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High-impact FN1 mutation decreases chondrogenic potential and affects cartilage deposition via decreased binding to collagen type II

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Osteoarthritis is the most prevalent joint disease worldwide, yet progress in development of effective disease-modifying treatments is slow because of lack of insight into the underlying disease pathways. Therefore, we aimed to identify the causal pathogenic mutation in an early-onset osteoarthritis family, followed by functional studies in human induced pluripotent stem cells (hiPSCs) in an in vitro organoid cartilage model. We demonstrated that the identified causal missense mutation in the gelatin-binding domain of the extracellular matrix protein 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 propensity to a procatabolic osteoarthritic state. Together, we demonstrate that binding of fibronectin to collagen type II is crucial for fibronectin downstream gene expression of chondrocytes. We advocate that effective treatment development should focus on restoring or maintaining proper binding between fibronectin and collagen type II.

INTRODUCTION

Osteoarthritis (OA) is an age-related, degenerative, heterogeneous disease of the articular joints, characterized by pathological changes of the articular cartilage, synovium, and subchondral bone (1). Currently, OA is the most prevalent joint disease worldwide and affects more than 40 million people in Europe alone (2, 3). Patients with OA suffer pain and stiffness in the articular joints, resulting in significant disability in everyday life (4). Despite these detrimental consequences, to date, no effective treatment is available except pain relief medication and, in the final stage, joint replacement surgery. Lacking insight into underlying OA pathophysiology has considerably contributed to failures in disease-modifying OA drug development.

For that matter, next-generation exome sequencing has been highly successful in identifying likely causal mutations in patients with familial, more severe phenotypes (5) or Mendelian disorders (6). Because of their strong effect, these mutations can provide direct clues to genotype-phenotype relations and are more actionable to express a particular disease state in experimental human in vitro tissue models. Since the pathways in which these genes operate are likely extrapolated to confer risk to common OA, these mutations are destined to elucidate OA-specific aspects of the disease. Using cells carrying high-impact mutations in tailored human in vitro tissue models may thus elucidate previously unknown, likely causal underlying pathways of OA (7, 8). Moreover, human induced pluripotent stem cell (hiPSC) technology allows reprogramming of primary cells into a sustainable pluripotent cell source (9), which is subsequently used for the directed differentiation into specialized cells of interest (10, 11). Even more, because of novel advancements in genomic engineering with CRISPR-Cas9 technology, cells are now easily edited or modulated (12). As a result, the creation of isogenic lines can give insight in the functional characterization of genetic variation. Since OA-relevant cells, such as chondrocytes, are not readily available, the combination of hiPSCs and CRISPR-Cas9 holds immense potential for translational human OA disease modeling to uncover the role of high-impact mutations (8).

By applying a genome-wide linkage scan in seven extended early-onset (EO)–OA families without dysplasia, we previously identified significant linkage on chromosome 2q33.3, with one family (family 2) that substantially contributed to this linkage area (13). However, Sanger sequencing of 20 positional candidate genes located in the confined linkage region of these seven families failed to definitively identify the causal mutation in these families. More recently, an exome sequencing dataset was established in these OA families, resulting in the successful identification of a high-impact mutation in one of the families (14). By applying a pathogenic prioritization scheme to the exome sequencing dataset of family 2, we identified a previously unidentified mutation and used hiPSCs and CRISPR-Cas9 technology to develop an in vitro organoid cartilage model to functionally characterize the effects of the causal mutation in this family.

RESULTS

Identification of heterozygous missense mutation in FN1

Whole-exome sequencing was applied to an affected individual of an EO generalized OA family (Fig. 1A, subject 10) with an average age of onset at 37 years and without dysplasia (table S1). This resulted in the identification of 73,407 variants after quality control, after which a prioritization scheme was applied to identify pathogenic variants (table S2). Hence, intergenic variants, intron variants, synonymous variants, and tolerated missense variants as determined by PolyPhen or SIFT (Sorting Intolerant From Tolerant) were excluded,
as well as variants present in dbSNP build 124 (including 1000 Genomes pilot project data). In addition, variants were excluded that were previously detected by whole-genome sequencing projects, in-house \( (N = 221) \), and by means of the BBMRI-Genome of The Netherlands project \( (N = 473) \). As a consequence, the number of variants was reduced to 122 missense variants \( (\text{table S3}) \) that were predicted to have a functional impact on the gene.

Previously, our group showed strong linkage on 2q33.3 with seven extended EO-OA families, including the family described in this study \((\text{LOD (logarithm of odds) score, 2.93})\). This family showed substantial contribution to this linkage area \( [\text{LOD (logarithm of odds) score, 2.93}] \); hence, we investigated the chromosomal location of the previously mentioned 122 missense variants. Of these variants, three were located around the linkage area on chromosome 2, namely, ALS2, FN1, and ABCB6. To explore functionality of these genes in OA-relevant tissue, gene expression levels in our previously published RNA sequencing data of lesioned and preserved cartilage and bone samples of patients undergoing joint replacement surgery due to end-stage OA, from the Research Osteo-Arthritis and Articular Cartilage study \((\text{RAAK study})\), were examined \((16, 17)\). FN1 showed the highest gene expression in both cartilage and bone samples and showed a high, significant up-regulation \((\text{fold change, 2.12; false discovery rate, } \times 10^{-3})\) in lesioned compared with preserved OA cartilage \( (\text{table 1}) \). Furthermore, ALS2 was expressed at low levels in both cartilage and bone samples, while ABCB6 expression was even lower in cartilage and not detected in bone. Therefore, de novo genotyping was performed for the ALS2 and FN1 variant, showing that the ALS2 variant was not detected, yet confirming that the FN1 variant was carried in seven affected individuals, but not in six unaffected family members. These data indicate complete linkage to robustly affected and unaffected subjects in the family \( (\text{table S4}) \). The FN1 variant was neither present in the additional 1467 OA cases \([\text{Genetics Osteoarthritis and Progression (GARP), RAAK, and Patients Prospectively Recruited in Knee and hip Arthroplasty (PAPRIKA study)}]\) and 744 random controls \([\text{Leiden Longevity Study (LLS)}]\), nor in the Genome Aggregation Database \((v2.1.1)\) and is therefore likely specific for this EO-OA family. Together, these data indicate that the G-to-T nucleotide change \((c1,819)\) resulting in an amino acid change from cysteine to phenylalanine \((\text{C518F}; \text{fig. 1B})\) in the FN1 gene is likely causal to the EO phenotype in this family.

FN1 encodes fibronectin, which is a dimeric glycoprotein, highly abundant in the extracellular matrix \((\text{ECM})\) of articular cartilage and more specifically localized in the pericellular matrix surrounding the chondrocyte. Fibronectin can bind to matrix proteins by multiple binding domains, such as the gelatin-binding domain, which binds to collagen type II, as well as to cell surface integrins, thereby functioning as an adhesion molecule facilitating signals from the ECM to chondrocytes \((18, 19)\). Next, we aimed to investigate the molecular implications of the identified FN1 mutation for the pathophysiology of OA.

**Targeted gene editing of FN1 mutation using CRISPR-Cas9 in hiPSCs**

To elucidate the potential pathogenic mechanism of the identified FN1 mutation, we sought to introduce it into wild-type hiPSCs, resulting in an isogenic pair of mutated and nontargeted hiPSCs. Gene editing was achieved by using CRISPR-Cas9, a guide RNA \((\text{gRNA})\) binding in close proximity to the target site, as well as a 141-mer single-stranded oligodeoxynucleotide \((\text{ssODN})\) containing the specific FN1 mutation \( (\text{table S5}) \). The creation of a Hinc II target site by introducing the mutation was used for screening of successfully targeted clones \( (\text{fig. 2A}) \). The presence of the introduced mutation was confirmed by Sanger sequencing \( (\text{fig. 2B}) \). A total of 274 hiPSC clones were screened, of which 14 \((5.1\%)\) were heterozygous and 3 \((1.1\%)\) were homozygous for the FN1 mutation. Last, one heterozygous and one homozygous FN1 mutant clone were selected for further experiments. Both clones had a normal karyotype, and the predicted off-target site allowing two mismatches was not altered \( (\text{fig. S1})\).
Table 1. Reproduced gene expression analysis of detected previously unidentified variants located around linkage area 2q33.3 in osteoarthritic cartilage and bone. AA sub.*, amino acid substitution; Base, base change; FC, fold change; FDR, false discovery rate of differential expression; ND, not detected.

| Position     | Gene   | Codon    | AA sub. | Base | Quartile | Cartilage FC | FDR       | Bone Quartile | FC | FDR       |
|--------------|--------|----------|---------|------|----------|--------------|-----------|--------------|----|-----------|
| 202590176    | ALS2   | TAT-gAT  | Y1084D  | A/C  | Q3       | 1.08         | 4.95 × 10⁻¹ | Q3           | 0.98 | 8.14 × 10⁻¹ |
| 216285518    | FN1    | TGC-Tcc  | C518F   | C/A  | Q4       | 2.12         | 1.17 × 10⁻³ | Q4           | 1.19 | 6.04 × 10⁻¹ |
| 220075737    | ABCB6  | CGT-gGT  | R688G   | G/C  | Q1       | 1.05         | 8.72 × 10⁻¹ | ND           | ND   | ND        |

*Variants are novel, missense damaging, nonsense, readthrough, frame error, or splice sites not present in dbSNPv124, as well as not detected in in-house genome sequencing projects (LLS; N = 221) and analyzed human genomes by means of the Genome of The Netherlands (N = 473) (15). †Expression levels in quartiles, with Q1 being the lowest and Q4 being the highest, fold changes, and false discovery rate of differential expression levels of identified variants reproduced from RNA sequencing dataset of articular cartilage (16) and subchondral bone (17).

Effect of missense mutation FN1 on chondrogenesis
The wild-type, heterozygous, and homozygous FN1 hiPSC clones were differentiated stepwise to the mesodermal lineage and further toward human induced chondroprogenitor cells (hiCPCs). Subsequently, hiCPC aggregates were manually picked, and chondrogenesis was initiated in our organoid cartilage model. After 5 weeks of chondrogenesis, the chondrogenic pellets were collected, where we observed different morphologies of the pellets. We categorized the pellets in groups based on shape (round or protrusive) and opaqueness of the matrix (Fig. 3A). The abundance of round, dense pellets (Fig. 3A, group 1) was 53% of the pellets in the wild-type group, while this decreased substantially to 15% in the heterozygous group and to 18% in the homozygous group (Fig. 3B). Furthermore, the abundance of protrusive pellets with more transparent matrix (Fig. 3A, group 3) in the heterozygous (60%) and homozygous (54%) groups was increased compared with the wild-type group (25%). When considering group 1 as “normal” pellets and groups 2 to 4 as “not-normal” pellets, we found a significant association between the occurrence of not-normal pellets and the presence of the FN1 mutation for both the heterozygous group [Beta = 1.84; 95% confidence interval (CI), 2.8 to 14.1; P = 9 × 10⁻⁶] and the homozygous group (Beta = 1.63; 95% CI, 2.4 to 11.1; P = 3.1 × 10⁻⁵). These data show that there is a dominant effect of the mutation on the formation of dense, round pellets, thus indicating an inhibited chondrogenic potential of the mutant hiCPCs.

To investigate whether the chondrogenic potential of the mutant hiCPCs was also inhibited before the chondrogenesis, we measured expression of the previously identified chondroprogenitor and chondrogenic markers MCAM, PDGFRβ, and COL2A1 of hiCPCs at day 0 of chondrogenesis (Fig. 3C) (10, 20–22). MCAM was not differentially expressed between the groups, yet PDGFRβ and COL2A1 expression was significantly down-regulated in the homozygous group, compared with wild type. Together, these data show that the chondrogenic potential is decreased as a result of the FN1 mutation, with the effect being most pronounced in the homozygous hiCPCs.

To characterize the differences between the normal and the not-normal chondrogenic pellets, we performed histological and immunohistochemical analyses (Fig. 4A). Hematoxylin and eosiin staining of chondrogenic pellets from group 3 showed that there was a clear difference in structure and composition between the dense and transparent matrix. Moreover, Alcian blue, fibronectin, and collagen type I and II staining revealed that the transparent matrix was non-cartilaginous tissue. When only considering normal pellets (group 1), visually there was no clear difference in matrix intensity staining of...
collagen type II and fibronectin (Fig. 4B). However, quantification of the Alcian blue staining in the chondrogenic parts of pellets from groups 1 to 3 revealed a significant decrease in intensity in the FN1 mutant chondrogenic pellets (Fig. 4C). Quantification of the neo-cartilage sulfated glycosaminoglycan (sGAG) deposition normalized to DNA content showed a significant linear reduction (Beta = −1.44; 95% CI, 0.07 to 0.86; P = 2.8 × 10⁻²) with the presence of the FN1 mutation (Fig. 4D). Moreover, fibronectin concentration was decreased in the medium of the FN1 mutant pellets, albeit not significantly in the homozygous group (Fig. 4E). Together, our data show that the FN1 mutation results in a negative effect on chondrogenic potential and neo-cartilage deposition.

**Mechanism of reduced chondrogenesis in mutant chondrogenic pellets**

The identified C518F mutation is located in the gelatin-binding domain of fibronectin, which is particularly involved in binding to collagen type II (19). The cysteine is involved in intramolecular disulfide bond formation and was shown to be highly conserved among multiple species, highlighting the importance of this amino acid (fig. S2A). The change from a polar cysteine to a nonpolar phenylalanine resulted in a predicted conformational change of the protein as determined by RaptorX (fig. S2B) (23). When replaced by the phenylalanine, the disulfide bond in this part of the domain cannot be formed. Therefore, we hypothesized that the mutation resulted in a conformational change in the gelatin-binding domain, with concurrent decreased binding of fibronectin to collagen type II. To explore this, we performed a collagen type II binding assay with wild-type, heterozygous, and homozygous mutant fibronectin. As a result, we observed a linear reduction (Beta = −0.26; 95% CI, 0.71 to 0.84; P = 2.3 × 10⁻⁹) in binding of mutant fibronectin to collagen type II relative to wild-type fibronectin, thereby confirming our hypothesis (Fig. 5A). To further investigate whether we could identify structural differences in the neo-cartilage as a result of this decreased binding between fibronectin and collagen type II, we visualized cartilage by transmission electron microscopy. We observed electron dense particles that were present both attached to the plasma membrane and a branching fibrillar network obviously devoid of striations emanating from the plasma membrane. The fibrillar network suggests a network of hyaluronic acid aggregates (24), and the electron dense particles are proteoglycans containing granules (Fig. 5B) (25). However, we could not identify any clear differences in the abundance and structure of observed structures between the wild-type and FN1 mutant.

To study the effect of the mutation on markers reflecting chondrocytes in health or disease state, we next explored chondrocyte gene expression in the pellets as a result of the mutation by performing reverse transcription quantitative polymerase chain reaction (RT-qPCR).
for 10 relevant genes. Relative gene expression levels were first calculated by normalizing to expression levels of GAPDH and SDHA (fig. S3). The observed difference in formation of neo-cartilage between the genotypes (Fig. 4A) was potentially a confounding factor in the gene expression analysis. Therefore, we aimed to correct for the differences in efficiency of the neo-cartilage formation between the genotypes. Hence, we selected cartilage-specific markers that were highly expressed in our lesioned and preserved osteoarthritic cartilage samples (RAAK study), but without significant differential expression (16), and that we showed to be specific markers for the neo-cartilage (Fig. 4A). As a result, COL1A1 and COL2A1 were selected as reference genes. The anabolic cartilage markers ACAN and FN1 were significantly down-regulated in the FN1 mutant chondrocytes as compared with wild type, while the catabolic and hypertrophic markers ADAMTS-5, ALPL, and RUNX2 were significantly up-regulated in the FN1 homozygous group (Table 2 and Fig. 6). These data indicate that the mutant chondrocytes were in a more OA disease state, where the effect was stronger in the homozygous mutant group. To investigate the downstream effects of the mutation on the fibronectin-binding chondrocyte cell surface integrins, we investigated the gene expression levels of ITGA5, ITGB1, ITGA3, and ITGA5. Integrin α5β1 is the main fibronectin-binding integrin, while α5β1 and αvβ1 can also bind fibronectin (26). ITGA3 and ITGB1 were significantly up-regulated in FN1 homozygous chondrocytes (Fig. 6). Notably, ITGA5 was down-regulated in the heterozygous group, while its expression was not changed in the homozygous group.

**DISCUSSION**

In the current study, we identified a heterozygous missense mutation (c1,819 G>T; C518F) in FN1 that is likely causal to the EO phenotype in an extended EO-OA family (Fig. 1). By introducing this high-impact mutation in hiPSCs using CRISPR-Cas9 gene editing and using them in an established in vitro organoid cartilage model, we showed that the chondrogenic potential (Fig. 3) and deposition of neo-cartilage (Fig. 4) of the FN1 mutant chondrocytes were decreased. Moreover, we demonstrated that the underlying pathogenic mechanism of the mutation was caused by a decreased binding of fibronectin to the surrounding ECM protein collagen type II (Fig. 5).

As a downstream effect, the formed neo-cartilage was prone to a hypertrophic and procatabolic (OA) state, as reflected by the changed chondrocyte gene expression pattern, particularly of ADAMTS-5 and RUNX2 (Fig. 6). This is further confirmed by the up-regulation of ITGA3 and ITGB1, which is comparable to an OA disease state of
chondrocytes (27). Since fibronectin is an important adhesion protein between ECM and chondrocytes via binding to the cell surface integrins, we advocate that the identified C518F mutation in fibronectin hampers essential interaction between chondrocytes and the ECM; hence, plasticity of chondrocytes to adequately respond to cues from the ECM is decreased. In osteoarthritic joints, it has been shown that fibronectin is degraded proteolytically into fragments (FN-fs), which amplify catabolic processes in articular cartilage (28–31). These FN-fs have distinct activities due to different binding capacities and thereby lead to changes in communication between the ECM and the chondrocyte. Therefore, it is tempting to speculate that the phenomenon of changed binding of fibronectin with ECM and chondrocytes could be directly contributing to the onset of age-related OA in the population.

One of the findings in this study is that the FN1 mutant fibronectin showed a dose-response decrease in binding capacity to collagen type II. (A) Solid-phase binding assay with collagen type II–coated wells and wild-type and C518F mutant fibronectin isolated from chondrogenic pellet cultures. Heterozygous (Hetero) and homozygous (Homo) mutant fibronectin showed linear reduction (Beta = −0.26, P = 2.26 × 10⁻⁹) of binding to collagen type II compared to wild-type fibronectin. Data are means ± SD (N = 3). P value was determined by generalized estimation equations, with absorbance as dependent variable and genotype as covariate. ***P < 0.005. (B) Representative transmission electron microscopy images showing a cell surrounded by cartilaginous matrix of wild-type and FN1 mutant hiCPC-derived chondrogenic pellets. The fibrillar network surrounding the cells is suggestive of hyaluronic acid aggregates, and the dense particles are proteoglycans containing granules. Scale bars, 2 μm.

Table 2. Differences in gene expression between C518F FN1 heterozygous and homozygous hiCPC-derived chondrogenic pellets compared with wild type after 5 weeks of chondrogenesis.

| Gene   | HETERO vs. WT* | HOMO vs. WT* |
|--------|----------------|--------------|
|        | FC  | Beta | SE | P            | FC | Beta | SE | P            |
| ACAN   | 0.57 | −0.81 | 0.23 | 4.52 × 10⁻⁴ | 0.57 | −1.10 | 0.24 | 2.62 × 10⁻⁴ |
| ADAMTS-5 | 1.55 | 0.63 | 0.48 | 1.93 × 10⁻¹ | 5.14 | 2.36 | 0.68 | 5.45 × 10⁻⁴ |
| ALPL   | 1.41 | 0.50 | 0.56 | 3.79 × 10⁻¹ | 2.45 | 1.30 | 0.63 | 4.03 × 10⁻² |
| COL10A1 | 1.03 | 0.05 | 0.41 | 9.11 × 10⁻¹ | 1.86 | 0.90 | 0.56 | 1.09 × 10⁻¹ |
| FN1    | 0.25 | −1.98 | 0.16 | 1.00 × 10⁻⁴ | 0.33 | −1.59 | 0.13 | 1.00 × 10⁻⁴ |
| ITGA3  | 1.18 | −0.41 | 0.77 | 5.93 × 10⁻¹ | 3.91 | 1.97 | 0.65 | 2.51 × 10⁻³ |
| ITGA5  | 0.23 | −2.13 | 0.23 | 1.00 × 10⁻⁴ | 1.01 | 0.02 | 0.24 | 9.34 × 10⁻³ |
| ITGAV  | 0.66 | −0.60 | 0.37 | 1.03 × 10⁻¹ | 1.53 | 0.61 | 0.37 | 1.03 × 10⁻¹ |
| ITB1   | 0.92 | −0.12 | 0.33 | 7.20 × 10⁻¹ | 2.09 | 1.07 | 0.33 | 1.11 × 10⁻³ |
| RUNX2  | 1.42 | 0.50 | 0.28 | 6.75 × 10⁻² | 1.79 | 0.84 | 0.26 | 9.48 × 10⁻⁴ |

*Fold change (FC), Beta value, SE, and P value for expression levels (−ΔCt) of 10 genes as determined by generalized estimation equations, with gene expression levels (−ΔCt) as dependent variable and genotype as factor, when comparing FN1 heterozygous (HETERO) and FN1 homozygous (HOMO) pellets to wild-type (WT) pellets.
that the presence of fibronectin matrix is essential for mesenchymal stromal cell condensation and chondrogenic differentiation (32, 33). The results of our study therefore suggest that decreased binding between fibronectin and collagen type II may negatively affect these processes. To elucidate the exact mechanism during differentiation, longitudinal analysis of the differentiation would have to be performed in future studies.

The less efficient formation of dense, round pellets as a result of the mutation (Fig. 3), hence variable quantity of neo-cartilage formed in the FN1 mutant pellets, was a confounding factor in the gene expression data analyses. We observed a lower amount of cartilage-producing cells relative to the total amount of cells in FN1 mutated pellets compared with wild type, as well as a decrease in the deposition of neo-cartilage (Fig. 4). As a result, an overall stepwise decrease in cartilage-specific gene expression was observed in the FN1 mutant pellets. To adjust for the relatively lower amount of cartilage formed in the FN1 mutant chondrogenic pellets, we used the cartilage-specific markers COL2A1 and COL1A1 for their typically high expression in cartilage, as well as their stability in ongoing OA pathophysiology, as reflected by a previously published large RNA sequencing dataset of autologous cartilage (16), and that we showed to be specific markers for the neo-cartilage (Fig 4A). Although we are confident that the observed significant increase in ADAMTS-5, ALPL, and RUNX2 and the significant decrease in ACAN and FN1 expression reflected true biological effects (Fig. 6), the accuracy of the fold changes provided may be affected. There are different possibilities to overcome this problem in the future. Recently, Adkar et al. (10) engineered a knock-in COL2A1-GFP reporter system in hiPSCs to purify chondroprogenitors, consequently improving chondrogenic capacity during differentiation. To be able to further investigate the effect of the mutation on chondrogenesis without the decrease in chondrogenic potential, the COL2A1 reporter system could be used for our FN1 OA disease model to circumvent this effect. On the other hand, single-cell RNA sequencing could identify the chondroprogenitor subpopulation in the heterogeneous pellets, so that gene expression analysis of only that subpopulation would be possible (21).

Notably, even after correcting for COL2A1 and COL1A1 as cartilage-specific markers, FN1 and ACAN expression was significantly decreased in FN1 mutant pellets, likely affecting quality of deposited cartilage matrix. Moreover, ITGA3 and ITGB1 were significantly up-regulated in the FN1 homozygous pellets. Since αβ1 integrin was shown to be up-regulated in OA chondrocytes (27), the here observed switch from αβ1 to αβ1 integrins in FN1 homozygous chondrocytes may reflect an unfavorable disease state of the FN1 homozygous chondrocytes (27). Conversely, ITGA5 was down-regulated only in the FN1 heterozygous pellets, which could have been a direct result of the formation of wild type–mutant fibronectin dimers, thereby also potentially being responsible for the decreased FN1 and ACAN expression. Recently, it has been shown that the fibronectin-αβ1 adhesion is essential for cartilage regeneration in mice (34). These data indicate that the mutation results in aberrant chondrocyte gene expression, potentially via a change in integrin subunit expression levels toward a more hypertrophic, catabolic state, possibly due to decreased binding between fibronectin and collagen type II. However, this should be confirmed by more investigative studies on protein level, e.g., by Western blot to quantify protein levels of the integrin subunits.

Previous studies regarding familial, high-impact FN1 mutations have been associated with glomerulopathy with fibronectin deposits (GDND), which is a hereditary kidney disease with proteinuria, microscopic hematuria, and hypertension, as well as spondylometaphyseal dysplasia with "corner fractures" (35, 36). These mutations were located in multiple heparin-binding domains, which play an essential role in regulating fibronectin assembly into organized fibrils in ECM. When comparing the phenotypes associated to the FN1 mutations, it is notable that the phenotype in our family is less severe since glomerulopathy results in renal failure and the spine and growth plates are affected in spondylometaphyseal dysplasia,
resulting in, among others, shortened trunk and sciotic posture. Apparently, a mutation in the gelatin-binding domain results in a more specific pathology involving only the joints. The previously identified mutations also often involved cysteine residues, confirming that proper folding of fibronectin, e.g., via disulfide bonds, is crucial for the binding of fibronectin to other matrix components. Together, these data highlight the importance of proper binding of fibronectin to collagen in articular cartilage.

Together, by combining exome sequencing and linkage analysis, we demonstrated that the identified FN1 mutation is causal to human primary OA at an age of onset around 20 to 40 years. Moreover, the mutation does not cause severe developmental aberrations; yet, it has sufficient impact on cartilage tissues to allow measurable functional in vitro effects. Furthermore, by precise genetic engineering of a registered hiPSC line without off-target effects (fig. S1), we established both a hetero- and homozygous isogenic clone, with the OA causing FN1 mutation (Fig. 2). By subsequently applying an established differentiation protocol able to produce biomimetic human neo-cartilage, we generated multiple reliable biological replicates on how aberrant function of fibronectin in cartilage is causal to OA onset. Hence, 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.

In conclusion, our approach highlights the immense potential of combining exome sequencing, hiPSCs, CRISPR-Cas9, and organoid disease modeling to uncover previously unknown underlying disease mechanisms that are readily extrapolated to novel therapeutic avenues in common, complex human genetic diseases. Particularly, we here show that the underlying pathogenic mechanism of the mutation was caused by a decreased binding of fibronectin to the surrounding ECM protein collagen type II. Moreover, as reflected by the changes in chondrocyte gene expression, particularly of ADAMTS-5 and RUNX2, such impaired binding results in the cartilage becoming prone to enter an OA disease state. In addition, we observed a switch in expression levels of the main fibronectin-binding receptor integrin α5β1 to integrin α5β1, reflecting a more OA disease state of the chondrocytes. Together, our work merits further exploration of fibronectin as potential target for therapeutic interventions. We advocate that restoring or maintaining proper binding between fibronectin and collagen type II should be the focus of such a quest.

MATERIALS AND METHODS

Experimental design

The objective of the current study was to identify a likely causal pathogenic mutation in an EO-OA family and underlying disease pathways by functional analyses of isogenic hiPSCs. Exome sequencing was applied to identify a pathogenic mutation, whereas linkage analysis was applied to confirm cosegregation of the mutation and the OA phenotype. To study underlying mechanisms, the mutation was introduced in hiPSCs using CRISPR-Cas9 genome engineering, after which they were used in an in vitro organoid cartilage model, followed by functional analyses. A solid-phase binding assay was performed to quantify binding capacity of mutant and wild-type fibronectin to collagen type II.

Study populations

EO-OA family members

Previously, we reported on an EO family with primary generalized OA without dysplasia (fig. S4) (13, 37). The proband was collected through a rheumatology outpatient clinic; family members were recruited via the proband. The age of onset of OA was between 23 and 45, and the phenotype is characterized by progressive OA with symptoms at multiple joints simultaneously. Two family members reported OA-related complaints; however, no radiographic evidence was available to confirm the diagnosis. An overview of the phenotypes is shown in table S1. The study was approved by the medical ethical committee, and written and informed consent was obtained from all participants.

Controls

Occurrence of the FN1 missense mutation was tested in the general population by de novo genotyping in healthy participants and OA cases. Middle-aged partners (N = 744) of the offspring of nonagenarian siblings from the LLS (38) were considered as controls for OA cases and were named “random controls.” OA cases included those selected from the GARP study (N = 177) (39), the PAPRIKA study (N = 1137) (40), and the ongoing RAAK study (N = 153) (41). For more details on ascertainment of RAAK study biospecimen, see Ramos et al. (14). Ethical permission for the described studies was obtained from the appropriate medical ethical committee under protocol numbers P08.239 and P19.013. Written informed consent was obtained from all participants.

Exome sequencing

Exome sequencing of an affected EO-OA family member was performed by Illumina HiSeq 2000 technology (Beijing Genome Institute). The sequences were generated as 100-base pair paired-end reads, after enrichment of 44-Mb exonic sequences by NimbleGen EZ (Roche NimbleGen). Raw imaging files were processed by Illumina base calling software v1.7 with default parameters. SOAPaligner/SOAP2.21 was used to align reads to the GRCh37 reference genome at the UCSC Genome Browser website (http://genome.ucsc.edu/). Approximately 70% of bases originated from the targeted exome, resulting in a mean coverage of 55.7-fold for the proband. More than 83% of the targeted exons were covered more than 10 times. Single-nucleotide variants were subsequently called by the SOAPsnp variant-filtering scheme, which resulted in 122 pathogenic mutations, is detailed in table S2.

Genotyping

Single-nucleotide polymorphisms were genotyped by mass spectrometry (MassARRAY System; Sequenom, San Diego, CA) using standard conditions as described by Meulenbelt et al. (42). Variants were genotyped across available EO-OA family members (see table S4), OA case studies (GARP, PAPRIKA, and RAAK study), and random controls (LLS).

hiPSC line and cell culture

The hiPSC line LUMC0004iCTRL10 (“LUMC0004” here) was generated from skin fibroblasts of a male donor without known genetic diseases (“wild type”), as described previously (43), and registered at the Human Pluripotent Stem Cell Registry. The generation of the hiPSC line was approved by the Leiden University Medical Center ethical committee under protocol P13.080. The hiPSCs were maintained under standard conditions (37°C, 5% CO2) on Vitronectin-XF (STEMCELL Technologies)—coated plates and refreshed daily with TeSR-E8 medium (STEMCELL Technologies). Cells were passaged in aggregates using Gentle Cell Dissociation Reagent (STEMCELL Technologies) upon reaching approximately 80% confluency.
Genome editing of hiPSCs and clonal isolation

hiPSCs were dissociated with Gentle Cell Dissociation Reagent (STEMCELL Technologies), and 1 × 10^5 cells were transfected with the Alt-R Cas9 RNP complex and the ssODN (both IDT) using the Neon Transfection System (Invitrogen) at 1200 V/30 ms/1 pulse (for gRNA and ssODN sequence, see table S5). Cells were plated in a Synthemax II-SC (Corning)—coated plate using TeSR-E8 supplemented with CloneR (STEMCELL Technologies). After recovery, 1000 cells were plated in a Synthemax II-SC—coated 10-cm dish in TeSR-E8 supplemented with CloneR and refreshed daily. After ~14 days, single cell–derived hiPSC colonies were manually picked, and one-half was used for DNA isolation with QuickExtract Solution (Lucigen). Positive clones were identified by PCR screening of the region of interest, followed by successful digestion by restriction enzyme Hinc II (New England Biolabs), according to the manufacturer’s instructions. Primer sequences are shown in table S6. The introduced mutation was confirmed by Sanger sequencing, performed by the Leiden Genome Technology Center.

Characterization of gene-edited hiPSCs

In silico analysis with the Cas-OFFinder tool (44) was used to identify most likely sites for off-target mutations. When allowing two mismatches, one potential off-target was identified, which was sequenced as described above (for primer sequences, see table S6). G-banding analysis was conducted at the Laboratory of Clinical Genetics Leiden according to standard procedures. A total of 18 metaphases were analyzed for one heterozygous clone, and 20 metaphases were analyzed for one homozygous clone.

hiPSC differentiation to induced chondroprogenitor cells

Generation of induced chondroprogenitor cells (hiCPCs) was based on a protocol previously described (11), which was shown to produce similar neo-cartilage to that produced by human primary articular chondrocytes (11). When hiPSCs reached 60% confluence, the culture medium was switched to mesodifferentiation (MD) medium, composed of IMDM Glutamax (IMDM; Thermo Fisher Scientific) and Ham’s F12 Nutrient Mix (F12; Sigma-Aldrich) with 1% chemically defined lipid concentrate (Gibco), 1% insulin/human transferrin/selenomethionine (ITS+; Corning), 0.5% penicillin-streptomycin (P/S; Gibco), and 450 μM 1-thioglycerol (Sigma-Aldrich). Before induction of anterior primitive streak (day 0), hiPSCs were washed with wash medium (IMDM/F12 and 0.5% P/S) and then fed with MD medium supplemented with activin A (30 ng/ml; Stemgent), 4 μM CHIR99021 (CHIR; Stemgent), and human fibroblast growth factor (20 ng/ml; GFG-2; R&D Systems) for 24 hours. Subsequently, the cells were washed again with wash medium, and paraxial mesoderm was induced on day 1, by MD medium supplemented with 2 μM SB-505124 (Tocris), 3 μM CHIR, GFG-2 (20 ng/ml), and 4 μM dorsomorphin (Tocris) for 24 hours. Before induction of early somite (day 2), cells were washed with wash medium, and then cells were fed with MD medium supplemented with 2 μM SB-505124, 4 μM dorsomorphin, 1 μM C59 (Collagen Technology), and 500 nM PD173074 (Tocris) for 24 hours. Subsequently, cells were washed with wash medium, and for induction of sclerotome, cells (days 3 to 5) were fed daily with MD medium supplemented with 2 μM pumorphamine (Stemgent) and 1 μM C59. To induce chondroprogenitor cells (days 6 to 14), cells were washed briefly with wash medium and fed daily with MD medium supplemented with human bone morphogenetic protein 4 (BMP-4; 20 ng/ml; Miltenyi Biotec). Five independent differentiations were done per clone.

Chondrogenic differentiation

Monolayer cultured hiCPC aggregates present at day 14 of the differentiation were washed with MD medium, manually picked, and centrifuged for 4 min at 1200 rpm. Cell aggregates were subsequently maintained in Dulbecco’s modified Eagle’s medium/F12 (Gibco), supplemented with 1% ITS+, 55 μM 2-mercaptoethanol (Gibco), 1% non-essential amino acids (Gibco), 0.5% P/S, l-ascorbate-2-phosphate (50 μg/ml; Sigma-Aldrich), l-proline (40 μg/ml; Sigma-Aldrich), and transforming growth factor–β1 (10 ng/ml; PeproTech) for 35 days while refreshing medium every 3 to 4 days. The remaining hiCPC aggregates in the well were pooled and lysed with 500 μl of TRizol reagent (Thermo Fisher Scientific) and stored at −80°C until further processing.

sGAG measurement

sGAG concentration in the chondrogenic pellets was measured with the 1,9-dimethylmethylene blue (DMMB) assay (45). Pellets were digested with papain from papaya (Sigma-Aldrich) at 60°C overnight. Shark chondroitin sulfate (Sigma-Aldrich) was used as a reference standard. The absorbance was measured at 525 and 595 nm in a microplate reader (SpectraMax iD3; Molecular Devices).

Histology and immunohistochemistry

Chondrogenic pellets were fixed in 4% formaldehyde overnight and stored in 70% ethanol at 4°C. They were then embedded in paraffin and sectioned at 5 μm. After sectioning, slides were deparaffinated and rehydrated. Overall cellular and tissue structure was visualized with hematoxylin and eosin staining. Sections were stained with 1% Alcian blue 8GX (Sigma-Aldrich) and Nuclear Fast Red (Sigma-Aldrich) to visualize glycosaminoglycan deposition. To detect fibronectin (1:400; ab2413, Abcam), collagen type I (1:1000; ab34710, Abcam), and collagen type II (1:100; ab34712, Abcam), immunohistochemistry was performed with 3-diaminobenzidine (DAB) solution (Sigma-Aldrich) and hematoxylin (Klinipath) as described before (46). Alcian blue staining was quantified by loading the images in Fiji and splitting the color channels. Subsequently, gray values were measured of three to five separate squares per pellet and corrected for the gray value of the background.

RNA isolation and RT-qPCR

For RNA isolation, two pellets were pooled and lysed in 200 μl of TRIzol reagent (Thermo Fisher Scientific) and homogenized using micro pestles. RNA was extracted with chloroform and purified from the supernatant using the RNeasy Mini Kit (QIAGEN). Synthesis of cDNA was performed with 150 ng of total RNA using a First Strand cDNA Synthesis kit according to the manufacturer’s protocol (Roche Applied Science). cDNA was diluted five times, and preamplification with TaqMan PreAmp master mix (Thermo Fisher Scientific) was performed for eight genes of interest. Primer sequences are shown in table S6. Gene expression was measured with the Fluidigm Biomark HD machine using a 96.96 IFC (Integrated Fluidic Circuit) chip or with QuantStudio 6 Real-Time PCR system (Applied Biosystems) using FastStart SYBR Green Master reaction mix (Roche Applied Science). Quality control of the data was performed, and nondetected values were removed. Relative gene expression levels were calculated with the 2 −ΔΔCt method as follows: The average of triplicate raw cycle threshold (Ct) values of the genes were averaged. Ct values of GAPDH and SDHA were averaged to function as reference genes (RG). Next, ΔCt values of the gene of interest (GOI) were calculated.
as Ct\textsubscript{GOI} – Ct\textsubscript{RG}. Subsequently, we averaged the ΔCt values of COL1A1 and COL2A1 as chondrogenic controls (CC) to calculate final ΔCt values of the GOI as follows: ΔCt\textsubscript{GOI\_new} = ΔCt\textsubscript{GOI} – ΔCt\textsubscript{CC}. Fold changes were calculated with 2\(^{-ΔCt}\), with the wild type as reference.

**Solid-phase binding assay**

Conditioned medium of wild-type and FN1 mutant pellets was collected and concentrated in preparation for the binding assay. To this end, 450 μl of medium was collected in 100 K molecular weight cut-off Pierce Protein Concentrators (Thermo Scientific) and centrifuged for 10 min at 12,000g. Subsequently, fibronectin concentration was determined using the Human Fibronectin ELISA Kit (Invitrogen) according to the manufacturer’s protocol.

Clear multiwell plates (R&D Systems) were coated overnight with 100 μl of purified human collagen type II (10 μg/ml; Merck) in phosphate-buffered saline (PBS) at 4°C, followed by four wash steps with wash buffer (0.05% Tween 20 in PBS). Nonspecific binding was blocked for 1 h with 3% (w/v) bovine serum albumin (BSA) in PBS. After washing with wash buffer, the plates were incubated with 100 μl of concentrated medium samples at FN1 concentration of 40 ng/ml in assay buffer (0.05% Tween 20 and 0.5% BSA in PBS) for 2 h. Plates were then washed four times with wash buffer and incubated with rabbit anti-fibronectin biotin-conjugated antibody (Rockland) at 0.2 μg/ml in assay buffer for 1 h. Plates were washed, after which the plates were incubated with streptavidin-horseradish peroxidase (Thermo Scientific) at 0.1 μg/ml in assay buffer for 1 h. After washing, color development was performed with 100 μl of tetramethylbenzidine substrate (Thermo Fisher Scientific) for 10 min, reaction was stopped with 100 μl of 1 M HCl, and absorbance was measured at 450 nm. Assays were performed in triplicate. Error bars are SDs.

**Transmission electron microscopy**

Chondrogenic pellets that were embedded in paraffin were melted together into a big image as previously described (90 nm) were made on a Reichert Ultracut S (Leica Microsystems). After the poststaining of the sections with uranyl acetate and lead with Epon and polymerized at 70°C during 48 hours. Ultrathin sections (90 nm) were cut on a Leica EM UC6 (Leica Microsystems) and placed on lacey carbon grids. The grids were imaged with a transmission electron microscope (Thermo Fisher Scientific, formerly FEI mission electron microscope) operating at 120 kV, using a Gatan OneView camera (Gatan, Pleasanton). Overlapping images were collected and stitched together into a big image as previously described.

**Statistical analysis**

Statistical analyses were performed using SPSS version 25 (IBM). Data are shown as means ± SD. With respect to RT-qPCR gene expression data, individual –ΔCt values are shown, as well as box plots per genotype. The box plots represent 25th, 50th, and 75th percentiles, and whiskers represent the lowest and largest data point lying within 1.5 times the interquartile range. Individual samples are depicted by dots in each graph. The reported P values were determined by applying a generalized estimating equation (GEE) to the experimental readout, i.e., –ΔCt values, relative Alcian blue intensity, and FN1 concentration, to effectively adjust for dependencies of the independent differentiations per genotype. In general, we followed a linear GEE model, with the readout data as dependent variable, genotype as factor, and exchangeable working matrix: Read-out ~ Genotype + (1|Differentiation). To determine a dose-response effect, we applied a GEE to the experimental readout, i.e., absorbance and SGAG/DNA, with the readout as dependent variable, genotype as covariate, and exchangeable working matrix. For the analysis of the pellet morphology, we followed a binary logistic GEE model, with normal and not-normal pellets as readout and genotype as factor, and adjusted for independent differentiations. P values <0.05 were considered statistically significant. *P < 0.05, **P < 0.01, and ***P < 0.005.
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High-impact *FN1* mutation decreases chondrogenic potential and affects cartilage deposition via decreased binding to collagen type II

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