Dissecting cellular function of fibronectin in osteoarthritic cartilage

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Chapter 5

Discussion

Summary

In the field of osteoarthritis (OA), development of effective drugs is considerably hampered by lacking insight into underlying OA pathophysiology and etiology. The aim of this thesis was to combine transcriptomics, genetics and human disease modeling to obtain further insight into molecular processes underlying OA. Performing transcriptome-wide analyses of OA relevant tissue, such as cartilage, has been shown to be a successful method to identify previously unknown genes that mark OA pathophysiology [1-5]. To further expand on this knowledge, in this thesis we aimed to elucidate the role of long noncoding RNAs (lncRNAs) expression changes as aberrant epigenetic mechanism in regulating gene expression in chondrocytes in **chapter 2**. Consequently, we identified previously unknown lncRNAs associated with the OA process in samples obtained from the Research osteoArthritis and Articular Cartilage (RAAK) study. Upon integrating messenger RNA (mRNA) sequencing data, we showed that intergenic and antisense lncRNAs demonstrate high, positive correlations with their respective flanking or sense genes. We functionally validated this *cis*-regulation for the antisense lncRNAs *P3H2-AS1* and its sense gene *P3H2*.

To provide insight in the etiology of OA, causal pathways can be identified by unravelling the substantial genetic component of OA. In **chapter 3**, we identified a high-impact causal mutation in *FN1* in an early-onset OA family, after which we set up an OA disease model to identify underlying pathways. To this end, we introduced the *FN1* mutation in human induced pluripotent stem cells (hiPSCs), followed by chondrogenic differentiation to neo-cartilage producing chondrocytes. We demonstrated that the missense mutation in the gelatin-binding domain of fibronectin resulted in significant decreased binding capacity to collagen type II. Further analyses of formed hiPSC-derived neo-cartilage tissue highlighted that mutated fibronectin affected chondrogenic capacity and enhanced propensity to a procatabolic OA state.

Finally, the common function of *FN1* in cartilage was investigated, since it is also highly up-regulated in lesioned compared to preserved OA cartilage. Moreover, *FN1* can give rise to 27 transcripts, of which 13 are protein coding, which raises the question whether specific *FN1* transcripts play a role in OA pathophysiology. In **chapter 4**, we identified migration-stimulating factor (MSF or *FN1-208*), a truncated isoform of fibronectin, associated with OA pathophysiology and not previously identified in OA cartilage. Down-regulation of full length *FN1* was unbeneficial for neo-cartilage deposition by human primary chondrocytes obtained from the RAAK study in our 3D in vitro chondrogenesis model.

Role of lncRNAs in osteoarthritic cartilage

OA pathophysiology in cartilage is marked by alterations in gene expression regulation in chondrocytes. Since chondrocytes remain in a maturational arrested state, they rely heavily on epigenetic mechanisms to regulate dynamic changes in gene expression in response to intrinsic and external challenges such as microtraumas and mechanical stress. As a response to these processes, chondrocytes need to become temporarily metabolically active and adjust expression levels of anabolic and catabolic genes, which is controlled by multiple levels of control including DNA methylation, histone modifications and noncoding RNAs [6]. Unraveling aberrant epigenetic mechanisms in chondrocytes thus provides another important level of insight into OA pathophysiology. One of the least characterized levels of epigenetic mechanisms in articular cartilage are lncRNAs. Potentially, lncRNAs could be candidate targets in OA, since their expression can be highly tissue specific [7].

Identifying long noncoding RNAs associated with OA pathophysiology

Hypothesis-free profiling of lncRNAs in healthy and OA cartilage was first based on microarray data [8-10], but as a consequence of decreasing costs of and significant technical advances in RNA sequencing, studies using this technique gained traction [11-13]. RNA sequencing greatly improved the ability to detect and identify lncRNAs, since they are structurally highly similar to mRNAs but relatively lower expressed. However, annotating lncRNAs remain challenging, since their sequence-function relationship is poorly understood and the number of experimentally characterized lncRNAs is low, namely <1% of identified loci [14]. Therefore, in **chapter 2**, we used a new RNA sequencing in-house pipeline to robustly detect lncRNAs in OA cartilage samples from the RAAK study. Recently, ribosome profiling and bioinformatic studies showed that a large proportion of transcripts has unknown protein coding potential [14]. In order to filter transcripts with unknown protein coding potential, we integrated two machine learning methods, Coding Potential Assessment Tool (CPAT) and the LncFinder R package. Transcripts with protein coding potential predicted by both tools were removed from the dataset. As a result, we identified 5,053 lncRNAs to be robustly expressed in OA cartilage, 191 of which were significantly differentially expressed lncRNAs between lesioned and preserved OA cartilage [15]. Notably, we observed an increase in the percentage of intergenic lncRNAs (lincRNAs), highlighting their general involvement in the OA pathophysiology process. Potential interactions were identified between the differentially expressed lncRNAs and differentially expressed protein coding genes in the same OA cartilage samples, where we observed an enrichment between lincRNAs and their flanking genes and between antisense lncRNAs and their sense genes, implying *cis*-regulation. In vitro functional validation of this *cis*-regulation revealed that the antisense lncRNA *P3H2-AS1* regulates its sense gene *P3H2*.

Of the 191 identified lncRNAs that associated with OA pathophysiology, multiple lncRNAs have been previously identified, such as *MEG3*, *LINC01614*, and *PART1* [12, 16]. However, multiple lncRNAs previously found associated with OA, including *MALAT1*, *HOTAIR*, and *GAS5*, were not significantly differentially expressed in our study. One explanation could be that our study design comprises a within patient comparison between lesioned and preserved cartilage, as opposed to a cross-sectional design comparing healthy and preserved OA cartilage. The cross-sectional design can give insight into which lncRNAs are involved in the early phase of OA and therefore potentially causal to the process, while our design allows for detection of lncRNAs specific to the OA pathophysiological process, independent of confounding factors such as genetic background, sex, and age. We were able to validate and replicate the direction of effect for five lncRNAs, indicating robustness of our lncRNA mapping strategy. However, upon applying a filter with a cutoff of ≥2 counts per lncRNAs, the number of detected lncRNAs was drastically decreased by ~ 83%. LncRNAs are known to be expressed at very low levels, yet can still be functional. To perform exploratory analyses of lowly expressed lncRNAs, deeper sequencing would have to be performed, with a read-depth of 50-100 million reads per sample. Furthermore, in our study poly-A enrichment was performed for the RNA sequencing library prep, meaning that lncRNAs without a poly-A tail could not be identified in our analysis. To capture transcripts both with and without a poly-A tail, future studies should enrich for poly-A RNAs yet keep the other fraction to obtain non-poly-A lncRNAs, followed by ribosomal RNA depletion, similar to what was done by Yang *et al.* [17].

Identifying downstream targets of long noncoding RNAs

To be able to potentially use lncRNAs as druggable targets, it is necessary to identify their downstream targets. Currently, no lncRNA-targeting therapeutics have entered clinical development. However, lncRNAs have increasingly been investigated and show promise as RNA interference or CRISPR targets [18]. Amodio *et al.* [19] investigated the function of *MALAT1* in multiple myeloma, where locked nucleic acid-GapmeR (LNA-GapmeR) antisense oligonucleotide (ASO) technology was used to target *MALAT1* expression. Down-regulation of *MALAT1* resulted in antitumor activity in a humanized myeloma mouse model, providing preclinical evidence for the use of this new ASO-targeting of lncRNAs for the treatment of multiple myeloma. In this study the effect of *MALAT1* down-regulation was measured by cell proliferation and viability, however, in this thesis we aimed to identify specific mRNAs downstream of the identified 191 differentially expressed lncRNAs in OA cartilage.

Unlike conserved miRNAs, there is no clear understanding yet of the sequence-function relation of lncRNAs. Functions of lncRNAs can be based on two elements; the base pairing in linear form in direct physical interaction with nucleic acids, proteins or lipids, and the chemical interactions as a consequence of secondary or tertiary structures [18]. Furthermore, lncRNAs can be classified based on whether they regulate the expression of neighboring genes in *cis* or more distant genes in *trans* [20]. *Cis*-acting lncRNAs comprise a considerable portion of known lncRNAs and can be positioned at various distances and orientations relative to their target genes. Examples are lincRNAs around transcription factor start sites, as well as sense and antisense lncRNAs that overlap with their sense genes [20]. To explore potential regulatory interactions we generated a lncRNA-mRNA coexpression network in cartilage based on correlations. This showed an enrichment of high correlations between lincRNAs and their flanking genes and between antisense lncRNAs and their sense genes, implying *cis*-regulation of these lncRNAs. However, these correlations do not provide evidence for downstream effects of the lncRNAs on the mRNAs.

To functionally validate the observed *cis*-regulation we selected lncRNA *P3H2-AS1* as proof of concept to establish whether it regulates its sense gene. *P3H2-AS1* is an antisense lncRNA and showed the highest correlation with its sense gene *P3H2* (**Figure 1A**). To down-regulate *P3H2-AS1* expression, we used LNA-GapmeR ASO technology, also used by Amodio *et al.* [19]. As a result, *P3H2* expression was also down-regulated, thereby confirming that *P3H2-AS1* positively regulates the expression of its sense gene in *cis*. Antisense lncRNAs can affect biogenesis or mobilization of target mRNA on multiple levels, such as transcription, splicing and translation [21]. *Cis*-acting antisense lncRNAs are known to function at nearly all levels of gene regulation: pre-transcriptional, transcriptional and post-transcriptional [21]. *P3H2-AS1* and *P3H2* have no linear sequence similarities, so it is likely that *P3H2-AS1* regulates gene expression not by binding to *P3H2* mRNA, but functions at the pre-transcription level, e.g. by influencing chromatin state, influencing DNA methylation, or modulating transcription factor activity (**Figure 1B**). Visualization of subcellular localization of lncRNAs by RNA fluorescence in situ hybridization can provide insight into potential function of lncRNA [14, 22]. To investigate more specific lncRNA-protein interactions, RNA immunoprecipitation or crosslinked immunoprecipitation can be performed, which can show whether a lncRNA targets chromatin-modifying enzymes or transcription factors. The more recent development of CRISPR-mediated interference and activation can modulate expression of lncRNAs from their endogenous promoter by blocking or activating transcription, respectively [23]. In this way, lncRNA function can be determined including the production of *cis*-acting RNA transcripts and *cis*-mediated regulation related to lncRNA transcription itself. Liu *et al.* [24] developed a large CRISPR interference platform in multiple cell lines and hiPSCs and identified many lncRNA loci required for robust cellular growth. It would be interesting to perform a comparable study for lncRNAs in chondrocytes. Functionality of lncRNAs can be assigned with more confidence when RNA interference techniques, such as LNA-GapmeRs, are complemented with CRISPR-based experiments [23].

P3H2 was shown to be significantly up-regulated in lesioned versus preserved OA cartilage samples from the RAAK study [25]. *P3H2* encodes an enzyme that catalyzes post-translational 3-hydroxylation of proline residues and plays a critical role in collagen chain assembly, stability, and crosslinking. Therefore, it seems likely that up-regulation of *P3H2-AS1* with

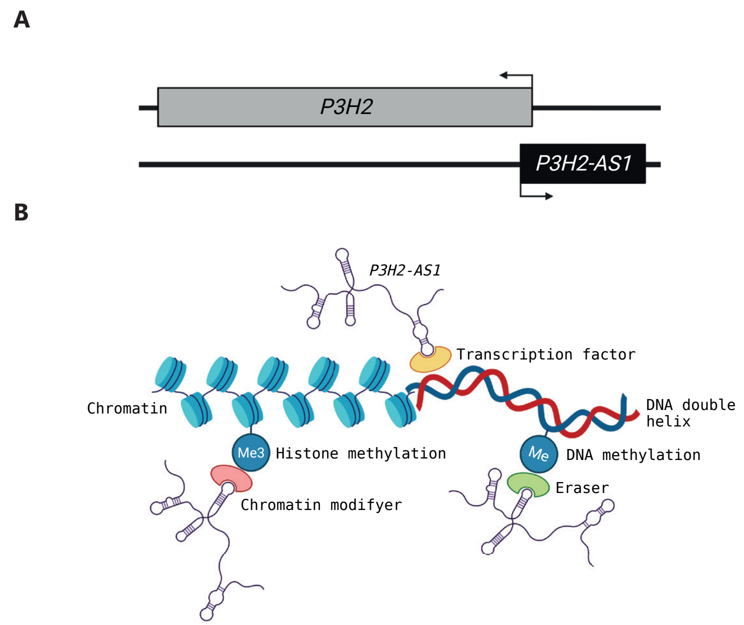

Figure 1 | The antisense long noncoding RNA *P3H2-AS1* regulates gene expression of its sense gene *P3H2* (**A**) Relative genomic location of *P3H2* and the antisense lncRNA *P3H2-As1*, the 5' end of *P3H2-AS1* is near the 5' end of *P3H2*, where the arrows indicate direction of transcription. (**B**) Potential mechanisms by which *P3H2-AS1* (purple) pre-transcriptionally regulates *P3H2* gene expression, binding to chromatin modifying enzymes (red), facilitating histone modifications thereby influencing chromatin state, binding to transcription factors (yellow) thereby modulating transcription, or binding to an eraser (green) and removing DNA methylation thereby inducing gene transcription. (Created with Biorender.com)

concurrent up-regulation of *P3H2* is a response to the OA process and beneficial in articular cartilage. This hypothesis could be tested by using CRISPR activation to up-regulate *P3H2-AS1* expression and investigate *P3H2* expression and further downstream effects on neo-cartilage deposition in human primary chondrocytes. Furthermore, additional targets of *P3H2-AS1* can be identified by performing transcriptome-wide analyses after CRISPR activation of *P3H2-AS1*.

Overall, we show that generating coexpression networks between lncRNAs and mRNAs can provide insight in potential regulatory function of lncRNAs. However, future studies regarding lncRNAs in relation to OA should be complemented by functional validation in order to confirm whether a correlation signifies a biologic causal relation between lncRNA and mRNA or is rather consequential. As a result of quickly advancing techniques involving CRISPR, the possibilities to determine the function of lncRNAs are growing steadily, indicating exciting future perspectives for identifying druggable targets for preclinical trials in OA.

Genetic disease modeling for osteoarthritis

In an effort to elucidate the complex genetic architecture of OA, genome wide association studies have provided evidence for susceptibility loci in common OA pathophysiology [26-28]. It has been shown that developing new drugs with genetic support can double the success rate in clinical development [29]. However, translation to clinically druggable targets is lacking for OA, among others as a result of the small effects of the associated genetic variants. For that matter, identifying rare mutations with large effects in early-onset OA patients can provide insight into genotype-phenotype relations and thereby can elucidate causal OA pathways. However, functional follow-up studies of earlier identified high-impact mutations in OA patients have often not been performed. The quickly developing progress in genomic engineering with CRISPR/Cas9 technology has advanced the field greatly in this aspect, of which we readily took advantage of. Hence, in **chapter 3** we investigated the biological functionality of the high-impact, pathogenic mutation identified in *FN1* in an early-onset OA family [30]. To this end, we introduced the C518F *FN1* mutation in hiPSCs using CRISPR/Cas9 gene editing, thereby creating *FN1* heterozygous and homozygous hiPSC lines. Subsequently, the mutant and isogenic control hiPSCs were used in an established in vitro organoid cartilage model, where we observed a decrease of both chondrogenic potential and neo-cartilage deposition of the *FN1* mutant cells. Moreover, we demonstrated that the underlying pathogenic mechanism of the mutation was caused by a decreased binding of mutant fibronectin to collagen type II.

Identification of high-impact mutation in early-onset OA family

By applying whole exome sequencing to an affected individual of an early-onset OA family, we obtained over 73,000 candidate variants after quality control. As the phenotype showed a dominant Mendelian inheritance pattern, we hypothesized the causal variant results in an amino acid change, thereby affecting protein structure and functioning. Consequently, we applied a pathogenic prioritization scheme to exclude intergenic, intronic, synonymous, common and tolerated missense variants. Common genic variants were filtered out when they were present in various population-scale variant databases, resulting in over 1,000 variants. Variant prioritization tools Sorts Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen) were used to remove tolerated missense variants, further reducing the number of variants predicted to have a functional impact on the gene produced to 122 missense variants. SIFT and PolyPhen were shown to have moderate sensitivity and their accuracy is dependent on whether loss-of-function or gain-of-function are being tested, indicating that further evidence to support causality is necessary [31]. Previously, our group showed strong linkage on 2q33.3 with multiple extended early-onset OA families, to which the current family contributed substantially, giving us a further indication of the chromosomal location of the pathogenic variant [32]. Of the 122 variants, three variants were located around the previously mentioned linkage area, namely *ALS2*, *FN1*, and *ABCB6*. Firstly, we investigated relevance of these three genes to OA by exploring gene expression levels in our previously published RNA sequencing data of lesioned and preserved cartilage and bone samples from the RAAK study [25, 33]. Only *ALS2* and *FN1* were expressed in both cartilage and bone, suggesting these genes are functional in these tissues. Furthermore, *FN1* was significantly up-regulated in lesioned OA cartilage compared to preserved, revealing that this gene is also sensitive to the OA process. Subsequently, de novo genotyping was performed for the *ALS2* and *FN1* variants. Since the investigated family is rather extended, genotyping showed complete linkage of the *FN1* variant in affected individuals, while the *ALS2* variant was not detected, thereby confirming that the C518F mutation in the *FN1* gene is likely causal to the early-onset phenotype in this family. Identifying a causal pathogenic mutation in rare Mendelian disease is not always successful, as the human exome contains thousands of variants [34]. However, in this thesis we exhibit the powerful combination of exome sequencing followed by linkage analysis in an extended family, allowing us to identify the causal mutation to the early-onset phenotype in the family [35]. Consequently, we aimed to set up a relevant in vitro OA disease model to investigate downstream biological pathways.

In vitro OA disease modeling

In this study, we choose to use hiPSCs in our OA disease model, as opposed to human primary articular chondrocytes. Disadvantages of primary chondrocytes include limited availability and representing end-stage disease state, as they are often obtained from patients who underwent joint replacement surgery due to OA. Moreover, since we sought to introduce a specific mutation, the selection process for the correct clone without off-target effects would result in substantial 2D culturing, which in primary chondrocytes results in significant dedifferentiation and loss of chondrogenic potential [36, 37]. Studies that performed CRISPR/Cas9 genome editing in chondrocytes did so either in a rat chondrosarcoma cell line [38], or performed gene knockout, which has a higher efficiency than precise gene editing [39, 40]. We obtained both hetero- and homozygous *FN1* hiPSC clones which in essence were two separate clones. After chondrogenic differentiation we observed a dose response as a result of the mutation at the molecular level, thereby providing robustness to our obtained results. As hiPSCs can be expanded substantially, we acquired a sustainable cell source, which can be readily used for future experiments.

Differentiation of hiPSCs to chondrocytes has been shown to give variable efficiency, yet progress has been made in establishing reproducible step-wise differentiation protocols [41-43]. In our group we showed that neo-cartilage from hiPSC-derived chondrocytes was almost 70% similar to that of neo-cartilage from human primary articular chondrocytes based on gene expression profiles, indicating suitability of our hiPSC-derived organoid neo-cartilage model [44]. In this thesis we observed that *FN1* mutated organoids contained less cartilage-producing cells relative to the total number of cells compared to wild type organoids, indicating decreased chondrogenic potential. Furthermore, we observed a decrease in the deposition of neo-cartilage, altogether indicating a less efficient formation of neo-cartilage. However, we could not separate the effect of the *FN1* mutation on the decreased chondrogenic potential and decreased neo-cartilage deposition, confounding our analyses. Dicks *et al.* demonstrated that heterogeneity of the chondroprogenitor cell population is partly due to mesenchymal and neurogenic lineage cells [45]. To circumvent the issue of heterogeneity in the chondroprogenitor population, a *GFP* reporter hiPSC line could be engineered with a specific chondrogenic marker to be able to purify chondroprogenitors, similar to Adkar *et al.* [41]. In this way, the effect of the mutation on the deposition of neo-cartilage can be investigated without the confounding factor of decreased chondrogenic potential. Furthermore, longitudinal analyses of the differentiation would have to be performed to elucidate how the *FN1* mutation affects hiPSC differentiation to chondroprogenitors and thereby chondrogenic potential. Previous findings showed that homogenous inactivation of fibronectin in mice resulted in early embryonic lethality and that fibronectin plays an essential role in mesodermal migration [46]. Additionally, the presence of fibronectin matrix was shown to be essential for mesenchymal stromal condensation and chondrogenic differentiation. Possibly, the mutation negatively affects these processes during the differentiation from hiPSCs to chondrocytes [47].

For decades, animals have served as the most common models of human disease, however, use of animal models is also limited due to genetic background differences, which has led to high rates of translational failure between human and animal models [48, 49]. Moreover, costs, housing and length of experiments are generally more costly with animal experiments and ethical guidelines are to be considered, since usually animals need to be sacrificed for OA studies, while there are no ethical issues regarding hiPSCs. In this thesis, we created isogenic hiPSC clones with the *FN1* mutation lacking off-target effects by precise genetic engineering, after which we applied an established differentiation protocol producing biomimetic human in vitro neo-cartilage. Hence, we consider our conditions near optimal and we are confident that our approach was able to create reliable data highly translating to the human in vivo situation, while contributing to the societal need to reduce animal studies. Taken together, we show the immense potential of combining exome sequencing, hiPSCs, CRISPR/Cas9 and organoid disease modeling in common, complex human genetic diseases such as OA.

Role of fibronectin in osteoarthritis pathophysiology

FN1 mutation in gelatin-binding domain

In **chapter 3** we showed that the C518F mutation in the gelatin-binding domain of fibronectin resulted in a linear reduction in binding of mutant fibronectin to collagen type II. The change from a polar cysteine to a nonpolar phenylalanine was predicted to result in a conformational change of the protein, as determined by RaptorX, whereby the formation of a conserved disulfide bond is abrogated. The gelatin-binding domain of fibronectin consists of six modules, namely 6FnI, 1-2FnII, and 7-9FnI, which were all shown to contribute to the interaction with gelatin and collagen, either directly or indirectly [50, 51]. As the C518F mutation is located in the 8FnI module, the predicted conformational change as a result of the mutation is likely directly causal to the decreased binding of mutant fibronectin to collagen type II. Collagen and fibronectin fibrillogenesis are thought to be interdependent processes [52], however, we did not observe obvious differences in collagen type II deposition when comparing wild type and mutant neo-cartilage pellets. We therefore hypothesized that the mutation induced structural differences at the fibril level. Unfortunately, we could not observe collagen fibrils by means of transmission electron microscopy in our neo-cartilage model, but it is possible that the conformation of collagen and fibronectin fibrils was affected by the mutation and thereby mechanical properties of the neo-cartilage. Consequently, determining mechanical properties of the wild type and mutant neo-cartilage as a measure of quality could provide insight in the effects of the decreased binding between collagen and fibronectin. Altogether, in **chapter 3** we highlight the importance of the proper binding of fibronectin to collagen type II in articular cartilage (**Figure 2**).Since fibronectin functions as a transducer of biomechanical signals to chondrocytes from the ECM to chondrocytes via integrins, the decreased binding to collagen type II likely results in changed interactions between ECM and chondrocytes. Thus, determining threshold strains of mechanical loading that result in catabolic responses and cartilage degeneration of neo-cartilage produced by wild type and mutant chondrocytes can provide insight into whether the mutation potentially affects mechanotransduction and in that way responsible for the early-onset OA phenotype.

The *FN1* mutation resulted in aberrant chondrocyte gene expression, where anabolic markers were down-regulated and catabolic markers were up-regulated in *FN1* mutant chondrocytes. Moreover, integrin subunits *ITGA3* and *ITGB1* were significantly up-regulated in the homozygous *FN1* mutant chondrocytes. It has been known that integrin expression changes

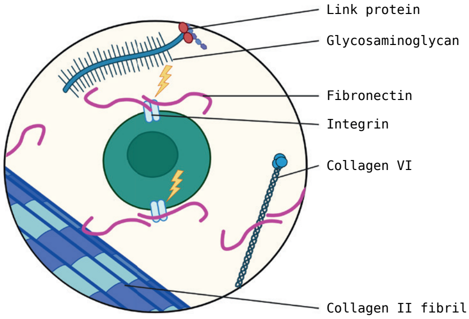

Figure 2 | Potential mechanism of how the conformational change of C518F mutant fibronectin induces unbeneficial responses in chondrocytes. Mutant fibronectin has decreased binding to collagen type II and potentially binds to integrin α5β1 as well as α3β1, where it induces unbeneficial gene expression changes, represented by the yellow bolt. (Created with Biorender.com).

during the development of OA, where α3β1 was shown to be up-regulated in OA chondrocytes [53]. Therefore, the up-regulation of *ITGA3* and *ITGB1* may reflect un unbeneficial state of the *FN1* mutant chondrocytes. Co-immunoprecipitation of wild-type and mutant fibronectin with integrins can provide insight into whether the shift in gene expression also resulted in changed interactions of fibronectin with these integrin subunits. Conversely, down-regulation of *ITGA5* occurred solely in the heterozygous *FN1* mutant chondrocytes. We hypothesized that the down-regulation could be a response to wild type-mutant fibronectin dimers binding to integrin α5β1. *ITGA5* was found to be down-regulated in OA cartilage compared to healthy [4], implying that down-regulation is likely unbeneficial for the heterozygous *FN1* mutant chondrocytes. It has been shown that fibronectin-α5β1 adhesion is essential for cartilage remodeling in mice, so quantitative binding assays between (mutant) fibronectin and integrin α5β1 could shed light on whether the mutation affects binding to integrin α5β1 [54]. Furthermore, integrin activation can occur via “outside-in” and “inside-out” signaling, thus up-regulation of integrin α3β1 in homozygous *FN1* mutant chondrocytes could also affect matrix homeostasis by changing chondrocyte adhesion to the ECM via “inside-out” signaling [55, 56].

Identifying *FN1* transcripts associated with OA pathophysiology

Studies have shown that genes with a larger number of transcripts play biologically more fundamental roles [57]. Fibronectin is a ubiquitous protein in the human body as part of the extracellular matrix, as well as a major component of blood plasma, where it is involved in wound healing [58]. Alternative splicing of *FN1* mRNA can give rise to many transcripts that encode protein molecules with different binding capacities [59]. However, it has not been completely clear what changes occur at the transcript level with OA with respect to *FN1*. As such, in **chapter 4** we aimed to identify *FN1* transcripts annotated in the Ensembl database associated with OA pathophysiology. As a result, we identified sixteen *FN1* transcripts to be significantly up-regulated in lesioned compared to preserved OA cartilage obtained from the RAAK study, of which five were protein coding and eleven non-protein coding. The non-protein coding transcripts are classified as retained introns, which were shorter in length and generally lower expressed in cartilage than protein coding transcripts. Intron retention has recently been getting more attention as alternative splicing mechanism and is mostly associated with down-regulation of gene expression via nonsense-mediated decay of the intron-retaining transcript [60]. In the case of fibronectin this seems unlikely, since the retained intron transcripts are so much smaller than the protein coding transcripts. However, it is suggested that intron retention potentially regulates noncoding RNAs, for example if the retained introns encode miRNAs or contain noncoding RNA-response elements thereby affecting miRNA or lncRNA functioning [61]. Future studies regarding the function of retained intron *FN1* transcripts should address whether they regulate gene expression levels of the protein coding *FN1* transcripts, thereby acting as noncoding RNAs. Regarding the protein coding *FN1* transcripts, we found EDA$^-$, EDB$^-$ and EDB$^+$ variants to be present in cartilage, while EDA$^+$ variants were less abundant, which is in line with previous findings [62]. The EDA domain has been associated with many functions ascribed to fibronectin, including cell adhesion, matrix assembly, and dimer formation [63]. However, the low abundance suggest that the EDA domain is not essential for proper functioning of fibronectin in cartilage.

Furthermore, we found *FN1-208*, encoding migration-stimulating factor (MSF), to be the most significantly up-regulated protein coding *FN1* transcript, which has not been previously identified in OA cartilage. MSF is a 3' truncated isoform of full length fibronectin of 70 kDa, containing the heparin- and gelatin-binding domain of full length fibronectin. It has been shown to be a potent motogenic factor, meaning it promotes cell motility, and it has been associated with cancer pathogenesis [64]. Consequently, we aimed to functionally investigate MSF in our established human 3D in vitro neo-cartilage model from primary chondrocytes. We could not achieve MSF overexpression in our model, therefore, we aimed to down-regulate full length *FN1*. As such, we were mimicking cartilage in an OA affected state by obtaining an up-regulation of MSF relative to all other *FN1* transcripts. Down-regulation of full length *FN1* transcripts was unbeneficial for neo-cartilage deposition, implying that the observed up-regulation in lesioned versus preserved OA cartilage from the RAAK study is a response to the OA process. Furthermore, *ADAMTS-5*, *ITGB1* and *ITGB5* expressoin levels were increased as a result of *FN1* down-regulation, suggesting a more disease state of the chondrocytes. Both *ADAMTS-5* and *ITGB1* showed similar responses in our *FN1* mutant hiPSC-derived neo-cartilage model, robustly indicating that *ADAMTS-5* and *ITGB1* are part of the fibronectin downstream signaling response. As MSF does not contain the classical arginine-glycine-aspartate (RGD) binding site to bind integrin α5β1, we hypothesize that decreased availability of this fibronectin domain is unbeneficial for chondrogenesis. However, this remains to be confirmed e.g. by down-regulating full length *FN1* transcripts in parallel to up-regulating MSF and investigating the downstream effects on neo-cartilage deposition. Furthermore, up-regulation of fibronectin in our in vitro neo-cartilage model could confirm whether the observed up-regulation in lesioned versus preserved OA cartilage is a response to the OA process and not causal.

Fibronectin fragments and migration-stimulating factor

Fibronectin can be cleaved by proteinases into fragments (FN-fs), which have catabolic activities in OA joints [65]. These FN-fs have obtained cryptic binding sites, resulting in altered binding to integrins and disharmonious downstream signaling. There are three main fragments that have been identified in this respect, comprising the 29 kDa N-terminal heparin-binding domain containing fragment, the 45 kDa gelatin-binding domain containing fragment, and the 110-140 kDa cell-binding domain fragment [66]. It has been shown that FN-fs increase aggrecan degradation via up-regulation of MMPs and ADAMTS-5 [67, 68]. Since

MSF is the length of the 29 kDa and 45 kDa fragment, it seems likely that this cell-produced fibronectin isoform has detrimental consequence for cartilage homeostasis. This hypothesis can be tested by adding MSF to chondrocyte pellet cultures and investigating downstream effects on neo-cartilage deposition.

Future perspectives

In this thesis we showed that identifying rare, high-impact variants and their biological functionality can give insight into underlying pathways of OA in articular cartilage. The usefulness of modifiable human in vitro hiPSC models such as the one established in this thesis can be expanded by using it to test potential therapeutics that act against the molecular pathways that are disrupted in the model (**Figure 3**). The 3D neo-cartilage pellets can relatively easily be scaled up to perform high throughput drug screening.

The complexity of OA pathophysiology is partially because it is a disease of the whole joint. In our current model we focused on cartilage but excluded investigating any effects in bone tissue. For that matter, human ex vivo osteochondral explants can be considered the most accurate 3D model of OA and have been shown to be useful in OA pathophysiology models, potentially for pre-clinical studies [69]. However, one of the drawbacks of explants is the fact that cells cannot be genetically modified. Considering that hiPSCs can be differentiated into any cell type, differentiation of genetically modified cells to both cartilage- and bone-producing cells can overcome this drawback. By seeding multiple hiPSC-derived cell types in microfluidic chips, so-called joint-on-a-chip technology, cross-talk between OA-relevant tissue can be investigated (**Figure 3**).

Apart from rare high-impact mutations, it is also valuable to perform functional follow-up studies of more common genetic variants identified in genome-wide association studies. Finding biological functional consequences, other than expression quantitative loci analyses, and causality of these loci has been shown to be challenging in the field of OA. Partly because of accessibility of disease relevant tissue, as well as the fact that these variants usually have small effect sizes and that multiple, independently associated risk alleles may be responsible for occurrence of the disease [70]. hiPSC technology creates the possibility to use large cohorts of hiPSCs with known genotypes and perform genome-wide analyses of genetic variant-driven cellular phenotypes, both in hiPSCs and hiPSC-differentiated cells. A suitable cohort of participants needs to be considered with relevant phenotypes and known genotypes, after which hiPSC lines can be reprogrammed from participant primary cells. Subsequently, transcriptome, proteome, and metabolome approaches can be applied to correlate genotype to phenotype [71]. This "humanity in a dish" approach could drastically accelerate the elucidation of the molecular basis of common OA (**Figure 3**).

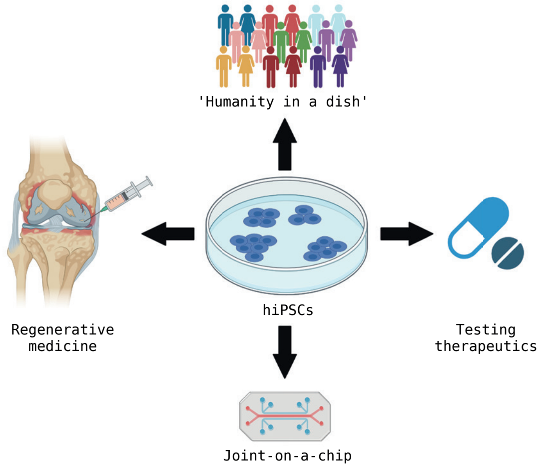

Figure 3 | Overview of future applications of human induced pluripotent stem cells (hiPSC) in preclinical models (regenerative medicine, testing therapeutics) and disease models ('humanity in a dish', joint-on-a-chip) for osteoarthritis (OA). For regenerative medicine, differentiated hiPSCs, either genetically engineered or not, can be used to repair damaged tissue by implantation in the osteoarthritic joint. Potential therapeutics that act against specific disrupted molecular pathways can be tested in modifiable human in vitro hiPSC models. Functional follow-up of more common genetic variants associated with OA can be performed in large cohorts of hiPSCs with known genotypes, so-called 'humanity in a dish'. Seeding genetically engineered hiPSC-derived OA-relevant cell types in microfluidic chips can provide valuable insight into the cross-talk between OA-relevant tissues. (Created with Biorender.com).

Next to the applicability of hiPSCs in OA disease modeling, they show promising potential for regenerative medicine, such as stem cell therapy. Regenerative medicine integrates cell biology, materials science and gene therapy, potentially resulting in cell-based implantation methods to repair damaged tissue in OA joints (**Figure 3**) [72]. The current problem is that hiPSC differentiation to chondroprogenitors results in a heterogenous cell population, thereby tempering progress to clinical applications. Gaining insight into chondrogenic lineage commitment of the hiPSCs can provide identification of modifiable factors that determine hiPSC cell fate to chondrogenic lineage. More specifically, insight at the single cell level of hiPSC differentiation gives more information regarding inter-cell variability. Wu *et al.* investigated gene regulatory networks regulating hiPSC differentiation at single-cell level during chondrogenesis, identifying *WNT* and *MITF* as hub genes governing the generation of off-target differentiation [73]. However, a multi-omics approach including epigenetic and proteomic analyses will allow an even more accurate characterization of factors regulating chondroprogenitor cell fate.

Regarding the role of fibronectin in osteoarthritic cartilage, in this thesis we highlighted the importance of proper binding between fibronectin and the ECM in articular cartilage, specifically via collagen type II. Furthermore, decreased deposition of full length fibronectin was unbeneficial for neo-cartilage deposition. Our work merits further exploration of therapeutic interventions focusing on fibronectin as potential target. Engineering recombinant fibronectin fragments that compensate unbeneficial interactions between the ECM and chondrocytes can be a starting point for tissue engineering [74]. Fibronectin conformational change can influence integrin specificity and we showed that ECM interactions can also be influenced by specific conformational structures of fibronectin, thereby regulating cell behavior. Further functional analyses of the role of MSF in cartilage can provide initial clues for functional recombinant fragments.

The observed extensive changes in the *FN1* transcriptome with OA pathophysiology suggests that there are changes in regulation of these transcripts. The question that arises is how these transcripts are regulated by epigenetic mechanisms. As previously mentioned, the non-protein coding transcripts could act as noncoding RNAs or influence noncoding RNAs. Thus, it would be interesting to use LNA-GapmeR ASO technology to elucidate the function of the non-protein coding transcripts. Furthermore, generating coexpression networks between fibronectin and miRNAs or lncRNAs could provide initial clues for how fibronectin expression is epigenetically regulated.

Looking back on the past years of OA research, it has become clear that fast progress has been made by many exciting technical advancements. This sparks hope for the future of OA research and therapy development. In this thesis we performed multifaceted studies, which can be used as starting points for future OA disease modeling and towards development of new therapeutic strategies.

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