,000 revolutions per minute for 15 minutes at 4°C. Supernatants (450 μl) were obtained and mixed with 450 μl isopropanol. This mixture was incubated for 10 minutes at room temperature and subsequently centrifuged at 11,000 rpm for 10 minutes at 4°C. The RNA pellets obtained were washed twice with 75% ethanol, dried, and dissolved in 10 μl RNase-free water (Fresenius Kabi). To remove chromosomal DNA contamination in the isolated mRNA samples, the samples were treated with DNase I (Invitrogen).
Reverse transcription–polymerase chain reaction (RT-PCR).

Isolated mRNA was transcribed into cDNA for quantitative PCR (qPCR). Eleven microliters (1 μg) of mRNA was mixed with 9 μl RT-PCR mix (1.9 μl distilled water, 2.4 μl 10× DNase buffer, 2.0 μl 0.1M dithiothreitol, 0.8 μl dNTPs (each at 12.5M), 0.4 μl oligo(dT) primer (0.5 μg/μl), 1.0 μl reverse transcriptase (200 μg/μl; Invitrogen), and 0.5 μl RNasin (Promega). The RT-PCR protocol was as follows: 5 minutes at 25°C, followed by 60 minutes at 39°C and a final step of 5 minutes at 94°C. After the RT-PCR procedure, the cDNA was diluted 20 times for further use in qPCR analysis.

Quantitative PCR

For qPCR, a primer mix consisting of 1 μl (2.5 μM) forward and reverse primer was added to 5 μl SYBR Green Master Mix (Applied Biosystems). Thereafter, 3 μl cDNA was added and the qPCR was run on a StepOnePlus Real-Time PCR System according to manufacturer’s protocol (Applied Biosystems). The used primer sets are displayed in Table 1. Ct values of the genes of interest were corrected for reference gene GAPDH (delta Ct) and normalized against the delta-Ct of the non-stimulated sample (delta delta Ct).

Table 1. Primers used for Q-PCR. Primers were accepted if the deviation from slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. The optimal annealing temp of each primer pair was 57 ±5 °C. (h = human primer, m = murine primer)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’—3’</th>
<th>Reverse primer 5’—3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCTGF</td>
<td>GTGCGGACCCCTACTACATCC</td>
<td>AGCAGCACCTGAGATGATAATCTC</td>
</tr>
<tr>
<td>hPLOD2b</td>
<td>TAAAGGAAGACCTCCTGAGATGAGA</td>
<td>AATGTTTCCGAGATGAGGAGTCTTTT</td>
</tr>
<tr>
<td>mCol1a1</td>
<td>TGACTGGAAGAGCGGAGAGTACT</td>
<td>CTTTGATGGCGTCCAGGTT</td>
</tr>
<tr>
<td>mCol5a1</td>
<td>GGCCTCGAGGAAATCGGAAAC</td>
<td>GACTCGGAGGCAAATCGG</td>
</tr>
<tr>
<td>mGapdh</td>
<td>GGCAAATTCAACGCGACA</td>
<td>GCTTTGGGATTCGCTCCAGT</td>
</tr>
<tr>
<td>mLox</td>
<td>TTCTCTGCTGCTGAGAACACC</td>
<td>GAGAAACAGCTGTTGAAACAG</td>
</tr>
<tr>
<td>mPlod2b</td>
<td>CGGCAATGCTAGATGACTACCTT</td>
<td>CATTTGGAATGTTCCGGAGTAG</td>
</tr>
<tr>
<td>mTgfβ</td>
<td>GCAGTCGCTGACCAAGGA</td>
<td>AAGAGCAGTGAGCCCTGAAATC</td>
</tr>
<tr>
<td>mTimp1</td>
<td>CAACCTCGACCTGTCATAAGG</td>
<td>CATCTTGATCTTATAACGCTGATAAGG</td>
</tr>
</tbody>
</table>

Cross-link measurement

Synovial tissue samples (n = 6 per group) were hydrolyzed in an oven with 6 M HCl at 110°C for 20 h. After drying (Speed Vac) samples were dissolved in 100 μl internal standard. The amount of hydroxyproline (Hyp) and the cross-links lysylpyridinoline (LP) and hydroxylysyl pyridinoline (HP) in these samples were determined by reversed-phase high-performance
liquid chromatography (HPLC) as described by Bank et al. (11). To calculate the amount of HP per triple helix the amount of HP (pmol) in the sample was divided by the total amount of collagen (=Hyp/300). Values are expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

**Histology and immunohistochemistry**
The murine knee joints were processed and sectioned in 7 µm frontal sections as previously described (12). Subsequently the sections were stained with Safranin O–fast green. The thickness of the synovium was measured as a parameter for fibrosis, by drawing a line from the bone covering the synovium to the outer layer of the synovium whilst omitting parts that do not cover the synovium. The total width of the line covering the synovium is used as a measure for synovial thickness. The location of measurement was lateral of the growth plates in the femur on three locations evenly distributed along the width of the growth plate. We measured these 3 locations in 2 sections per knee joints (Figure 1b). The average value per knee joint was used for further calculations.

**Statistical analysis**
First all data were checked for normality with the Shapiro-Wilk test. To determine significant (P<0.05) differences between groups that were normally distributed a One-Way ANOVA with Bonferroni post hoc test for multiple comparison was performed when equal variances were assumed otherwise the Games-Howell post hoc test for multiple comparison was performed. For the correlations a Pearson correlation of ≥ 0.6 was chosen as cutoff value, as we were only interested in strong correlations The statistical analyses were performed with the statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA).
Results

Before we examined whether and how hPlod2b influences CTGF-induced synovial fibrosis we checked whether the adenoviruses induced expression of hPlod2b and hCTGF in the synovium of murine right knee joint where the adenoviruses were intra-articular injected. This showed a clear but moderate expression of both hPlod2b and hCTGF until at least day 27 (Table 2).

Table 2. Ct values of hPlod2b and hCTGF in the synovium after overexpressing hCTGF, hPlod2b or a combination of both. Mice were injected i.a. with an adenovirus encoding either hPlod2b or hCTGF or a control virus (Luciferase (Luc)). Five, 13 and 27 days after injection of the adenovirus synovial biopsy punches were isolated for mRNA extraction. Primers detecting only human Plod2b and human CTGF were used to evaluate whether expression was sustained over a period of time. Ct values were corrected for Gapdh. Higher Ct values represent a lower expression. Nd = not detectable. Human Plod2b was only found in synovial biopsy punches of mice injected with Ad-hPlod2b whereas hCTGF was only detected in synovial biopsy punches of mice injected with Ad-hCTGF. The expression levels declined in time, but was still detectable at day 27.

<table>
<thead>
<tr>
<th></th>
<th>Human PLOD2B</th>
<th>Human CTGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Luc day 5</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-Luc day 13</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-Luc day 27</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-Luc day 5</td>
<td>13,2</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-Luc day 13</td>
<td>14,9</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-Luc day 27</td>
<td>16,4</td>
<td>Nd</td>
</tr>
<tr>
<td>Ad-CTGF + Ad-Luc day 5</td>
<td>Nd</td>
<td>15,5</td>
</tr>
<tr>
<td>Ad-CTGF + Ad-Luc day 13</td>
<td>Nd</td>
<td>19,0</td>
</tr>
<tr>
<td>Ad-CTGF + Ad-Luc day 27</td>
<td>Nd</td>
<td>17,5</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-CTGF day 5</td>
<td>11,3</td>
<td>12,2</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-CTGF day 13</td>
<td>14,5</td>
<td>19,5</td>
</tr>
<tr>
<td>Ad-PLOD2B + Ad-CTGF day 27</td>
<td>14,8</td>
<td>19,0</td>
</tr>
</tbody>
</table>

CTGF increases the thickness of the synovium at both day 13 and 27

We compared the thickness of the synovium between the Plod2b, CTGF and the combination group to examine whether Plod2b can make fibrosis, induced by CTGF, persistent (Figure 1). As expected, there were no differences in synovial thickness between the Ad-Plod2b and Ad-Luc control group. In the Ad-CTGF group the synovium was thicker compared to the control group on day 13 (p = 0.001). However, when comparing CTGF groups in time we found
that on both day 13 (p = 0.002) and 27 (p = 0.020) the synovium was thicker compared to
day 5. Also in the CTGF + Plod2b group the synovium was significantly thicker on day 13
(p = 0.036) compared to the control group. There were no significant differences between
CTGF and the CTGF + Plod2b group at any of the measured time points. Moreover, we only
observed a limited induction of fibrosis by CTGF and this was not reversible within the 27
days of the experiment.

* Figure 1. [a] Thickness of the synovium in µm, average of three measurements lateral of the growth
plate as indicated on figure 1 [b]. * p = ≤ 0.05 ** p = ≤ 0.005

Col5a1 was significantly upregulated by overexpressing hPlod2b, hCTGF or the
combination of both.

In our prior studies, we found there were 5 genes of high importance in synovial fibrosis and
related to OA. Therefore, we investigated whether these “OA-related fibrotic genes” (Plod2b,
Col1a1, Col5a1, Lox and Timp1) were affected by CTGF, Plod2b or the combination of both
(Figure 2). Col5a1 mRNA was significantly upregulated at day 5, after overexpressing Ad-
hPlod2b (p = 0.024) and the combination of Ad-hPlod2b and Ad-CTGF (p= 0.006) compared
to Ad-Luc. At day 13 Col5a1 mRNA was still significantly upregulated when Ad-Plod2b and
Ad-CTGF (p = 0.024) were co-expressed, but no longer by Plod2b alone. Although Ad-CTGF
did not have an impact on Col5a1 at day 5, we did observe a significant upregulation by day
13 (p = 0.046). None of the measured genes was significantly different at day 27 between the
Ad-Plod2b, Ad-CTGF or Ad-Plod2b + Ad-CTGF group and the control group (Ad-Luc).
Furthermore, there were no significant differences between the Ad-Plod2b, Ad-CTGF and
Ad-Plod2b + Ad-CTGF group at any of the measured time points.
Figure 2. mRNA expression of Col1a1, Col5a1, Plod2b, Lox and Timp1 in murine synovium 5, 13 and 27 days after i.a. injection of Ad-CTGF, Ad-Plod2b or Ad-CTGF + Ad-Plod2b. * p = ≤ 0.05
**Col5a1 significantly correlates with both Ad-Plod2b and Ad-CTGF expression.**

Because there was quite some spread in the hPlod2b and hCTGF gene expression levels in the synovial samples, which could have a major impact on the final outcome of the experiments, we decided to investigate whether the height of the measured fibrotic genes was affected by the expression of the overexpressed genes. Ad-hPlod2b significantly correlated with mCol5a1 and mTimp1 whereas Ad-hCTGF correlated with mCol5a1 and mPlod2b (Table 3). These findings are in line with the elevated Co51a1 gene expression levels due to the Plod2b and/or CTGF overexpression.

**Table 3.** Pearson correlation between Ad-hPlod2b or Ad-hCTGF and the fibrotic genes Plod2b, Col1a1, Col5a1, Lox and Timp1.

<table>
<thead>
<tr>
<th></th>
<th>mCol1</th>
<th>mCol5</th>
<th>Plod2b</th>
<th>LOX</th>
<th>Timp1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-hPlod2b</td>
<td>Pearson Correlation</td>
<td>0.426</td>
<td>0.618</td>
<td>0.506</td>
<td>0.582</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.78</td>
<td>0.006</td>
<td>0.032</td>
<td>0.11</td>
<td>0.004</td>
</tr>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Ad-hCTGF</td>
<td>Pearson Correlation</td>
<td>0.202</td>
<td>0.638</td>
<td>0.667</td>
<td>0.468</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.488</td>
<td>0.14</td>
<td>0.009</td>
<td>0.092</td>
<td>0.086</td>
</tr>
<tr>
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<td>14</td>
<td>14</td>
<td>14</td>
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<td>14</td>
</tr>
</tbody>
</table>

**Correlation of Mouse with Mouse genes.**

To get more insight in whether our selected OA-related fibrotic genes are associated with each other, we studied how their endogenous gene expression levels correlated. Because the correlations between mPlod2b or mCtgf and the other genes can be influenced by the presence of hPlod2b and hCTGF these correlations were not investigated. With the exception of the correlation between mCol5a1 and mTimp1, all other correlations between mCol1a1 mCol5a1, mTgfβ, mLox and mTimp1 had a Pearson correlation above 0.6, and therefore strongly correlate with each other (Table 4).

**Table 4.** Pearson correlation between the endogenous levels of our top of OA-related fibrotic genes (Col1a1, Col5a1, Lox and Timp1).

<table>
<thead>
<tr>
<th></th>
<th>mCol1</th>
<th>mCol5</th>
<th>mTgfβ</th>
<th>mLox</th>
<th>mTimp1</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCol1</td>
<td>Pearson Correlation</td>
<td>-</td>
<td>0.672</td>
<td>0.686</td>
<td>0.701</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>-</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>mCol5</td>
<td>Pearson Correlation</td>
<td>0.672</td>
<td>-</td>
<td>0.602</td>
<td>0.840</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>66</td>
<td>-</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
</tbody>
</table>
The amount of pyridiniline cross-links is not changed by overexpressing Ad-hPlod2b, Ad-CTGF or the combination of both.

On histology we expected that the combination of Plod2b and CTGF would give the highest amount of fibrosis at least from day 13 on. However, the CTGF group with the exception of day 5, did not differ from the combination of CTGF with Plod2b. Therefore, we measured whether the increased Plod2b expression indeed resulted in more pyridinoline cross-links in the synovium as anticipated (Figure 3). In the Ad-Plod2b group, the amount of pyridinoline cross-links at day 13 was lower compared to day 5 (p = ≤ 0.0005) and day 27 (p = ≤ 0.0005), but also compared to day 13 (p = 0.016) of the Ad-CTGF group. These results were unexpected as we expected an increase of pyridinoline cross-links in the Ad-Plod2b groups. However, this does explain the lack of anticipated effects of Plod2b on synovial thickness.

<table>
<thead>
<tr>
<th></th>
<th>mCol1</th>
<th>mCol5</th>
<th>mTgfβ</th>
<th>mLox</th>
<th>mTimp1</th>
</tr>
</thead>
<tbody>
<tr>
<td>mLox Pearson Correlation</td>
<td>0.701</td>
<td>0.840</td>
<td>0.719</td>
<td>-</td>
<td>0.603</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>-</td>
<td>66</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>mTimp1 Pearson Correlation</th>
<th>0.793</th>
<th>0.550</th>
<th>0.759</th>
<th>0.603</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sig. (2-tailed)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>66</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3. HPLC cross-link measurement in synovial punches, obtained from mice after overexpression of Ad-Luc, Ad-Plod2b, Ad-CTGF, and Ad-Plod2b + Ad-CTGF (n = 6 per group). only at day 13 the amount of pyridinoline cross-links in the Ad-Plod2b group was significant lower compared to day 5 and day 27 of the Ad-Plod2b group and compared to day 13 of the Ad-CTGF group.

* p = ≤ 0.05 ** p = ≤ 0.005 *** p = ≤ 0.0005.
Discussion

In this study we set out to investigate the role of Plod2b in the onset and maintenance of synovial fibrosis. We expected to find an increase in synovial fibrosis when combining CTGF and Plod2b when compared to CTGF alone. Unfortunately, this did not happen, which we consider due to technical issues. First of all, we did not observe the same fibrotic pattern we found in our previous Ad-CTGF experiment (10). In the current study CTGF resulted in less synovial thickening and was not reversed by day 27, as observed in the previous experiments (6, 10). We do not expect the slight differences in timing between the current experiment and our prior study to be an issue here. The fact that we used half of the viral load of Ad-CTGF compared to our prior study, could play a role as this resulted in less than half of the CTGF gene expression found in the previous experiment. We could not circumvent this as this was necessary to keep all groups comparable to the Ad-Plod2b + Ad-CTGF in terms of total viral load of $1 \times 10^7$ pfu. We expected an increased pyridinoline cross linking when Plod2b was added to CTGF, but this was not the case, which suggests that the hPlod2b was not overexpressed high enough or did not function properly. This might be due to the improper protein translation of the hPlod2b gene or incorrect protein folding resulting in an inactive enzyme. Another, less likely possibility is that the human Plod2b does not function in mice. Given the high homology of human and mouse Plod2b this is rather unexpected. The Adenovirus only target the synovial lining, which is only one till three cell layers thick and thus a small proportion of the synovium. Because Plod2b is an intracellular enzyme, Plod2b will only be overexpressed in the synovial lining and can only have an effect in the transfected cells. This may not be enough to make a substantial difference in the amount of pyridinoline cross-links in the synovium.

We propose that we can answer this question by a slight modification in the future setup. First we need to ensure that the Ad-Plod2b is transcribed in an active enzyme. This can be achieved by overexpressing it in murine fibroblasts and measure the amount of pyridinoline cross-links after several weeks of culturing. When the Ad-Plod2b proves to be functional, the in vivo experiment can be repeated but with double the viral load and with the same time points as the previous CTGF experiment (day 3, 7 and 28). This doubling of the viral load is required to obtain comparable CTGF expression levels as in the previous experiment, which showed the desired fibrotic profile (6, 10). The only potential problem with this is that the higher total viral load might lead to unwanted side effects.

Despite the unexpected limitations of our study, we were able to identify that Plod2b, Col1a1, Col5a1, Lox and Timp1 strongly correlates with each other in this experiment. These strong correlations suggest that these fibrotic genes are most likely regulated via the same factors. TGFββ is one of these factors and is assumable the main cause of the correlations as
all the measured fibrotic genes are regulated via TGFβ-signaling and as TGFβ is the main inducer of these genes. However, because Ad-Plod2b and Ad-CTGF were able to induce Col5a1 expression and both are strongly correlated with Col5a1 expression, there might also a direct effect of CTGF and Plod2b on Col5a1 expression. Also Ad-CTGF and Plod2b were highly correlated, but we know from this and previous experiment that Plod2b is not induced by CTGF, suggesting that these two genes are probably correlated via TGFβ.

We show, for the first time, that CTGF and Plod2b are able to induce Col5a1. These results indicate that inhibition of Plod2b or CTGF also may lower the Col5a1 expression in some extend, as they are both inducers of Col5a1. Type V Collagen is a regulatory fibril-forming collagen that forms heterotypic fibrils with collagen I (13). Moreover, depletion of type V Collagen leads to dysfunctional regulation of fibrillogenesis. However, the importance of type V Collagen in collagen fibril formation its role in fibrosis is not yet examined, there are some clues that it may play a role in the maintenance of fibrosis. This because it is published that α1(V)-N-propeptide domain of type V collagen is able to bind TGFβ and MMP2 among other things. This may lead to a local increase of active TGFβ, as more of these α1(V)-N-propeptide domain will be present in the synovium due to the increased Col5a1 expression, and sustaining the fibrotic cascade.

Unfortunately, valid conclusions about the role of Plod2b in synovial fibrosis can only be drawn when Plod2b can specifically en functionally overexpressed or inhibited and result in more or less pyridinoline crosslinks in the synovium.
References


Chapter 5

TGFβ induces Lysyl hydroxylase 2b in human synovial osteoarthritic fibroblasts through ALK5 signaling

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2 Medical Biology Section, Stem Cell and Tissue Engineering Group, University Medical Center Groningen, Groningen, The Netherlands.
Abstract

Lysyl hydroxylase 2b (LH2b) is known to increase pyridinoline cross-links, making collagen less susceptible to enzymatic degradation. Previously, we observed a relationship between LH2b and osteoarthritis-related fibrosis in murine knee joint.

For this study, we investigate if transforming growth factor-beta (TGFβ) and connective tissue growth factor (CTGF) regulate procollagen-lysine, 2-oxoglutarate 5- dioxygenase 2 (PLOD2) (gene encoding LH2b) and LH2b expression differently in osteoarthritic human synovial fibroblasts (hSF). Furthermore, we investigate via which TGFβ route (Smad2/3P or Smad1/5/8P) LH2b is regulated, to explore options to inhibit LH2b during fibrosis.

To answer these questions, fibroblasts were isolated from knee joints of osteoarthritis patients. The hSF were stimulated with TGFβ with or without a kinase inhibitor of ALK4/5/7 (SB-505124) or ALK1/2/3/6 (dorsomorphin). TGFβ, CTGF, constitutively active (ca)ALK1 and caALK5 were adenovirally overexpressed in hSF. The gene expression levels of PLOD1/2/3, CTGF and COL1A1 were analyzed with Q-PCR. LH2 protein levels were determined with western blot.

As expected, TGFβ induced PLOD2/LH2 expression in hSF, whereas CTGF did not. PLOD1 and PLOD3 were not affected by either TGFβ or CTGF. SB-505124 prevented the induction of TGFβ-induced PLOD2, CTGF and COL1A1. Surprisingly, dorsomorphin completely blocked the induction of CTGF and COL1A1, whereas TGFβ-induced PLOD2 was only slightly reduced. Overexpression of caALK5 in osteoarthritic hSF significantly induced PLOD2/LH2 expression, whereas caALK1 had no effect.

We showed, in osteoarthritic hSF, that TGFβ induced PLOD2/LH2 via ALK5 Smad2/3P. This elevation of LH2b in osteoarthritic hSF makes LH2b an interesting target to interfere with osteoarthritis-related persistent fibrosis.
Introduction

Osteoarthritis (OA) is the joint disease with the highest incidence. The hallmarks of osteoarthritis are cartilage degeneration, osteophyte formation and fibrosis, with as main symptoms joint pain and joint stiffness. Osteoarthritis-related fibrosis is characterized by fibroblast proliferation and excessive deposition of extracellular matrix components such as collagen. This excessive deposition of collagen leads to thickening and stiffening of the synovial membrane making synovial fibrosis a major contributor to joint stiffness and pain in osteoarthritis-affected joints (1). The response by fibroblasts toward soluble mediators, in particular transforming growth factor beta (TGFβ) and connective tissue growth factor (CTGF), is thought to be the key event in the onset and progression of fibrosis (2). TGFβ was found to be highly expressed in fibrotic organs suggesting a major role in fibrosis (3, 4). Furthermore, tissue-specific overexpression of TGFβ1 in transgenic mice resulted in lung, liver or skin fibrosis, whereas blocking TGFβ in osteoarthritis resulted in strong reduction of synovial fibrosis (5, 6).

When TGFβ binds the TGFβ type II receptor (TGFβR-II), they form a complex that recruits the TGFβ type I receptor, activin receptor-like kinase 5 (ALK5) (7). ALK5 then phosphorylates the receptor-Smads (R-Smads), Smad2 and Smad3. Activated R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus, to regulate the expression of target genes. Other than phosphorylation of Smad2/3 (Smad2/3P) via ALK5, TGFβ can also give rise to Smad1/5/8P (8). There is evidence that, depending on the cell type, TGFβ can induce Smad1/5/8P via ALK5 alone or via a complex containing both ALK5 and ALK1 (9, 10). Importantly, depending on the ALK1/ALK5 ratio, TGFβ will induce different downstream effects (8, 11).

It has been previously shown by our group and other groups that overexpressing TGFβ leads to persistent fibrosis, whereas CTGF induces transient fibrosis (12-16). We recently investigated what caused this difference in persistence between TGFβ- and CTGF-induced fibrosis in the synovium of mice. Changes in collagen synthesis levels and protease expression could not explain this difference (17). In fact, TGFβ stimulation resulted in elevated expression levels of MMP3, 9 and 13, whereas CTGF had a minor influence on MMP expression. Since alterations in collagen cross-linking can have an impact on collagen degradation, we investigated the lysyl hydroxylases. Lysyl hydroxylases are enzymes that catalyze the hydroxylation of lysine into hydroxylysine and, therefore, fulfill a crucial role in collagen modification and cross-linking (18, 19). Comparing TGFβ- and CTGF-induced synovial fibrosis revealed that TGFβ overexpression resulted in a major increase in Lysyl hydroxylase 2b (LH2b) expression, whereas CTGF did not change LH2b expression in murine knee joints (17). LH2b is responsible for the overhydroxylation of collagen telopeptides, which will result in more hydroxy-
allysine-derived cross-links such as pyridinolines (18). These cross-links are more resistant to degradation than lysine-associated cross-links and have been found elevated in various fibrotic diseases such as systemic sclerosis, lipodermatosclerosis, alcoholic cirrhosis, glomerulosclerosis and interstitial fibrosis (20, 21). Furthermore, in experimental osteoarthritis, we found an increase in LH2b expression as well as an increase in the number of pyridinoline cross-links per triple helix. Therefore, a causal relationship between LH2b and irreversible fibrosis in osteoarthritis is plausible.

In this study, we investigate whether PLOD2 (the gene encoding for splice variants LH2a and LH2b) expression and LH2b protein were upregulated by TGFβ in human osteoarthritic fibroblasts. Furthermore, we investigate which TGFβ signaling route induces LH2b expression in human synovial fibroblasts. This could enable identification of potential targets to block osteoarthritis-related fibrosis.
Materials and methods

Cell culture
Synovial fibroblasts were isolated from synovial tissue obtained from osteoarthritis patients that underwent knee joint arthroplasties. This synovial tissue was separated from fat, tendon and ligamental tissue and then cut into small pieces of approximately 3 mm³, which were placed into a six-well plate (3 pieces per well). The fibroblasts were allowed to grow out of the tissue and proliferate for 4 weeks before they were transferred into a culture flask. The fibroblasts were cultured in monolayer in Basal Medium Eagle (BME) medium (Invitrogen, Gibco, Carlsbad, CA, USA) enriched with 10 % FCS, gentamycin (50 μg/ml) and 1 % pyruvate up to passage 10 (22) (see supplementary Figure S1). We measured type1 collagen (COL1A1) and propyl-4-hydroxylase (P4H) expression as markers for fibroblasts. Before every experiment, the fibroblasts were cultured in serum-free medium for 24 h. Each experiment was performed in duplo using 4 different donors.

Blocking of TGFβ induced ALK1 and ALK5 signaling
To distinguish between ALK4/5/7 and ALK1/2/3/6 kinase activity, we used SB-505124 (Sigma, St. Louis, MO, USA) and dorsomorphin (BIOMAL International, Exeter, UK), which inhibits the kinase activity of these ALKs, respectively (23, 24). We tested dorsomorphin on its ability to inhibit ALK1 signaling in synovial fibroblasts by adding dorsomorphin to fibroblasts that overexpressed constitutively active (ca)ALK1 and subsequently performed a western blot on Smad1/5/8P levels (see supplementary data Figure S2). Human synovial fibroblasts were stimulated for 30 min, 24 and 48 h with 10 ng/ml TGFβ with and without 4 h pre-incubation with 5 μM SB-505124 or dorsomorphin. Subsequently, the cells were lysed in TRI Reagent (Sigma) for mRNA isolation and subsequent Q-PCR analyses or in lysis buffer for western blot analysis.

Adenoviral transduction
Human synovial fibroblasts were transduced with a multiplicity of infection (MOI) of 50 for 2 h with adenoviruses overexpressing either TGFβ (Ad-TGFβ) or CTGF (Ad-CTGF) to examine the effect of TGFβ on LH1, LH2b and LH3 mRNA expression. To study whether these effects were ALK1- or ALK5-dependent, the fibroblasts were transduced with constitutively active (ca)ALK1 (Ad-caALK1) or caALK5 (Ad-caALK5). CTGF was used to compare transient fibrosis to TGFβ-induced persistent fibrosis. Ad-Luc, an overexpressing luciferase, was used as a control virus. After 2 h, the transductionmix was replaced by serum-free medium and the cells were cultured for 24 h. Subsequently, the cells were lysed in TRI Reagent (Sigma) for Q-PCR analysis or in lysis buffer for western blot analysis.
Quantitative PCR

Total RNA was isolated from the fibroblasts and treated with DNase I (Invitrogen) to remove potential DNA contamination. The mRNA was reverse transcribed into cDNA and subjected to quantitative real-time PCR (Q-PCR) on StepOnePlus (Applied Biosystems, Darmstadt, Germany). Each cDNA sample was amplified using specific primers for PLOD1/2b/3, CTGF and collagen type 1A1 (COL1A1) (Table 1). The total reaction volume of 20 μl contained 3 μl cDNA, 5 μl SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 μl (2.5 μM) forward and reverse primer. The primer for PLOD2 specifically detected splice variant LH2b. All primers were accepted if the deviation from the slope of the standard curve was not higher than 0.3 compared to the slope of the GAPDH standard curve. In addition, the melting curve was required to show only one product. Ct values of the genes of interest were corrected for reference gene GAPDH (delta Ct) and normalized against the delta-Ct of the non-stimulated sample (delta delta Ct).

Table 1. Primers used for Q-PCR. Primers were accepted if the deviation from the slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. Efficiencies for all primer sets were determined using a standard curve of 5 serial cDNA dilutions in water in duplicate

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLOD1</td>
<td>5'-CAAGCGCTCAGCTCAGTTCTTC-3'</td>
<td>5'-CTTCAGCAGCCGGACCTTCT-3'</td>
</tr>
<tr>
<td>PLOD2 (LH2b)</td>
<td>5'-TTAAAGGAAAGACACTCCGATCAGAGATGA-3'</td>
<td>5'-AATGTTTCCGGAGTAGGGGAGTCTTTTT-3'</td>
</tr>
<tr>
<td>PLOD3</td>
<td>5'-CTGGGGCCTGGGAGAGGAGTG-3'</td>
<td>5'-TCACGTGCTAGCTATCCA-CAACAT-3'</td>
</tr>
<tr>
<td>COL1A1</td>
<td>5'-GTGGAAACCCCGAGCCCTGCC-3'</td>
<td>5'-TCCCTTGGGTCCTCGACGC-3'</td>
</tr>
<tr>
<td>CTGF</td>
<td>5'-GCCCCTCGCGGGCTTACC-3'</td>
<td>5'-AGGCAGTTGGCTCTAATCATTGTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ATCTTTTTTTCGTCGC-CAG-3'</td>
<td>5'-TTCCCCATGTTGTCTGAGC-3'</td>
</tr>
</tbody>
</table>

Western blot analysis

For western blot analysis, the cells were washed twice with 0.9 % NaCl and lysed with Lysis Buffer containing: 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4 and complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Soluble proteins and cell membranes were separated by centrifugation (15,000g, 15 min, 4 °C). For
each sample, the protein concentration was measured with the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Equal amounts of each protein sample were loaded under reducing conditions on a 7.5 % SDS-PAGE gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen). The membrane was blocked according to the manufacturer’s protocol. Subsequently, the membrane was incubated overnight at room temperature with antibodies directed against Smad2/3P or Smad1/5/8P (1:1,000) (Cell Signaling Technology, USA). For LH2 detection, the membrane was incubated for 1 h with anti-LH2 (1:1,000) (Proteintech, Manchester, UK). The secondary antibody used for α-Smad2/3P, α-Smad1/5/8P and α-LH2 was goat-anti-rabbit HRP (1:1,500) (Dako, Copenhagen, Denmark). Proteins were detected using the ECL Plus western blotting detection system (GE Healthcare, Buckinghamshire, UK).

**Statistical analysis**

First, all data were checked for normality with the Shapiro–Wilk test. To determine significant (P<0.05) differences between groups that were normally distributed, a one-way ANOVA with Bonferroni post hoc test for multiple comparison was performed. Significant (P<0.05) differences between groups that were not normally distributed, were determined with the Wilcoxon Signed Ranks Test. The statistical analyses were performed with the statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA).
Results

TGFβ induces both LH2b mRNA and LH2 protein expression in human synovial osteoarthritic fibroblasts.

Previously, we have shown that TGFβ induces LH2b expression in the synovium of murine knee joints (17). To investigate whether TGFβ had similar effects in humans, we isolated fibroblasts from synovial tissue obtained from osteoarthritis patients that underwent knee joint arthroplasties. TGFβ was compared to CTGF to examine if there were differences between these pro-fibrotic factors regarding PLOD gene expression in human synovial osteoarthritic fibroblasts. Adenoviral TGFβ overexpression significantly induced PLOD2 (LH2b mRNA) expression, whereas PLOD1 and PLOD3 were not significantly altered compared with the adenoviral control Ad-Luc (Figure 1a). As expected, overexpression of adenoviral CTGF had no effect on PLOD1, PLOD2 or PLOD3 in human synovial fibroblasts. To confirm whether TGFβ also enhances LH2 protein expression, fibroblasts were stimulated with 10 ng TGFβ and subsequently harvested for western blot analysis. The LH2 protein levels were strongly increased after both 24 and 48 h of TGFβ stimulation confirming that the increased LH2b mRNA expression results in elevated LH2 protein levels (Figure 1b). In compliance with our previous results in mice, PLOD2 and LH2 protein expression were also induced in human synovial fibroblasts by TGFβ and not by CTGF stimulation.

Figure 1.

TGFβ induces both PLOD2b mRNA and LH2 protein expression levels in human synovial fibroblasts.

[a] PLOD2b mRNA expression in human synovial fibroblasts 24 h after transduction with Ad-TGFβ or Ad-CTGF. TGFβ strongly induces PLOD2b, whereas PLOD1 and PLOD3 were not significantly affected by TGFβ. CTGF did not alter mRNA expression of the different PLOD isoforms.

[b] Human synovial fibroblasts were cultured in the presence or absence of TGFβ for 24 and 48 h and harvested for western blot analysis. A strong increase in LH2 protein level was observed after both 24 and 48 h TGFβ stimulation.
SB-505124 inhibits TGFβ-induced Smad2/3P and Smad1/5/8P.

We investigated whether Smad2/3P or Smad1/5/8P were required for TGFβ-induced LH2b expression. First, we investigated the capacity of SB-505124 and dorsomorphin to inhibit TGFβ-induced Smad2/3P and Smad1/5/8P in human synovial fibroblasts, by western blot analysis. In the absence of exogenous TGFβ, a moderate basal protein level of Smad1/5/8P was observed, whereas no basal protein levels for Smad2/3P were detected (Figure 2a). TGFβ stimulation increased both Smad2/3P and Smad1/5/8P protein levels. In contrast to the basal Smad1/5/8P levels, which were completely inhibited by dorsomorphin, TGFβ-induced Smad1/5/8P was only partly reduced by dorsomorphin. SB-505124 strongly decreased TGFβ-induced Smad1/5/8P and completely blocked TGFβ-induced Smad2/3P. As expected, dorsomorphin did not alter Smad2/3P protein levels. These results indicate that ALK4/5/7 kinase activity is involved in both TGFβ-induced Smad2/3 and Smad1/5/8 phosphorylation.

Inhibition of ALK4/5/7 kinase activity prevents induction of LH2b in human synovial fibroblasts.

We determined whether ALK4/5/7 or ALK1/2/3/6 kinase activity was responsible for increased PLOD2, CTGF and COL1A1 expression. Therefore, the human synovial fibroblasts were stimulated with TGFβ alone or TGFβ with SB-505124 or dorsomorphin. TGFβ stimulation significantly induced LH2b, COL1A1 and CTGF mRNA expression in human synovial fibroblasts (Figure 2b). PLOD1 and PLOD3 mRNA expression were not significantly affected by TGFβ. Also, blocking the ALK4/5/7 or ALK1/2/3/6 kinase activity with SB-505124 or dorsomorphin, respectively, had no significant effects on PLOD 1 or PLOD3 mRNA expression. SB-505124 completely blocked TGFβ-induced LH2b, COL1A1 and CTGF mRNA expression. Similar to SB-505124, dorsomorphin also fully blocked TGFβ-induced COL1A1 and CTGF. In contrast to SB-505124, LH2b mRNA expression was still significantly up-regulated by TGFβ in the presence of dorsomorphin (Figure 2b). These results demonstrate that TGFβ-induced LH2b is mainly dependent on ALK4/5/7 kinase activity.

Figure 2.

[a] TGFβ both induces Smad2/3P and Smad1/5/8P in human synovial fibroblasts. SB-5 blocks both TGFβ-induced Smad2/3P and Smad1/5/8P, whereas DM only partially blocks TGFβ-induced Smad1/5/8P and has no effect on Smad2/3P.
Overexpressing caALK5 but not caALK1 induces LH2b mRNA and LH2 protein in human synovial fibroblasts.

To confirm that LH2b is ALK5- and not ALK1-dependent, we investigated the effect of caALK1 and caALK5 on PLOD1, PLOD2 and PLOD3 gene expression. Overexpression of caALK5 significantly induced LH2b mRNA expression. In contrast, overexpressing caALK1 did not alter LH2b mRNA expression (Figure 3). Neither PLOD1 nor PLOD3 mRNA expression were affected by overexpressing either caALK1 or caALK5. Also, LH2 protein was elevated after overexpression of caALK5, whereas caALK1 had no effect. These results show that PLOD2/LH2 is induced via ALK5 and not via ALK1 signaling in human synovial fibroblasts.

Figure 2.
[b] mRNA expression of PLOD1, PLOD2b, PLOD3, CTGF and COL1A1 in human synovial fibroblasts 24 h after TGFβ stimulation with and without 4 h pre-incubation with SB-5 or DM. Inhibition of ALK4/5/7 kinase activity with SB-5, fully blocks the induction of PLOD2b by TGFβ. Blocking the ALK1/2/3/6 kinase activity with DM, only slightly reduces TGFβ-induced PLOD2b.

Figure 3. [a] mRNA expression of PLOD1, PLOD2b and PLOD3 in human synovial fibroblasts, 24 h after transduction with Ad-caALK1 or Ad-caALK5. Whereas overexpressing caALK5 induces PLOD2b, caALK1 has no effect on PLOD2b mRNA expression. Neither PLOD1 nor PLOD3 were affected by caALK1 or caALK5. [b] LH2 protein expression was elevated after Ad-caALK5 overexpression but not after overexpression of Ad-caALK1.
TGFβ dose-dependently induces Smad2/3P and Smad1/5/8P but this does not influence the height of the LH2b mRNA expression level.

We investigated dose-dependent induction of Smad1/5/8P and/or Smad2/3P levels and the subsequent effect on LH2b regulation. Therefore, human synovial fibroblasts were stimulated with a concentration range from 0 to 15 ng TGFβ. Smad1/5/8P increased along with the TGFβ concentration, whereas Smad2/3P decreased as the TGFβ concentration increased (Figure 4a). High TGFβ concentrations (7.5 ng and higher) favored Smad1/5/8P, whereas lower TGFβ concentrations of approximately 0.63–5 ng favored Smad2/3P in human osteoarthritic fibroblasts. To examine whether a low (1 ng) TGFβ concentration increased LH2b mRNA expression rather than a high (20 ng) TGFβ concentration, the LH2b mRNA expression was determined after 24 h stimulation with 1 and 20 ng TGFβ. To study if blocking the Smad2/3 or Smad1/5/8 route modulated the LH2b expression, the TGFβ stimulation was performed with and without the presence of dorsomorphin and SB-505124. No differences between 1 and 20 ng TGFβ were found on LH2b mRNA expression with or without the SB-505124 or dorsomorphin (Figure 4b).

![Figure 4.](image)

**Figure 4.** [a] Smad 1/5/8P increases with higher TGFβ concentrations whereas Smad 2/3P decreases at higher TGFβ concentration in comparison to 0.63 ng TGFβ. [b] No significant differences were observed in PLOD2b mRNA expression between 1 and 20 ng TGFβ stimulations.
Discussion

Fibrosis is characterized by an accumulation of collagen. In fibrotic lesions, collagen contains an increased amount of pyridinoline cross-links. LH2b is known to induce the formation of pyridinoline cross-links in collagen. Collagen containing pyridinoline cross-links is less susceptible to enzymatic degradation, which may lead to collagen accumulation (18). Furthermore, the amount of pyridinoline cross-links in the matrix is an important criterion in assessing the irreversibility of fibrosis (18).

In previous studies, we have shown that TGFβ-induced irreversible fibrosis, whereas CTGF-induced transient fibrosis (6, 12, 17). In this study, we demonstrated that both LH2b mRNA and LH2 protein expression were strongly elevated by TGFβ and not by CTGF in osteoarthritic human synovial fibroblasts. Van der Slot et al. studied the effect of TGFβ on skin fibroblasts and also found an induction of LH2b mRNA expression. This increase of LH2b was accompanied by an increase in pyridinoline cross-links (25). Since TGFβ was increased in the synovial fluid of osteoarthritis patients, an induction of LH2b and an increase of pyridinoline cross-links in the synovium is plausible (3). Elevated amounts of pyridinoline cross-links were reported in different fibrotic diseases: for instance, in systemic sclerosis, alcoholic cirrhosis and glomerulosclerosis (18). We propose that this induction of LH2b and therefore an increase in pyridinoline cross-links, may be responsible for persistent fibrosis in osteoarthritis. Besides LH2b, TGFβ also induced CTGF mRNA expression in human synovial fibroblasts. Some researchers propose that CTGF is necessary for the persistence of fibrosis, since they found that only subcutaneous injection of TGFβ plus CTGF into the tissue of newborn mice produced long-term fibrotic tissue (15). However, in our previous paper, we showed that TGFβ-induced persistent fibrosis for at least 3 months, whereas CTGF-induced fibrosis was resorbed by day 28 (17). CTGF might still be an important factor in the maintenance of TGFβ-induced fibrosis but in itself it was not capable of producing persistent fibrosis. TGFβ alone could be sufficient to produce persistent fibrosis due to the collagen accumulation and subsequent cross-linking by LH2b. However, since CTGF is always expressed by exposure to TGFβ, we cannot rule out an additional role for CTGF in TGFβ-dependent persistent fibrosis. Since we proposed that LH2b is the factor responsible for the persistence of TGFβ-induced synovial fibrosis in osteoarthritis, we investigated how LH2b expression was regulated via TGFβ signaling. This knowledge could be of considerable importance for finding a target to prevent the induction of LH2b and thereby persistent fibrosis in osteoarthritis.

We investigated whether LH2b, CTGF and COL1A1 mRNA expression were specific for ALK4/5/7 or ALK1/2/3/6 signaling. Dorsomorphin and SB-505124 were used to inhibit the kinase activity of ALK1/2/3/6 and ALK4/5/7, respectively. Dorsomorphin completely
inhibited the basal “ALK1/2/3/6-mediated” Smad1/5/8P level but could only slightly reduce TGFβ-induced Smad1/5/8P. This minor reduction by dorsomorphin might be due to the abolished basal Smad1/5/8P level, since the TGFβ-induced Smad1/5/8P signal was an accumulation of both the basal Smad1/5/8P and TGFβ-induced Smad1/5/8P. This suggests that ALK1/2/3/6 kinase activity is not required for TGFβ-induced Smad1/5/8P in human synovial fibroblasts. Blocking the ALK4/5/7 kinase activity with SB-505124 inhibited both TGFβ-induced Smad2/3P and Smad1/5/8P. This indicated that TGFβ-induced Smad2/3P and Smad1/5/8P were both dependent on ALK4/5/7 kinase activity in human synovial fibroblasts. This could potentially explain why TGFβ-induced Smad1/5/8P could not be blocked by dorsomorphin. Involvement of TGFβ-mediated ALK4/5/7 kinase activity in both Smad2/3 and Smad1/5/8 phosphorylation has also been shown in C2C12 and HepG2 cells, where dorsomorphin did not abolish TGFβ-induced Smad1/5/8P, similar to our data (10). Blocking either ALK1/2/3/6 or ALK4/5/7 kinase activity in the human synovial fibroblasts prevented the induction of COL1A1 and CTGF after TGFβ stimulation. Since dorsomorphin is unable to abolish TGFβ-induced Smad1/5/8 phosphorylation, the inhibition of COL1A1 and CTGF by dorsomorphin could very well be reliant on a non-Smad-dependent pathway. Non-Smad pathways that can be blocked by dorsomorphin are the MAPKs p38, ERK1/2, Akt and the Src pathway (23, 26). In different articles and for different cell types, including fibroblasts, it has been shown that the Src pathway is necessary for TGFβ-related CTGF induction. Therefore, blockage of this non-Smad pathway might clarify the inhibition of CTGF by dorsomorphin that we found in the human synovial fibroblasts (27-29). Furthermore, it has been shown that CTGF was required for TGFβ-induced COL1A1 expression (30, 31). Since we blocked TGFβ-related CTGF induction by dorsomorphin, this would also prevent TGFβ-related CTGF-dependent COL1A1 elevation. For scleroderma fibroblasts, it has been reported that the TGFβR1-dependent induction of COL1A1 and CTGF did not involve Smad2/3 activation but was mediated by ALK1/Smad1 and ERK1/2 pathways (32). However, in Smad3-knockout fibroblasts, it was shown that normal dermal fibroblasts required Smad3 for TGFβ-induced CTGF expression (33). This difference between normal and scleroderma fibroblasts was thought to be due to the existence of an alternative TGFβ-dependent, Smad3-independent signaling pathway that might operate in chronic stages of SSc fibrosis. Thus, depending on the cell type and stage, the signaling by TGFβ might be different. Our results show that in osteoarthritic human synovial fibroblasts both the ALK4/5/7 and ALK1/2/3/6 route are required for induction of COL1A1 and CTGF.

Similar to COL1A1 and CTGF gene expression, inhibition of ALK4/5/7 kinase activity prevented TGFβ-induced LH2b mRNA expression. Strikingly, LH2b was still significantly upregulated by TGFβ when the ALK1/2/3/6 kinase activity was blocked. Furthermore, over-expressing caALK5 induced both LH2b mRNA expression and LH2 protein expression, whereas caALK1 did not alter LH2b expression. Whereas COL1A1 and CTGF depend on
both the ALK4/5/7 and ALK1/2/3/6 route, LH2b only required the ALK4/5/7 route. These results suggest that TGFβ-induced LH2b is governed by the canonical TGFβ type 1 receptor ALK5 and does not require ALK1 signaling. Since we found a shift towards Smad1/5/8P at higher TGFβ concentrations we expected lower LH2b mRNA expression at higher TGFβ concentrations due to the diminished Smad2/3P, in comparison to the lower TGFβ concentrations. The effects observed on western blot were after 30 min of TGFβ stimulation, whereas the samples for Q-PCR were stimulated for 24 h. Initially, we thought this might explain the lack of changes in LH2b mRNA expression. However, we repeated the experiment with shorter stimulations with TGFβ (data not shown), which did not show any change in LH2b expression. A possible explanation can be that a limited amount of Smad2/3 signaling was sufficient to fully induce LH2b expression.

Since we propose that LH2b is responsible for the persistence of fibrosis in osteoarthritis, blocking ALK5 may prevent persisted fibrosis. This is supported by a study that showed that progressive TGFβ1–induced pulmonary fibrosis in rats was inhibited when an ALK5 kinase activity inhibitor was administered at the time of initiation of fibrogenesis. Furthermore, they showed that blocking the ALK5 kinase activity even blocked progressive fibrosis in rats with established fibrosis (34). Although blocking LH2b by ALK5 inhibition sounds promising for prevention of fibrosis, in joints with osteoarthritis, extreme caution should be taken as ALK5 is indispensable in maintenance of cartilage. As such, blocking ALK5 in general will have adverse effects on the cartilage causing increased MMP-13 expression and decreased aggrecan and collagen type II synthesis by the chondrocytes. This will result in loss of homeostasis of the articular cartilage (11).

In this study, we showed that LH2b was induced by TGFβ in osteoarthritic human synovial fibroblasts. Overexpressing ALK5 induced LH2b, whereas blocking the ALK5 kinase activity prevented TGFβ-induced LH2b expression. In contrast to COL1A1 and CTGF, LH2b did not require both ALK1/2/3/6 and ALK5 signaling but relies on ALK5 signaling alone. In osteoarthritis, TGFβ is elevated in the knee joint, potentially causing elevated LH2b expression. LH2b increases the formation of pyridinoline cross-links in collagen, which are commonly found elevated in different fibrotic diseases. Therefore, TGFβ–induced LH2b may be responsible for the persistence of fibrosis in osteoarthritis. Blocking of LH2b in osteoarthritis may prevent the formation of the pyridinoline cross-links and consequently the formation of persistent fibrosis and chronic joint stiffness.
Acknowledgments

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References


Chapter 6

Concluding remarks
Synovial fibrosis is a synoviopathy that affects more than 50% of the OA patients and is an important cause for joint stiffness in OA. In this thesis we have investigated which factor is responsible for OA-related fibrosis. TGFβ is known for its fibrotic properties and is found elevated in the synovial fluid of OA patients. Because inhibition of TGFβ, due to its role in cartilage maintenance, will have severe side effects we searched for genes downstream of TGFβ which may be involved in synovial fibrosis and could be a target for therapy. The most promising gene, looking both at side effects and on its possible contribution to fibrosis, that came out of our exploration was PLOD2.

We found PLOD2 to be elevated in OA fibroblasts stimulated with TGFβ and in the synovium of mice with TGFβ-induced fibrosis, but not in conditions with the pro-fibrotic growth factor CTGF. Because CTGF induces transient fibrosis whereas TGFβ-induced fibrosis is persistent, this is highly suggestive for a key role of PLOD2 in the persistence of fibrosis. Especially because PLOD2 encodes for the collagen cross-linking enzyme lysyl hydroxylase 2b (LH2b), which activity results in the formation of pyridinoline cross-links. These pyridinoline cross-links make collagen harder to degrade and more rigid and are present at elevated levels in different fibrotic tissues. We found in an experimental OA model that is accompanied by fibrosis, besides elevated PLOD2 expression levels (both on gene expression and protein level), an increase in the amount of pyridinoline cross-links per triple helix.

For that reason it is plausible that PLOD2 is responsible for OA-related persistent fibrosis. Most importantly, we found that PLOD2 was also increased in the synovium of patients with end-stage OA (Figure 1). It would have been convenient if PLOD2 would have been regulated via TGFβ/ALK1 signaling, because ALK1 signaling can be deleterious for cartilage and therefore could potentially be blocked without negative side effects in an OA joint. Unfortunately, we found that LH2b was regulated via TGFβ/ALK5 signaling. Blocking ALK5 would be disastrous for the cartilage because this leads to elevated MMP13 expression and therefore cartilage breakdown.

The question that still remains after completion of this thesis is whether blocking the induction of LH2 in OA indeed will prevent or even reverse OA-related synovial fibrosis. For clinical application it is very important to make the distinction whether inhibition of LH2b can only prevent or also reverse OA-related fibrosis. When inhibition of LH2 can only prevent fibrosis, exclusively patients with onset OA, before fibrosis occurs, could be treated. However, we presume that blocking LH2 in OA will both prevent and reverse OA-related synovial fibrosis, because the collagen will not be longer highly cross-linked. Therefore, in time the collagen will be degraded by the elevated MMP activity in the OA joints. We expect that solving fibrosis in OA-patients will contribute to reduction of joint stiffness and even joint pain. This since the fibrotic synovium may impair joint movement by increasing the tension of the joint capsule and thereby also limiting smooth joint movement.

Next to potentially solve OA-related fibrosis, inhibition of LH2b could also be promising
to prevent arthrofibrosis, a disease whereby knee motion is limited due to massive fibrosis after joint injury or surgery.

Unfortunately, we could not find an effective method to inhibit LH2 activity within the time span of this project. Because, LH2 is intracellular and no small molecule inhibitors are available LH2 is a difficult target to inhibit. However, it still our goal to inhibit LH2 and we want to achieve this in the near future in experimental OA models, and if successful, in OA patients.

**Figure 1.** Schematic overview of our main findings and the proposed effect of PLOD2 on the synovium.
Chapter 7

Nederlandse samenvatting
List of abbreviations
Dankwoord
List of publications
Curriculum vitae
Osteoarthritis (OA) wordt gekenmerkt door kraakbeenschade, osteofytvorming en synoviale fibrose. Deze fibrose maakt het synovium dikker en stijver waardoor het gewricht minder bewegelijk wordt. Daarom wordt synoviale fibrose gezien als een van de hoofdoorzaken van gewrichtsstijfheid. Naar schatting heeft het merendeel van de OA patiënten last van synoviale fibrose.

TGFβ dat verhoogd aanwezig is in het gewricht tijdens OA staat bekend als de factor verantwoordelijk voor fibrose (hoofdstuk 1). In eerdere studies hebben we laten zien dat het verhoogd tot expressie brengen van TGFβ in muizen kniegewrichten resulteert in fibrose, waar het inhibitoren van TGFβ in experimentele OA in muizen juist fibrose voorkomt. Helaas zal het blokken van TGFβ in een OA gewricht te veel negatieve effecten hebben, aangezien TGFβ nodig is voor het onderhoud van het kraakbeen. Daarom hebben we gezocht naar een eiwit/gen lager in de TGFβ signaalroute dat verantwoordelijk is voor de synoviale fibrose (hoofdstuk 2). Om dit eiwit/gen te vinden hebben we de expressie van genen gemeten die coderen voor eiwitten die betrokken zijn bij de opbouw en afbraak van de extracellulaire matrix (ECM) of deze kunnen beïnvloeden. Deze genen hebben we gemeten in synoviale fibroblasten gestimuleerd met TGFβ, in synovium van muizen met TGFβ-geïnduceerde fibrose en collagenase-geïnduceerde OA, maar ook in het synovium van patiënten met eind-stadium OA. We vonden dat de genen PLOD2, LOX, COL1A1, Col5A1 en TIMP1 hoger tot expressie kwamen in al deze OA/fibrotische condities in vergelijking met de controles.

Vervolgens hebben we TGFβ geïnduceerde “blijvende” fibrose vergeleken met CTGF geïnduceerde “tijdelijke” fibrose (hoofdstuk 3). Dit omdat een verschil in genregulatie tussen TGFβ en CTGF geïnduceerde fibrose de factor verantwoordelijk voor de persistentie van TGFβ geïnduceerde fibrose kan zijn. Het meest opmerkelijke verschil tussen CTGF- en TGFβ-geïnduceerde fibrose was de expressie van PLOD2. PLOD2 codeert voor het enzym lysyl hydroxylase 2b (LH2b) dat zorgt voor een verhoging van het aantal pyridinoline kruisverbindingen tussen de collageen drievoudige helixen. Deze kruisverbindingen maken het collageen moeilijker afbreekbaar voor enzymatische reacties, wat resulteert in accumulatie van collageen en daardoor bijdragen aan fibrose. We hebben gemeten of deze pyridinoline kruisverbindingen ook daadwerkelijk verhoogd zijn in het synovium van muizen met collagenase-geïnduceerde OA dat gepaard gaat met LH2b en waar LH2b zowel op gen als op eiwit niveau verhoogd is. We vonden dat er vanaf dag 21 na artrose inductie inderdaad een verhoging aantoonbaar was van het aantal pyridinoline kruisverbindingen per collageen drievoudige helix. Dit maakt PLOD2/LH2 een zeer interessant doelwit om te remmen bij synoviale fibrose tijdens OA. Om te vast te stellen of LH2b inderdaad verantwoordelijk is voor de persistentie van fibrose hebben we LH2b in combinatie met CTGF verhoogd tot expressie
gebracht in muizenknietjes (hoofdstuk 4). Dit om te testen of LH2b, CTGF geïnduceerde fibrose persistent kan maken. Helaas konden we door technische problemen in dit experiment geen valide conclusies trekken.

Tevens hebben we onderzocht via welke TGFβ signaalroute, ALK1 of ALK5, LH2 gereguleerd word (hoofdstuk 5). Doormiddel van overexpressie en remming studies hebben we aangetoond dat LH2 gereguleerd wordt via TGFβ/ALK5 signalering. Aangezien ALK5 de route van TGFβ is die zorgt voor het onderhoud van het kraakbeen is het niet verstandig deze te onderdrukken in een gewricht van een OA patiënt. Daarom zal er naar een andere manier gekeken moeten worden om LH2b te remmen.

In dit proefschrift hebben we gezocht naar de oorzaak van OA gerelateerde synoviale fibrose. We hebben gevonden dat LH2b in verscheidene fibrotische/OA gerelateerde condities sterk verhoogd is ten opzichte van de controles. Ook hebben we gevonden dat de pyridinoline kruis verbindingen die door LH2b mogelijk gemaakt worden verhoogd zijn bij experimentele OA. Deze pyridinoline kruisverbindingen zijn verhoogd aangetroffen in verschillende fibrotische ziekten. Dit toont de potentie aan van het remmen van LH2b om synoviale fibrose te voorkomen. Daarom is het een aantrekkelijke optie om LH2b te remmen tijdens OA om te onderzoeken of dit een nieuwe behandeling kan worden om gewrichtsstijfheid bij OA patiënten te verminderen.
**List of abbreviations**

<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>A disintegrin and metalloproteinase domain 12</td>
<td>ADAM12</td>
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<tr>
<td>Activin receptor-like kinase</td>
<td>ALK</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Ad</td>
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<tr>
<td>Collagen, type x, alpha x</td>
<td>COLxAx</td>
</tr>
<tr>
<td>Complementary DNA</td>
<td>cDNA</td>
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<tr>
<td>Connective tissue growth factor</td>
<td>CTGF</td>
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<td>Constitutively active</td>
<td>ca</td>
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<td>Dorsomorphin</td>
<td>DM</td>
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<tr>
<td>Extracellular matrix</td>
<td>ECM</td>
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<tr>
<td>Fat-conditioned medium</td>
<td>FCM</td>
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<tr>
<td>high-performance liquid chromatography</td>
<td>HPLC</td>
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<td>Human synovial fibroblasts</td>
<td>hSF</td>
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<td>Intra-articularly</td>
<td>i.a.</td>
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<tr>
<td>Lysyl hydroxylase</td>
<td>LH</td>
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<tr>
<td>Lysyl oxidase</td>
<td>LOX</td>
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<tr>
<td>Matrix metalloproteinases</td>
<td>MMP</td>
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<tr>
<td>osteoarthritis</td>
<td>OA</td>
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<tr>
<td>Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2</td>
<td>PLOD2</td>
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<td>Prostaglandin F2α</td>
<td>PGF2α</td>
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<tr>
<td>Quantitative PCR</td>
<td>Q-PCR</td>
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<td>SB-505124</td>
<td>SB-5</td>
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<tr>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>TIMP-1</td>
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<tr>
<td>Transforming growth factor-beta</td>
<td>TGFβ</td>
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<tr>
<td>Unilateral ureteral obstruction</td>
<td>UUO</td>
</tr>
</tbody>
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Dankwoord

Als eerste wil ik de afdeling Experimentele Reumatologie in zijn geheel bedanken. Ik heb hier echt een hele leuke tijd gehad en deed met plezier mijn onderzoek. Iedereen is altijd zeer behulpzaam en betrokken of het nu om werk of privé gaat. Ook de dagjes uit, sinterklaas- en kerstvieringen, als ook de spelletjes- en film avonden waren altijd weer een succes. Verder heb ik veel plezier beleefd aan het mede organiseren van enkele kerst vieringen en het maken van filmsketches. Wat een top afdeling!
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List of publications

1. **Unraveling OA-related synovial fibrosis: A step closer to solving joint stiffness.**
   Remst DF, Blaney Davidson EN, van der Kraan PM.
   (Accepted for publication in Rheumatology)

2. **TGF-ß induces Lysyl hydroxylase 2b in human synovial osteoarthritic fibroblasts through ALK5 signaling.**
   Remst DF, Blaney Davidson EN, Vitters EL, Bank RA, van den Berg WB, van der Kraan PM.

3. **Gene expression analysis of osteoarthritis synovium reveals elevation of transforming growth factor-beta responsive genes in osteoarthritis-related fibrosis.**
   Remst DF, Blom AB, Vitters EL, Bank RA, van den Berg WB, Blaney Davidson EN, van der Kraan PM.

4. **Osteoarthritis-related fibrosis is associated with both elevated pyridinoline cross-link formation and lysyl hydroxylase 2b expression.**
   Remst DF, Blaney Davidson EN, Vitters EL, Blom AB, Stoop R, Snabel JM, Bank RA, van den Berg WB, van der Kraan PM.

5. **Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice.**
   Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM.
Curriculum vitae

Dennis Florian George Remst werd geboren op 7 december 1981 te Oss. Na het behalen van zijn MAVO diploma in 2000, startte hij in dat zelfde jaar een opleiding tot middenkader-functionaris laboratoriumtechniek met als afstudeerrichting biochemie op het ROC in Oss.

Nadat hij deze opleiding succesvol had afgerekend koos hij ervoor om verder te studeren en begon hij aan de hogere laboratoriumopleiding met als specialisatie biochemie op de Hogeschool van Arnhem en Nijmegen (HAN). Zijn eindstage deed hij binnen de onderzoeksgroep van prof. Wim van den Berg op de afdeling Experimentele Reumatologie van het Radboudumc in de werkgroep van dr. Peter van der Kraan onder directe begeleiding van dr. Esmeralda Blaney Davidson. Hier deed hij onderzoek naar de effecten van TGFβ-ALK5 en TGFβ-ALK1 signalering in chondrocyten op genen betrokken bij kraakbeen homestase.


Tijdens zijn promotietraject bestuurde hij de rol van lysyl hydroxylase 2b in synoviale OA-gerelateerde fibrose. Gedurende zijn promotieonderzoek heeft hij op diverse nationale en internationale congressen zijn werk gepresenteerd en won in 2012 de ORS New Investigator Recognition Award (NIRA).

Momenteel is Dennis werkzaam als huisvader en ziet hij met veel plezier zijn twee fantastische kinderen, Luca en Fenna, opgroeien. Hij mist het onderzoek wel en wil graag weer aan de slag op een onderzoekslaboratorium.