# Dissecting cellular function of fibronectin in osteoarthritic cartilage

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# Chapter 1

Introduction

## Osteoarthritis

Osteoarthritis (OA) is an age-related, heterogeneous, degenerative disease of the articular joints, characterized by pathological changes in amongst others cartilage, synovium, and subchondral bone [1]. OA may develop in any joint, but most commonly affects knees, hips, and hands [2]. Risk factors for OA can be divided in systemic factors, including increasing age, female sex, obesity, and genetic predisposition, and local factors, such as joint injury and abnormal loading on the joint [3, 4].

Currently, OA is globally the most prevalent joint disease and affected almost 1.5 million people in the Netherlands in 2019 [4-6]. The societal and economic burden of OA is expected to increase exponentially in the coming years, given the ageing of the population and the increasing prevalence of obesity [4, 5, 7-9]. The social burden is marked by pain and stiffness in the articular joints and decreased mobility, which can result in significant disability in everyday life [8]. The considerable cost of OA lies in the absence from work, social services, and medical costs, where the costs of OA were 1.4% of total health care costs in the Netherlands in 2017 [5, 6, 10]. Despite these detrimental consequences, to date no effective treatment to halt or reverse the progression of the disease is available except pain relief medication and, in a final stage, joint replacement surgery. One major reason for unsuccessful disease modifying OA drug development is inadequate insight into underlying pathophysiological mechanisms.



## Articular cartilage

Articular cartilage covers the end of long bones and is designed to withstand the forces that are exerted upon the joint by providing a low-friction, load-bearing surface between bones [11, 12]. It is aneural and avascular tissue, however, the cellular organisation and composition is complex [13, 14]. To be able to transfer the loads during mechanical loading and joint motion, the tissue consists of mainly water (>70%) and extracellular matrix (ECM) that is synthesized and maintained by the only cell type present: chondrocytes (**Figure 1A**). The major components of cartilage ECM are proteoglycans, mainly aggrecan, various types of collagen, the most abundant being type II collagen, and glycoproteins, such as fibronectin [14, 15]. Proteoglycan consists of a central core protein to which one or multiple negatively charged glycosaminoglycan (GAG) side chains are covalently linked. Generally, proteoglycans exist as aggregates consisting of non-covalently associated proteoglycans with hyaluronic acid and link protein [14]. Type II collagen fibrils form a network that entraps negatively charged proteoglycan aggregates, other small proteoglycans and cartilage matrix proteins. The arrangement of the collagen fibril network differs in the different layers of the cartilage, referred to as superficial layer, middle zone, and deep zone (**Figure 1A**). In the superficial layer the collagen fibrils are parallel to the surface. The distribution becomes more random in the middle zone and is perpendicular to the surface in the deep zone [16], together significantly enhancing tensile strength while the proteoglycan aggregates provide compressive resilience. Upon compression, water molecules

associated with the hydrophilic GAG chains of proteoglycans are dissipated from the cartilage and when compression is released, the proteoglycans attract the water molecules via osmosis back into the matrix, restoring original cartilage composition [15].

Chondrocytes have different morphologies and organization, depending on their localization in the articular cartilage layers. In the superficial layer, chondrocytes are closely together and more flattened, while in the middle zone there are less cells and they are rounder. Furthermore, in the deep zone they become larger and form strings parallel to the collagen fibrils (**Figure 1A**). In healthy adult cartilage the chondrocytes are in a maturational arrested state and there is an equilibrium between anabolic and catabolic processes. Turnover of collagen type II is low, while glycosaminoglycans and other non-collagen matrix proteins are more readily produced [15]. Calcified cartilage is the interface between the articular cartilage and subchondral bone, where there are few chondrocytes with a hypertrophic phenotype. The calcified cartilage is separated from the articular cartilage by a thin line marking the mineralization called the tidemark.

## Pericellular matrix of articular cartilage

One aspect of the complexity of the ECM of articular cartilage lies in the matrix heterogeneity, where interterritorial and pericellular matrices are recognized [17, 18]. The pericellular matrix (PCM) is a narrow matrix region of approximately 2-4 μm directly surrounding the chondrocytes, that is biochemically and biomechanically distinct from the interterritorial matrix (**Figure 1B**) [19]. The PCM is characterized by high concentrations of proteoglycans,

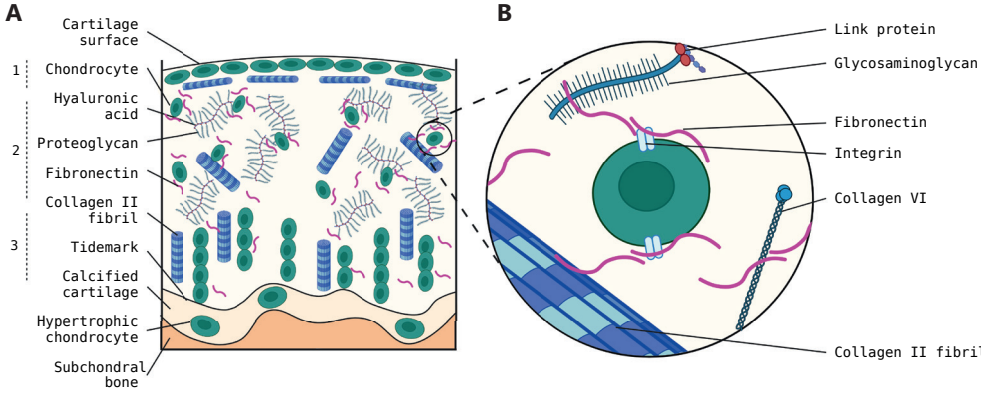


**Figure 1 | Overview of composition articular cartilage and the pericellular matrix directly surrounding the chondrocyte.** (**A**) Schematic representation of structure and morphology of chondrocytes and extracellular matrix proteins in articular cartilage, including collagen type II fibrils, proteoglycan aggregates, and fibronectin. Dotted lines represent the different layers in cartilage: 1) Superficial layer. 2) Middle zone. 3) Deep zone. (**B**) Schematic representation of the pericellular matrix surrounding a chondrocyte. The pericellular matrix is rich in proteoglycans, collagen type II, fibronectin and collagen type VI. (Created with Biorender.com).

collagen type II and fibronectin, as well as collagen type VI, which is exclusively present in the PCM and not in the interterritorial matrix [20]. Although the complete role of the PCM is still unknown, it has been shown to serve as a transducer of biomechanical and biochemical signals to the chondrocytes [17, 21]. The properties of the PCM can regulate mechanical and chemical surroundings of the chondrocyte, thereby influencing cartilage matrix homeostasis [22]. Homozygous inactivation of *COL6A1* in mice showed structurally intact PCM in the articular cartilage, but exhibited significantly reduced mechanical properties compared to wildtype controls. Moreover, the mice also showed accelerated development of OA joint degeneration, giving indirect evidence that alterations in PCM composition and properties can lead to the progression of OA [23].



Chondrocytes interact with the PCM via transmembrane receptors, such as integrins, that mediate cell function, including cell proliferation, cell survival and matrix remodeling. Integrins are heterodimeric receptors, consisting of α and β sununits, that connect the PCM with intracellular cytoskeletal structures and signaling complexes [24]. In humans, there are 18 different α and 8 different β subunits, which can be combined to 24 different integrin heterodimers with different ligand specificities [25]. Chondrocytes express multiple integrins, including α5β1, αVβ1, αVβ3, αVβ5, α3β1, α1β1, and α10β1, which bind to various matrix components [26]. Binding of ligands results in activation of integrins, leading to conformational changes that initiate downstream kinase-mediated intracellular signaling or binding to cytoskeletal proteins via focal adhesion complexes [25]. In this way, integrins play an important role in chondrocyte cell adhesion, ECM remodeling, and mechanotransduction [24]. One key protein involved in these processes linking the cartilage matrix and chondrocytes is fibronectin.

## Fibronectin

In articular cartilage fibronectin is mostly localized in the PCM, where it is organized in a fibrillar network [27]. Fibronectin mediates a wide variety of cellular interactions with the ECM by binding to matrix proteins via multiple binding domains, as well as interactions with chondrocytes via integrins that mediate intracellular signalling [28, 29]. In this way, fibronectin functions as an adhesion molecule facilitating signals from the ECM to the chondrocytes, such as cell adhesion, differentiation and survival [30]. Fibronectin is a glycoprotein, which is usually comprised of a dimer consisting of two high molecular weight subunits (230 – 270 kDa) linked covalently by two C-terminal disulfide bonds [27]. Each monomer consists of three types of repeating units: type I, which are ~40 amino acid residues, type II, which are ~60 amino acids in length, and type III, which are ~90 residues long and the most abundant repeats in the protein [31]. More specifically, fibronectin consists of 12 type I repeats, 2 type II repeats, and 15-17 type III repeats (**Figure 2**).

Fibronectin is encoded by the *FN1* gene, which can give rise to up to 27 transcripts in humans by means of alternative splicing [28]. Alternative splicing occurs at three major sites, called extra domain A (EDA), extra domain B, and variable region (V) [32]. Splicing at the EDA and EDB domain results in inclusion or exclusion of one exon, whereas splicing at the V region can occur at multiple splice sites [31, 33]. Based on solubility two forms of fibronectin can be distinguished, namely the soluble plasma form and the less-soluble cellular form. Plasma fibronectin lacks the EDA and EDB domains, while cellular fibronectin consists of a more heterogenous group of isoforms [28]. This splicing variation provides cells the capacity to generate large numbers of transcripts with different binding properties to precisely alter the ECM composition in a developmental and tissue-specific manner [28]. Fibronectin has been shown to be essential for mesodermal migration, adhesion and proliferation during embryogenesis, as homozygous inactivation of fibronectin caused early embryonic lethality in mice [34].

Fibronectin can bind multiple matrix proteins, including heparin, collagen, and fibrin by distinct structural and functional domains (**Figure 2**). There are two major heparin-binding domains, the strongest is located at the C-terminal side (repeats III 12-14) and the weaker at the N-terminal side (repeats I 1-5) of the protein. The gelatin-binding domain (repeats I 6-9 and II 1-2) can bind to gelatin and collagen. There are two major fibrin-binding domains, the N-terminal domain (repeats I 4-5) and the C-terminal domain (repeats I 10-12) [28]. However, the different specific domains of fibronectin can interact with multiple binding partners. As a result, the variable modular structure results in flexibility of the fibronectin protein and its binding capacities. The main integrin-binding domain contains the arginine-glycine-aspartate (RGD) motif (in repeat III 10), which binds multiple integrin heterodimers, including the classic fibronectin receptor α5β1 [35, 36]. The fibronectin-α5β1 adhesion has been shown to be critical for cartilage regeneration in mice [37], emphasizing the importance

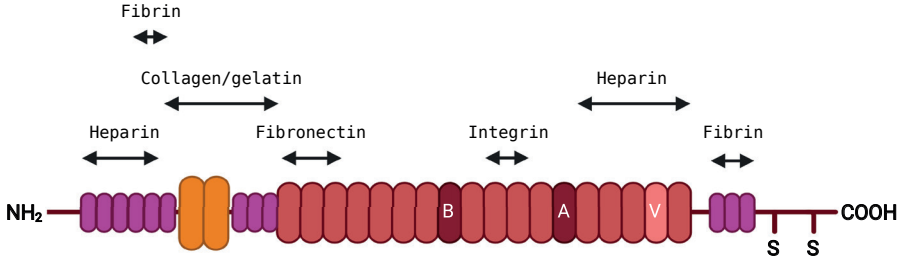


**Figure 2 | Structure of fibronectin.** Linear fibronectin monomer consisting of three types of repeats: type I (purple), type II (orange), and type III (red). Alternative spicing domains EDA (A) and EDB (B) are dark red and V region (V) is light red. Two C-terminal cysteine residues (S) are involved in dimerization of fibronectin via disulfide bonds. Main protein binding domains are indicated as arrow. (Created with Biorender.com).

of fibronectin binding to α5β1 in cartilage. However, in articular cartilage α3β1, αVβ1, αVβ3, and αVβ5 are known to also bind fibronectin [26].

## Pathophysiology osteoarthritic articular cartilage

Currently, OA is considered a disease of the whole joint, characterized by subchondral bone remodeling, formation of osteophytes, and inflammation of the synovium [13]. Moreover, a general hallmark of OA pathophysiology is the degeneration of articular cartilage, which is present to some extend in all OA affected joints (**Figure 3A**) [16]. In OA cartilage, chondrocytes become activated from their quiescent state, characterized by cell hypertrophy, cell clustering, and an increase of both matrix protein and matrix-degrading enzyme synthesis, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloprotease with thrombospondin motif (ADAMTS). The increased deposition of matrix proteins is insufficient to counteract the increased degradation by the matrix-degrading enzymes. Consequently, proteoglycan content is decreased and collagen is cleaved, resulting in disruption of the pericellular matrix and eventually degradation of the cartilage matrix (**Figure 3B**) [38].

Once the collagen network is affected, progression to irreversible cartilage degeneration is inevitable [39]. At the cartilage surface fissures form and chondrocytes become apoptotic, while the calcified zone increases, resulting in tidemark rupture. Moreover, fibronectin content increases in osteoarthritic cartilage due to both enhanced synthesis and enhanced retention [33, 40]. Concomitantly, fibronectin is proteolytically degraded into fragments (FN-fs), that amplify catabolic processes by up-regulating MMPs via the Toll-like receptor signaling pathway [41-43]. Fragmentation results in cryptic binding properties that naïve fibronectin does not share, thereby acquiring catabolic activities [44]. These alterations in the molecular composition and organization of the cartilage PCM and ECM as a result of abnormal matrix turnover lead to changes in the mechanical properties and structural integrity of the articular cartilage [16].

### Molecular pathophysiology of osteoarthritic cartilage

The exact underlying molecular mechanisms of OA pathophysiology are not completely understood. One way to elucidate these mechanisms is to identify genes and pathways involved in the development of OA by performing transcriptomic analyses of cartilage tissue. Comparisons of expression profiles between OA affected and healthy cartilage identified multiple relevant pathways to be differentially expressed. Various genes encoding ECM organisation, such as the collagen-encoding *COL1A1*, *COL2A1*, and *COL6A3*, and genes involved in matrix degradation, such as *MMP-3*, *MMP-13*, and *ADAMTS-5* were found to be up-regulated in OA cartilage (**Table 1**). Moreover, genes involved in skeletal development, such as *ALPL* and *DIO2* were found to be up-regulated, while the chondrogenic transcription

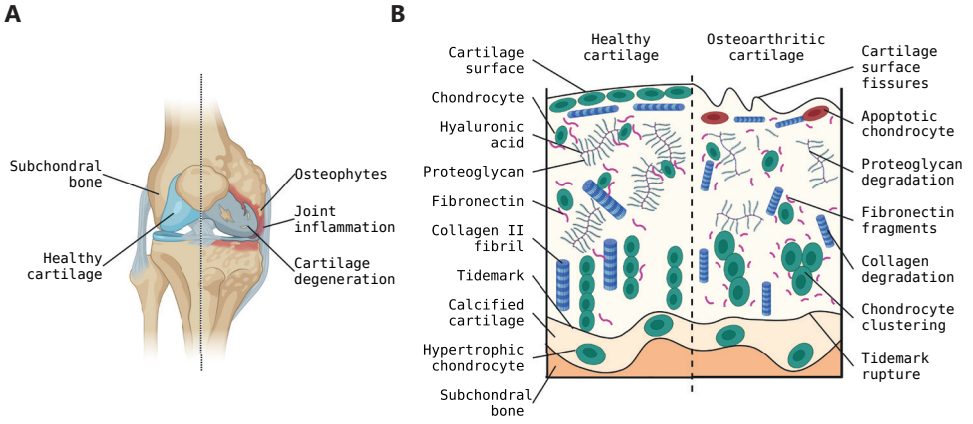


**Figure 3 | Overview of osteoarthritic pathophysiological changes in a joint and articular cartilage.** (**A**) Schematic representation of a knee joint, where the left side represents a normal joint, with cartilage covering the subchondral bone. The right side represents an OA affected joint, with cartilage degeneration, osteophyte formation, and joint inflammation. (**B**) Overview of structural differences in articular cartilage in healthy and osteoarthritic state, including collagen type II fibril and proteoglycan degradation, and fibronectin fragmentation. (Created with Biorender.com).

factor *SOX9* is down-regulated [45-47]. Multiple integrin subunits were found to be up-regulated, including *ITGAV*, *ITGB1*, and *ITGB5*, while *ITGA5* was down-regulated in OA cartilage compared to healthy.

Studies comparing expression profiles of lesioned and preserved cartilage areas from the same joints of patients with OA showed similar pathways to be differentially expressed [48-50]. However, there are numerous examples of genes that were only found differentially expressed in the comparison between lesioned and preserved, suggesting they play a role during the OA pathophysiologic process, but not in the initial phase. One such example is the gene *P3H2* encoding prolyl-3-hydroxylase 2, which is involved in collagen chain assembly and crosslinking [51]. Furthermore, genes such as *MMP-3*, *ITGA5*, and *COL9A1* showed opposite direction of effect in healthy versus OA cartilage compared to lesioned versus preserved cartilage. Therefore, these genes are likely mainly involved in the initial response of chondrocytes to cartilage degeneration and potential causal in the process. In our group, we collect OA relevant tissues, (cartilage, bone, and synovial fluid) and cells (bone-marrow derived mesenchymal stromal cells, primary chondrocytes) from patients undergoing joint replacement surgery due to end-stage OA as part of the Research osteoArthritis and Articular Cartilage (RAAK) study [48]. This study design allows for analyses independent of confounding factors such as sex, age, and joint site, while identifying expression changes specific to OA pathophysiology.

Notably, *FN1* was among the highest expressed genes in OA cartilage from the RAAK study and next to being highly up-regulated between lesioned and preserved OA cartilage, increased

**Table 1 | Pathways and a number of genes in those pathways involved in osteoarthritis pathophysiology, identified by transcriptome- wide profiles of healthy and osteoarthritic cartilage.**

| Pathway | Comparison | Joint | Dir | Gene symbol* | Ref |
|---|---|---|---|---|---|
| ECM organisation | Healthy vs OA | Knee | Up | *COL1A1* , *COL2A1* , *COL6A1* , *COL9A1*, *COL10A1* , *FN1* | [45-47] |
| | | | Down | *ACAN* | |
| | Lesioned vs preserved | Knee | Up | *COL1A1* , *COL6A3* , *FN1*, *P3H2* | [48, 51, 95] |
| | | | Down | *ACAN* , *COL9A1* | |
| Matrix degrading enzymes | Healthy vs OA | Knee | Up | *MMP-3* , *MMP-13*, *ADAMTS-5*, | [46, 47] |
| | Lesioned vs preserved | Knee | Up | *ADAMTS-5* | [51, 95] |
| | | | Down | *MMP-3* | |
| Chondrocyte signaling | Healthy vs OA | Knee | Up | *ITGAV* , *ITGB5*, *ITGB1* | [45, 47] |
| | | | Down | *ITGA5* , *SOX9* | |
| | Lesioned vs preserved | Knee | Up | *ITGA5* , *ITGAV* , *ITGB5* , *ITGB1* | [48, 51, 95] |
| | | | Down | *SOX9* | |
| Skeletal development | Healthy vs OA | Knee | Up | *ALPL* , *DIO2* | [46, 47] |
| | Lesioned vs preserved | Knee & Hip | Up | *TNFRSF11B* | [48, 96] |
| | | | Down | *FRZB* | |

* Gene symbols of genes related to the pathway.
Dir = direction of effect of genes in pathway. Ref = reference.

expression was also found in OA compared to healthy cartilage. (**Table 1**) [45, 48]. Next to investigating the effects of these changes in gene expression, it is of interest to gain better insight into how these gene expression changes are induced and controlled in OA cartilage by epigenetic mechanisms.

## Epigenetic mechanisms in osteoarthritic cartilage

Chondrocytes are maturational arrested cells, but are required to dynamically modify gene expression to maintain cartilage homeostasis upon environmental changes, such as aging, mechanical stress and microtraumas. Therefore, chondrocytes depend on multiple epigenetic mechanisms to regulate gene transcription and translation, including DNA methylation, histone modifications and noncoding RNAs, such as micro-RNAs (miRNAs) and long noncoding RNAs (lncRNAs) (**Figure 4**) [51-55]. DNA methylation is the most studied epigenetic mechanism in OA cartilage, which is a relatively stable epigenetic marker that comprises the addition of a methyl group to a cytosine at a CpG dinucleotide and influences binding of proteins to DNA, resulting in altered transcription [56]. In the RAAK study genome-wide methylation has been shown to characterize the cartilage based on joint site [57]. Furthermore, by combining genome, transcriptome, and methylome data from the same samples, epigenetically regulated genes involved in development and extracellular matrix maintenance pathways were identified in articular cartilage [52]. Therefore, it seems likely that the loss of epigenetic control in OA cartilage causes reactivation of developmental pathways in articular chondrocytes [56].

Previously thought to be transcriptional noise, noncoding RNAs represent the majority of the human genome, namely 80% versus 2% of coding-RNAs [58]. MiRNAs are a widely studied class of noncoding RNAs of < 22 nucleotides in length, which play a role in post-transcriptional regulation of gene expression by binding to complementary sequences in their target messenger RNA (mRNA) molecules, thereby reducing mRNA stability and/or inhibiting translation [53, 59]. In the RAAK study we aimed to uncover the miRNA interactome of the OA pathphysiological process in cartilage, by combining transcriptome-wide mRNA and miRNA data from the same OA cartilage samples. Pathway enrichment analysis revealed that genes involved in nervous system development are likely mediated by miRNA regulatory mechanisms [51]. Targeting dysfunctional miRNA-mRNA interactions shows promise for therapeutic development [60], but this is still ongoing work for OA.

### Long noncoding RNAs

More recently, lncRNAs have received increased attention, which are RNA transcripts of > 200 nucleotides and are similar to mRNAs in structure, but with little to no protein-coding potential. LncRNAs can regulate transcription and translation directly and indirectly by multiple mechanisms, including chromatin remodelling, mRNA processing, stability and

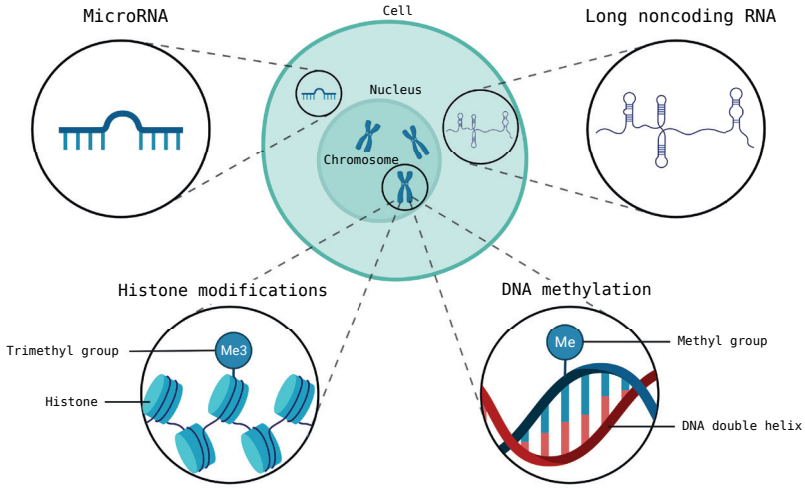


**Figure 4 | Schematic overview of levels of epigenetic regulation in chondrocytes.** Chondrocytes rely on multiple epigenetic mechanisms to regulate their gene expression, including noncoding RNAs, such as microRNAs and long noncoding RNAs, histone modifications, and DNA methylation. (Created with Biorender.com).

splicing, and regulating miRNA function [61, 62]. Based on the genomic location with respect to protein-coding genes, lncRNAs can be classified in biotypes, including antisense RNAs, sense RNAs, pseudogenes, and intergenic lncRNAs. Studies have shown that lncRNAs play an important role in cartilage matrix homeostatis [55, 63-66].

Transcriptome-wide profiles of OA cartilage compared to healthy cartilage resulted in the identification of lncRNAs involved in OA pathophysiology, among others *MEG3*, *MALAT1*, *HOTAIR*, and *GAS5* (**Table 2**) [67-70]. Notably, *MEG3* has been shown to be both up-regulated [68, 71], as well as down-regulated in OA cartilage samples compared to healthy [67, 69]. However, in the studies where *MEG3* was down-regulated, the donors of healthy cartilage were very young and not age-matched to the OA cartilage donors, e.g. 10-51 years, implying that up-regulation of *MEG3* is age-related and down-regulation is specific for the OA process [68, 71]. Moreover, 125 lncRNAs were differentially expressed after IL-1β stimulation of chondrocytes, including *PACER1*, suggesting they play a role in the inflammation-driven cartilage degeneration in OA [55]. These studies have focused primarily on intergenic lncRNAs, even though the proportion of genic and intergenic lncRNAs can be similar depending on the investigated tissue [72]. Therefore, many potentially functional lncRNAs are missing in these studies.

Functional studies of lncRNAs in cartilage showed that overexpression of *MEG3* induced cell proliferation and decreased ECM degradation in IL-1β induced chondrocytes [73].

**Table 2 | Robustly identified long noncoding RNAs in osteoarthritis pathophysiology, identified by transcriptome-wide profiles of healthy and osteoarthritic cartilage and functional studies that have been performed.**

| LncRNA | Biotype | Comparison | Joint | Dir | Ref | Functional studies | Ref |
|---|---|---|---|---|---|---|---|
| *MEG3* | Intergenic | Healthy vs OA | Knee | Up | [68, 71] | *MEG3* induces proliferation and relieves ECM degradation in IL-1β induced chondrocytes via targeting miR-93/TGFBR2 axis. | [73] |
| | | Healthy vs OA | Hip | Down | [67] | *MEG3* targets miR-361-5p/FOXO1 axis and elevates cell proliferation and impairs apoptosis in OA chondrocytes. | [97] |
| | | Healthy vs OA | Knee | Down | [69] | *MEG3* down-regulation leads to OA progression via miR-16/*SMAD7* axis. | [98] |
| *MALAT1* | Intergenic | Healthy vs OA | Hip | Down | [67] | *MALAT1* increases cell proliferation, inhibits apoptosis and inhibits ECM degradation via miR-150-5p/AKT3 axis. | [70] |
| | | Healthy vs OA | Knee | Down | [71] | *MALAT1* /miR-145 axis contributes to ECM degradation in IL-1β induced OA chondrocytes via *ADAMTS-5*. | [99] |
| *HOTAIR* | Intergenic | Healthy vs OA | Knee | Up | [68] | Knockdown of *HOTAIR* in IL-1β induced OA in vitro model reverse IL-1β stimulated expression of matrix-degrading enzymes and significantly reduce apoptosis rate. | [100] |
| | | Healthy vs OA | Knee | Up | [71] | *HOTAIR* promotes chondrocyte apoptosis and ECM degradation via miR-20/PTEN axis in IL-1β induced chondrocytes. | [101] |
| *GAS5* | Intergenic | Healthy vs OA | Knee | Up | [68] | *GAS5* induced apoptosis of chondrocytes through targeting miR-137. | [102] |
| | | Lesioned vs preserved | Knee | Up | [103] | *GAS5* increased expression of matrix-degrading enzymes and *GAS5* was found to act as a negative regulator of miR-21. | [103] |

Dir = direction of effect of genes in pathway. Ref = reference.

Others have shown that *MEG3* targets the TGF-β signaling pathway, which is also active in chondrocytes [74]. Together, these data imply that *MEG3* is involved in the development of OA. An overview of functional studies performed of previously mentioned lncRNAs associated with OA pathophysiology is shown in **Table 2**. These functional studies show that lncRNAs are often involved in miRNA-regulated pathways, however, many of these studies were performed in IL-1β stimulated chondrocytes as OA model, which only provides insight into inflammatory responses. Due to the pleiotropic functions of lncRNAs, functional studies with other OA models are necessary to elucidate the exact molecular mechanisms of OA-relevant lncRNAs.



## Insight into etiology of osteoarthritis

The identification of genes and their epigenetic regulation as markers in OA pathophysiology can help find potential therapeutic targets. However, these studies do not provide insight in the etiology of the disease. Causal pathways can be identified by unravelling the substantial genetic component of OA. The genetic background of common OA is highly polygenetic, where many genetic variants have been identified with small effects conferring risk to OA [75-78]. On the other hand, family-based linkage studies with more severe OA phenotypes result in the identification of high-impact mutations [79]. These rare variants are often located in the protein coding region of the gene and induce an amino acid change, thereby affecting protein function directly. To identify pathogenic variants a prioritization scheme has to be applied, where intergenic, intronic, synonymous, common variants, and tolerated missense mutations are filtered. Tolerated missense mutations are filtered based on sequence homology and physical properties of amino acids, thereby likely not affecting protein function. Filtering can result in up to 150 missense, damaging variants in one affected person, after which linkage analysis has to be performed, followed by genotyping in multiple affected and non-affected individuals to identify the likely causal mutation.

For that matter, whole exome sequencing combined with linkage in early-onset OA families with OA associated phenotypes has been highly successful in identifying likely causal mutations in genes in familial patients in mostly matrix protein encoding genes, including *TNFRSF11B* [80], *COL2A1* [81], *GDF5* [82], *COL11A1* and *COL11A2* [83], *COMP* [84], and *SMAD3* [85] (**Table 3**). Strikingly, functional follow-up of these mutations is mostly not performed. Nonetheless, because of their strong effect, these high-impact mutations can help in the characterization of causal underlying pathways, which can likely be extrapolated to confer risk to common OA. Moreover, they are more actionable to express a disease state in experimental human in vitro tissue models, in which they likely elucidate underlying pathways of OA [86]. Therefore, to understand how causality is explained by these high-impact mutations, functional disease modeling is necessary to increase translation to potential drug development.

**Table 3 | Previously identified high-impact mutations in early-onset osteoarthritis linkage studies and functional follow-up performed.**

| Gene | Protein function | Variant | Functional validation | Ref |
|---|---|---|---|---|
| *COL2A1* | Main collagen protein in articular cartilage | p.Gly204Ala | No functional validation. | [81] |
| *COL11A1*<br>*COL11A2* | Minor collagen protein in articular cartilage | p.Pro446Gln<br>p.Arg53Trp | No functional validation. | [83] |
| *COMP* | Glycoprotein in articular cartilage | p.Arg718Trp | No functional validation. | [84] |
| *GDF5* | Cytokine essential for cartilage development and homeostasis | p.Leu441Pro<br>p.Arg438Leu | Expressed mutant proteins in chicken limb bud micromass culture and treated ATDC5 and C2C12 cells with recombinant GDF5. Leu441Pro mutant was inactive, Arg438Leu mutant showed increased biological activity. | [82] |
| *SMAD3* | Intracellular signal transducer protein in TGF-β signaling | p.Cys89Tyr | No functional validation. | [85] |
| *TNFRSF11B* | Decoy receptor inhibiting osteoclastogenesis | p.Stop402Leu | Bone resorption assay showed mutant OPG had enhanced capacity to inhibit osteoclastogenesis. | [80] |

Ref = reference.

## In vitro OA disease modeling

After the identification of OA related genes, human 3D in vitro cell models can be applied to investigate the role of the gene in underlying pathophysiology. OA relevant cells include bone-marrow derived mesenchymal stromal cells and primary articular chondrocytes, which can subsequently be used in 3D chondrogenesis models. Lentiviral up- or down-regulation of a specific gene in these cells can mimick abberant gene function, after which the effect on cartilage deposition can be assessed. For example, *SOX9* overexpression resulted in increased *COL2A1* expression in chondrocytes in alginate bead culture, showing *SOX9* overexpressing could counteract the loss of chondropotential in 2D culture [87]. Moreover, *DIO2* up-regulation in a human 3D in vitro chondrogenesis model of bone marrow-derived mesenchymal stromal cells resulted in a significant reduced capacity to deposit ECM [88]. More recently, up-regulation of *TNFRSF11B* was shown to be detrimental for neo-cartilage production in a human 3D in vitro chondrogenesis model of primary articular chondrocytes [89]. Together, these studies show that abberant gene expression in vitro can reveal functional consequences of candidate genes. However, the drawback of these cell models is that the cells have limited availability and are generally obtained from OA affected tissue from donors receiving joint replacement surgery. Consequently, the material represents end-stage disease and effects in healthy and young chondrocytes remain mostly unknown.

To circumvent aforementioned issues, human induced pluripotent stem cell (hiPSC) technology has been groundbreaking, which allows reprogramming of readily available primary cells into a sustainable pluripotent cell source [90]. Subsequently, the hiPSCs can be used for directed differentiation into specialized cells of interest [91, 92]. To characterize genetic variation, accessible human donor cells can be reprogrammed into hiPSCs, which contain the unmodified genome of the donor. Functional studies after chondrogenic differentiation of healthy and OA-predisposed patient donors can elucidate underlying pathways of disease causing variants. The drawback of this method is that there is high variability of chondrogenic differentiation potential as a result of different genetic backgrounds among cell lines, which can not be corrected for. However, because of relative novel advancements in CRISPR/Cas9, precise genome editing of cells is easier than ever before. The main CRISPR system utilizes a guide RNA (gRNA) in complex with Cas9, an endonuclease. The gRNA contains the desired target sequence as well as the Cas9 binding sequence, so that Cas9 cleaves the DNA at the target site [93]. The resulting double stranded breaks are repaired by either the non-homologous end joining pathway or the homology directed repair pathway, if a double stranded DNA template is provided. In this way, either gene knockouts or specific mutations can be introduced in the cell's genome. The process of genome editing using CRISPR/Cas9 is infeasible in human primary articular chondrocytes, as they lose chondrogenic potential with prolonged 2D cell culturing [94]. Therefore, the creation of isogenic hiPSC lines followed by chondrogenic differentiation can give insight in the functional characterization of genetic variation. Taken together, the combination of hiPSCs and CRISPR/Cas9 holds immense potential for translational human OA disease modeling [86].



## Outline of this thesis

In this thesis, we aim to obtain insight into causal underlying pathways in OA pathophysiology by combining transcriptomics, genetics and OA disease modeling. In **chapter 2** we applied an improved detection strategy to detect robustly differentially expressed lncRNAs in OA cartilage and identify lncRNAs associated with the OA process in samples obtained in the RAAK study. Integration of these lncRNAs with differential expression levels in the same samples provided insight into their regulatory networks, which we functionally characterized. In **chapter 3**, a high-impact mutation in *FN1* was identified, after which disease modeling was applied to elucidate the underlying mechanism of the mutation. To this end, the mutation was introduced in hiPSCs using CRISPR/Cas9, after which functional studies were performed in our in vitro organoid cartilage model. In **chapter 4**, we sought out to identify *FN1* transcripts associated with OA pathophysiology and investigate downstream effect of modulation of *FN1* expression and relative transcript ratio in our 3D in vitro chondrogenesis model of primary articular chondrocytes obtained from the RAAK study. As such, studies performed in this thesis contributed to the translation from genetics to mechanisms of disease.

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