Elucidating Epigenetic Regulation by Identifying Functional cis-Acting Long Noncoding RNAs and Their Targets in Osteoarthritic Articular Cartilage

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Objective. To identify robustly differentially expressed long noncoding RNAs (lncRNAs) with osteoarthritis (OA) pathophysiology in cartilage and to explore potential target messenger RNA (mRNA) by establishing coexpression networks, followed by functional validation.

Methods. RNA sequencing was performed on macroscopically lesioned and preserved OA cartilage from patients who underwent joint replacement surgery due to OA (n = 98). Differential expression analysis was performed on lncRNAs that were annotated in GENCODE and Ensembl databases. To identify potential interactions, correlations were calculated between the identified differentially expressed lncRNAs and the previously reported differentially expressed protein-coding genes in the same samples. Modulation of chondrocyte lncRNA expression was achieved using locked nucleic acid GapmeRs.

Results. By applying our in-house pipeline, we identified 5,053 lncRNAs that were robustly expressed, of which 191 were significantly differentially expressed (according to false discovery rate) between lesioned and preserved OA cartilage. Upon integrating mRNA sequencing data, we showed that intergenic and antisense differentially expressed lncRNAs demonstrate high, positive correlations with their respective flanking or sense genes. To functionally validate this observation, we selected P3H2-AS1, which was down-regulated in primary chondrocytes, resulting in the down-regulation of P3H2 gene expression levels. As such, we can confirm that P3H2-AS1 regulates its sense gene P3H2.

Conclusion. By applying an improved detection strategy, robustly differentially expressed lncRNAs in OA cartilage were detected. Integration of these lncRNAs with differential mRNA expression levels in the same samples provided insight into their regulatory networks. Our data indicate that intergenic and antisense lncRNAs play an important role in regulating the pathophysiology of OA.

INTRODUCTION

Osteoarthritis (OA) is an age-related, heterogeneous, degenerative disease of the articular joints, characterized in part by cartilage degeneration and remodeling of subchondral bone, which results in stiff and painful joints and decreased mobility (1). Despite the fact that OA is the most globally prevalent joint disease, no effective treatment is currently available (2). It has been demonstrated that OA pathophysiology in cartilage is marked by altered gene expression regulation in chondrocytes (3,4). This alteration of gene expression regulation could be triggered by adaptation processes occurring due to aging, genetic predisposition, or environmental stimuli, and is in part caused by aberrant epigenetic mechanisms. These mechanisms include DNA methylation, histone modifications, and expression of microRNAs (<22 nucleotides) (4–6). More recently, long noncoding RNAs (lncRNAs; >200 nucleotides) have been shown to play an important role in the homeostasis of the extracellular matrix of cartilage (5,7–10).

LncRNAs are defined as RNA transcripts with little or no protein-coding potential and are known to regulate transcription

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and translation by numerous mechanisms, such as chromatin remodeling, messenger RNA (mRNA) stabilization, microRNA modulation, and recruitment of scaffolding proteins. One classification type of IncRNAs is based on the genomic location with respect to protein-coding genes, so-called biotypes, including antisense RNAs, sense RNAs, pseudogenes, and long intergenic noncoding RNAs (lncRNAs). Another type of classification is based on the location at which the IncRNA functions relative to its transcription site, which can be in trans or cis (11–13). Cis-acting lncRNAs comprise a considerable portion of known IncRNAs and can be positioned at various distances and orientations relative to their target genes, such as lincRNAs around transcription factor start sites, as well as sense and antisense IncRNAs that overlap with their sense genes (13,14). Potentially, IncRNAs could be candidate targets in OA treatment, since their expression can be highly tissue specific (9).

RNA sequencing (RNA-Seq) has improved the ability to detect IncRNAs, but mapping and annotating IncRNAs remains challenging. These challenges arise from the fact that they are usually expressed at very low levels and their sequence–function relationship is still poorly understood. Moreover, recent findings from studies on ribosome profiling and bioinformatics suggest that a large proportion of transcripts has unknown coding potential (15). Recent studies on OA have focused on intergenic IncRNAs, even though the proportion of genic and intergenic IncRNAs can be similar depending on the investigated tissue (15,16). To determine the complete IncRNA transcriptome, we used an in-house pipeline to robustly capture IncRNAs in a previously assessed RNA-Seq data set of lesioned and preserved OA cartilage samples (4). Subsequently, IncRNAs associated with OA pathophysiology were identified, and potential interactions with OA-specific mRNAs were investigated.

**MATERIALS AND METHODS**

**Sample collection.** Macroscopically lesioned and preserved articular cartilage samples were obtained from participants in the Research Osteoarthritis and Articular Cartilage (RAAK) study described by Ramos et al (3). In the present study, a total of 98 samples were used (65 knees, 33 hips) (see Supplementary Table 1, on the Arthritis & Rheumatology website at http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). Ethical approval was obtained from the medical ethics committee of the Leiden University Medical Center (no. P08.239/P19.013), and informed consent was obtained from all participants.

**RNA sequencing.** Total RNA from articular cartilage was isolated using a Qiagen RNeasy Mini Kit. Paired-end 2 × 100-bp read RNA sequencing (Illumina TruSeq RNA Library Prep Kit, Illumina HiSeq2000, and Illumina HiSeq4000) was performed. Strand-specific RNA-Seq libraries were generated, which yielded a mean of 20 million reads per sample. Quality control was performed as previously described (4), and reads were subsequently aligned to the GRCh38 reference genome with an RNA-Seq aligner STAR (version 2.6.0) (17). Thereafter, aligned reads were processed into individual transcripts using StringTie (version 1.3.4) (18). LncRNAs were identified by mapping the transcripts to GENCODE (version 29) (11) and Ensembl (version 94) (19).

In order to filter transcripts with unknown protein-coding potential, we integrated 2 sources of evidence: 1) predictions from the alignment-free Coding Potential Assessment Tool (CPAT, version 1.2.2) (20), and 2) predictions from the LncFinder R package (version 1.1.3) (21). CPAT is a machine learning–based method that analyzes the sequence features of transcript open-reading frames (ORFs) using a logistic regression model built from ORF size, Fickett TESTCODE statistic, and hexamer usage bias. In CPAT, a transcript with a coding probability of ≥0.364 was considered to be a coding sequence. LncFinder predicts IncRNAs using heterologous features and a machine learning model (21). Transcripts with protein-coding potential predicted by both tools were removed from the data set.

**Differential expression analysis and replication.** Differential expression analysis was performed on 32 paired samples (25 knees and 7 hips) (Supplementary Table 1A, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract), using the DESeq2 R package (version 1.24) (22). A general linear model assuming a negative binomial distribution was applied, followed by a paired Wald's test comparing lesioned OA cartilage samples and preserved OA cartilage samples, with the preserved samples as the referent. P values less than 0.05 (after Benjamini-Hochberg correction) were considered significant and are reported as the false discovery rate (FDR). This analysis was repeated for knee and hip samples separately.

Furthermore, to validate the results, 5 significantly differentially expressed IncRNAs were selected and measured by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) in 10 paired cartilage samples overlapping with the RNA-Seq samples (Supplementary Table 1B), and replication was performed in an independent cohort of 10 paired cartilage samples (Supplementary Table 1C). Total RNA was isolated using an RNeasy Mini Kit, followed by complementary DNA (cDNA) synthesis using 100-ng RNA with a First Strand cDNA Synthesis kit according to the instructions of the manufacturer (Roche Applied Science). Expression levels of AC025370.1, AC090877.2, MEG3, P3H2-AS1, TBILA, and GAPDH were determined using FastStart SYBR Green Master reaction mix (Roche Applied Science). Primer sequences are shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). Relative gene expression levels were calculated with the 2−ΔΔCt method, using GAPDH as internal control. A paired t-test was performed on the −ΔΔCt values, and P values less than 0.05 were considered significant.
**LncRNA–mRNA interactions.** To identify potential interactions, correlations were calculated between the identified differentially expressed lncRNAs and the previously reported differentially expressed protein-coding genes in the same samples. LncRNA expression data were normalized and variance stabilizing transformed using the DESeq2 R package (version 1.24) (22), and batch effect was removed using the limma R package (version 3.40.6) (23). Our previously published mRNA data (4) were equally normalized and transformed, and batch effect was removed. Subsequently, Spearman’s correlations were calculated between the significantly differentially expressed lncRNAs identified in the combined analysis of knee and hip samples and the differentially expressed protein-coding genes previously published (4), using the Hmisc R package (version 4.2.0) for OA cartilage samples (Supplementary Table 1D). Correlations with P values less than 0.05 were considered significant. Network visualization was performed using the RedeR package (version 3.10) (24).

**In vitro down-regulation of lncRNA using locked nucleic acid (LNA) GapmeRs.** Primary chondrocytes were isolated from 3 independent donors and passaged twice or thrice, as previously described (25). Chondrocytes were transfected in duplo with antisense LNA GapmeR (Qiagen) targeting P3H2-AS1 (TGAGCAACTAGGTGTA) or GapmeR negative control (AACACGTCTATACGC) at 10 nM final concentration using Lipofectamine RNAiMax Transfection Reagent according to instructions of the manufacturer (Invitrogen). Cells were lysed 30 hours posttransfection with TRIzol reagent (Thermo Fisher Scientific) for RNA isolation, which was done using an RNeasy Mini Kit. Synthesis of cDNA was performed with 150 ng of total RNA using a First Strand cDNA Synthesis kit according to the instructions of the manufacturer. Expression levels of P3H2-AS1, P3H2, and GAPDH were determined using FastStart SYBR Green Master reaction mix. Primer sequences are shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). Relative gene expression levels were calculated with the 2−ΔΔCt method, using GAPDH as internal control. A paired t-test was performed on the −ΔCt values, and P values less than 0.05 were considered significant.

**Data availability statement.** FASTQ files are available on ArrayExpress E-MTAB-7313.

**RESULTS**

**Characterization of lncRNAs in OA cartilage.** To characterize lncRNAs in OA cartilage, we used our previously assessed RNA-Seq data on 32 paired samples (25 knees, 7 hips) of lesioned and preserved OA cartilage (4) (Supplementary Table 1A, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). Our in-house pipeline was applied to capture lncRNAs from 2 databases (GENCODE and Ensembl). As shown in Figure 1, 30,354 lncRNAs were initially detected in our data set. To filter out possible transcripts of unknown coding potential, we integrated results from 2 machine learning approaches (CPAT [20] and Lncfinder [21]). After removing these transcripts, 29,219 lncRNAs remained in the data set and were considered for further analyses. To robustly detect lncRNAs expressed in OA cartilage, a cutoff of an average of ≥2 counts per lncRNA was applied, resulting in a total of 5,053 lncRNAs expressed in cartilage (Figure 1). Classification of these lncRNAs based on biotype showed that 1,989 were antisense RNAs (39.4%), 249 were sense RNAs (4.9%), 1,532 were pseudogenes (30.3%), and 900 were lincRNAs (17.8%) (Figure 2).

**Differential expression of lncRNAs between lesioned and preserved OA cartilage.** To identify lncRNAs associated with the OA process, differential expression analysis was performed on paired lesioned and preserved OA cartilage samples, resulting in 191 significantly differentially expressed lncRNAs (FDR < 0.05; Figure 1). Of these, 65 were antisense RNAs (34.0%), 10 were sense RNAs (5.2%), 33 were pseudogenes (17.3%), and 66 were lincRNAs (34.6%) (Figure 2 and Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). When comparing the biotypes of the total expressed lncRNAs to the biotypes of the differentially expressed lncRNAs (Figure 2), we observed an increase of lincRNAs and a decrease

![Figure 1. Overview of applied strategy. Numbers of genes or long noncoding RNAs (lncRNAs) represent significantly differentially expressed (DE) genes or lncRNAs (according to false discovery rate). OA = osteoarthritis; LNA = locked nucleic acid.](image-url)
of pseudogenes. The most significantly differentially expressed lncRNA was lincRNA AL139220.2 (fold change 2.2, FDR 2.0 × 10^{-10}). As depicted in Figure 3, 114 lncRNAs were down-regulated and 77 were up-regulated, with a fold change ranging from 0.3 (AC100782.1, FDR 6.5 × 10^{-4}) to 4.5 (LINC01411, FDR 2.6 × 10^{-6}). Notably, previously identified lncRNAs such as MALAT1 (fold change 1.3, FDR 0.4) (27), TUG1 (fold change 1.1, FDR 0.7) (28), HOTAIR (fold change 0.8, FDR 0.5), and GAS5 (fold change 1.1, FDR 0.8) (29) were not found to be significantly differentially expressed in the present study.

To validate the differential expression results, we selected 5 lncRNAs (AC025370.1, AC090877.2, MEG3, P3H2-AS1, and TBILA) based on the highest absolute fold change and genomic location, using RT-qPCR in a cohort consisting of 10 paired samples (Supplementary Table 1B, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract), overlapping with the RNA-Seq samples. All 5 lncRNAs were detected using RT-qPCR with equal direction of effect as those found in the RNA-Seq analysis (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). Furthermore, replication was performed in an independent cohort of 10 paired cartilage samples (Supplementary Table 1C), which also showed comparable effect sizes and directions (Supplementary Table 4).

To explore whether joint-specific lncRNAs could be detected, stratified analyses were performed for knee samples (25 pairs) and hip samples (7 pairs). Upon performing differential expression analysis on the knee samples, 90 significantly differentially expressed lncRNAs were identified (Supplementary Figure 3).

**Figure 2.** Distribution of biotypes of total long noncoding RNAs (lncRNAs) expressed in total cartilage compared to lncRNAs that were significantly differentially expressed (according to the false discovery rate) between lesioned osteoarthritis (OA) cartilage and preserved OA cartilage.

**Figure 3.** Differential expression analysis of long noncoding RNAs (lncRNAs) between lesioned osteoarthritis (OA) cartilage and preserved OA cartilage. Volcano plot shows differentially expressed lncRNAs, with down-regulated lncRNAs represented by blue circles and up-regulated lncRNAs represented by red circles. Top differentially expressed lncRNAs are labeled, as are known and novel OA-associated lncRNAs. FDR = false discovery rate; FC = fold change.
Table 5A, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract), of which 12 were not found in the previous combined analysis and therefore were unique to knee cartilage (Supplementary Table 6A, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/abstract). In the hip samples, 31 lncRNAs were significantly differentially expressed (Supplementary Table 5B), of which 13 were unique to hip cartilage (Supplementary Table 6B). The most significantly differentially expressed lncRNA unique to the knee was MSL3P1 (fold change 1.5, FDR $1.49 \times 10^{-2}$), while one of the most significantly differentially expressed lncRNAs unique to the hip was PAPPA-AS1 (fold change 9.4, FDR $2.77 \times 10^{-4}$). Notably, the most up-regulated lncRNA in the hip, AP001515.1 (fold change 21.5, FDR $2.8 \times 10^{-4}$), was also unique to the hip, while the most up-regulated lncRNA in the knee, LINC01411 (fold change 5.8, FDR $6.1 \times 10^{-6}$), was not unique to the knee.

**Potential interactions between IncRNAs and mRNAs relevant to OA pathophysiology.** We next aimed to investigate whether mRNAs associated with the OA process are regulated by differentially expressed IncRNAs. Based on the assumption that interactions between IncRNAs and mRNAs likely show coexpression (30) among lesioned and preserved OA cartilage samples, correlations were calculated between our previously reported differentially expressed protein-coding genes (4) and differentially expressed IncRNAs (Supplementary Table 1D), as shown in Figure 1. This resulted in 343 significant correlations ($r > 0.8$) (Supplementary Table 7, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/), comprising 47 unique IncRNAs, of which 17 were antisense (36%) and 14...
were intergenic (30%) (Supplementary Table 8, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/). This distribution is comparable to that found among all differentially expressed IncRNAs (Figure 2), supporting the notion that IncRNAs regulate mRNAs, independent of biotype. Notably, the most significantly differentially expressed IncRNA, AL139220.2 (fold change 2.2, FDR $2.0 \times 10^{-10}$), showed one of the highest correlations with COL6A3 ($r = 0.8, P = 2.2 \times 10^{-16}$), encoding a type VI collagen chain.

To visualize these interactions, an OA-specific IncRNA–mRNA coexpression network was generated. As shown in Figure 4, 3 relatively large clusters of interacting IncRNAs and mRNAs were observed. One cluster was characterized as being highly interlinked with a cluster of the same genes (e.g., ITGB1BP1 correlated with the 6 IncRNAs IER-AS1, AL355075.3, AC234917.1, AC091564.4, AC108449.3, and AL450306.1), whereas the other 2 were characterized by IncRNAs interlinked with mostly unique genes (e.g., LNCSRLR with 18 genes). In addition to the clusters, there were a number of singular interlinked IncRNAs, such as AC090877.2 (fold change 0.3, FDR $6.2 \times 10^{-5}$) with GREM1 ($r = 0.9, P = 2.2 \times 10^{-16}$), which encodes a cytokine of the bone morphogenetic protein antagonist family (Figure 4). Interestingly, GREM1 was the gene located closest to AC090877.2, suggesting that this IncRNA cis-regulates this gene.

One of our objectives in the present study was to generalize the identification of potential cis-regulation of differentially expressed IncRNAs (Figure 1). As shown in Figure 5A, we compared the distribution of significant correlations between differentially expressed IncRNAs and all genes and between differentially expressed IncRNAs and genes that lie within a 100-kb window of the transcription start site. The proportion of significant correlations $>0.5$ with all differentially expressed genes was 11%, but this increased to 44% when we only considered the 100-kb window. Since the percentage of differentially expressed antisense IncRNAs (34%) was comparable to that of intergenic IncRNAs (34.6%), we also aimed to identify potential cis-regulation of antisense IncRNAs. To this end, we compared the distribution of correlations between differentially expressed antisense IncRNAs and all protein-coding mRNAs and between differentially expressed antisense IncRNAs and their sense genes (Figure 5B). The percentage of correlations $>0.5$ was 10% with all genes and 61% with only the sense genes, showing that there is an enrichment for higher, positive correlations between antisense IncRNAs and their sense gene. Taken together, these data suggest that both intergenic and antisense IncRNAs are prone to regulate mRNAs in cis in OA cartilage.

**Figure 5.** Distribution of significant correlations between intergenic differentially expressed long noncoding RNAs (IncRNAs) and previously identified differentially expressed protein-coding genes or protein-coding genes in a 100-kb window (A), and between antisense differentially expressed IncRNAs and differentially expressed protein-coding genes or their sense genes (B). Correlations between IncRNA and mRNA data were calculated from the same osteoarthritis cartilage samples ($n = 98$).
ROLE OF DIFFERENTIALLY EXPRESSED lncRNAs IN OA

Down-regulation of lncRNA expression using LNA GapmeRs. To validate whether the previously identified cis-regulation between lncRNAs and their surrounding genes is caused by a direct effect, P3H2-AS1 was selected as a proof of concept for functional validation. P3H2-AS1 is an antisense lncRNA, which was found to be highly up-regulated in lesioned OA cartilage (fold change 2.7, FDR 4.1 × 10−4) (4), and the highest correlation was with its sense gene P3H2 (r = 0.63, P = 1.0 × 10−13) (Supplementary Figure 1, http://onlinelibrary.wiley.com/doi/10.1002/art.41396/). To this end, primary chondrocytes were transfected with a P3H2-AS1–targeting LNA GapmeR. As shown in Figure 6A, this resulted in a significant down-regulation of P3H2-AS1 compared to a nontargeting LNA GapmeR (fold change 0.28, P = 0.0035). Subsequently, P3H2 expression levels were measured, which showed that P3H2 expression was significantly down-regulated compared to cells transfected with nontargeting control LNA GapmeRs (fold change 0.36, P = 0.001) (Figure 6B).

DISCUSSION

To our knowledge, we are the first to report on robust differential expression of lncRNAs as related to OA pathophysiology, while integrating them with data on differential mRNA expression levels of the same samples using RNA sequencing. As a result, our new in-house pipeline identified 5,053 lncRNAs that were expressed in OA cartilage. However, after applying a filter with a cutoff of an average of ≥2 counts per lncRNA, the detected lncRNAs were reduced by ~83% to 5,053. Since lncRNAs are known to be expressed at very low levels this was to be expected, yet lncRNAs expressed at low levels can still be functional (12). To allow exploratory analyses with lncRNAs expressed at such low levels, deeper sequencing would be necessary, with a read depth of ~50 million reads per sample. Additionally, to be able to report on valid lncRNAs in OA articular cartilage and their potential target mRNAs, we prioritized reporting known lncRNAs with a predicted non–protein-coding potential. Nonetheless, by focusing on these known lncRNAs, we may have disregarded compelling novel OA-relevant lncRNAs.

We identified 29,219 lncRNAs that were expressed in OA cartilage. However, after applying a filter with a cutoff of an average of ≥2 counts per lncRNA, the detected lncRNAs were reduced by ~83% to 5,053. Since lncRNAs are known to be expressed at very low levels this was to be expected, yet lncRNAs expressed at low levels can still be functional (12). To allow exploratory analyses with lncRNAs expressed at such low levels, deeper sequencing would be necessary, with a read depth of ~50 million reads per sample. Additionally, to be able to report on valid lncRNAs in OA articular cartilage and their potential target mRNAs, we prioritized reporting known lncRNAs with a predicted non–protein-coding potential. Nonetheless, by focusing on these known lncRNAs, we may have disregarded compelling novel OA-relevant lncRNAs.

Given that we had a (within-patient) paired lesioned cartilage–preserved cartilage study design, with pairs sequenced on the same batch, we applied a paired Wald’s test as implemented in the DESeq2 R package. Since our data set also included lncRNAs expressed at low levels, the addition of a random effect to compensate for technical errors may have been a better, yet more
conservative approach. As such, the IncRNAs in our data set, particularly those with low read counts, could be subject to false positive results and therefore require replication and verification.

We observed a particular enrichment of IncRNAs in the differential expression analysis compared to the total data set (34.6% versus 17.8%) (Figure 2), showing that IncRNAs indeed play an important role in OA pathophysiology, as seen in previous studies (8, 16, 30). Nonetheless, in comparison to the fraction of significantly differentially expressed IncRNAs reported by Pearson et al. (8), this proportion is still relatively small. However, Pearson and colleagues performed RNA-Seq on samples from isolated chondrocytes in contrast to the RNA isolated from cartilage in our study and focused on profiling IncRNAs up-regulated by interleukin-1β. The activation of chondrocyte proliferation in tissue culture will likely induce expression of RNAs involved in transcriptional regulation, compared to the transcriptome of maturational arrested chondrocytes residing in cartilage.

Of the 191 IncRNAs that were significantly differentially expressed between lesioned and preserved OA cartilage (Figure 3), multiple IncRNAs have been previously identified, including MEG3, LINCO1614, and PART1 (16, 26). However, there were also examples of IncRNAs previously associated with OA (27–29), which were not significantly differentially expressed, such as MALAT1, HOTAIR, GAS5, and TUG1. A possible explanation could be that they were found to be differentially expressed between preserved OA and healthy cartilage, as opposed to our comparison between lesioned OA cartilage and preserved OA cartilage (7). The cross-sectional study design comparing OA cartilage and healthy cartilage provides insight into which IncRNAs are involved in the early phase of OA pathophysiology and are therefore potentially causal in the process and which IncRNAs are specific to healthy cartilage; this was not possible with our study design. Nonetheless, the paired analysis allowed for detection of IncRNA expression changes specific to the OA pathobiologic process, independent of confounding factors such as sex and age. At least 35 differentially expressed IncRNAs in our data set were previously found to be associated with OA (10, 16, 30), but the most significantly differentially expressed IncRNA, AL139220.2, and the most up- and down-regulated differentially expressed IncRNAs, LINCO1411 and AC100782.1, respectively, have not previously been found to be associated with OA (3), showing that a paired study design allows for the detection of many more IncRNAs involved in the OA pathobiologic process.

Previous studies have demonstrated differences in dysregulated pathways between knee and hip OA cartilage and epigenetic differences based on DNA methylation (8, 16, 31, 32); thus, we aimed to identify joint-specific IncRNAs. Differential expression analysis in knee samples resulted in a higher number of significantly differentially expressed IncRNAs (n = 90) than in hip samples (n = 31), which could be due to the smaller sample size of the hip samples (25 knee pairs versus 7 hip pairs). However, the number of unique IncRNAs per joint site was similar: 12 unique knee IncRNAs and 13 unique hip IncRNAs. This suggests that there is more heterogeneity in the processes in the knee, which could be due in part to anatomic joint site–specific differences. This is also supported by the fact that the average fold change of the up-regulated IncRNAs unique to the hip was 8.3, while it was 1.5 for knee. The unique IncRNAs with the highest fold change in the knee (AC068768.1 fold change 1.6, FDR 2.3 × 10−2) and hip (AP001615.1 fold change 21.5, FDR 2.8 × 10−10) were not previously found to be associated with OA. The identification of these joint-specific IncRNAs is useful for follow-up studies to determine potential joint-specific therapeutic targets.

Unlike conserved microRNAs, it is difficult to predict the function of IncRNAs based solely on nucleotide sequence, due to their lack of conservation of the primary sequence (15). To explore potential regulatory interactions between IncRNAs and mRNAs in cartilage, correlations were calculated between differentially expressed IncRNAs and differentially expressed protein-coding mRNAs (Figure 4). At the transcriptional level, IncRNAs can exert their function in trans or cis (13), both of which we observed in this study. The most significantly differentially expressed IncRNA, AL139220.2, showed one of the highest correlations with COL6A3 (r = 0.8, P = 2.2 × 10−10), encoding one of the type VI collagen chains as part of the complete type VI collagen molecule, which is mostly present in the pericellular matrix of cartilage. AL139220.2 is located on chromosome 1 and, at present, little is known about its function. Since COL6A3 is located on chromosome 2, it seems likely that AL139220.2 regulates COL6A3 expression in trans. Notably, AC090877.2 showed the highest correlation with its sense gene GREM1 (r = 0.9, P = 2.2 × 10−10), suggesting that this IncRNA cis-regulates this gene. In previous studies, it has been shown that IncRNAs often regulate flanking mRNAs in cis in OA, in which a positive correlation was found between the expression of mRNA-flanking IncRNAs and their nearest coding mRNA (8, 30). This observation was confirmed by our findings, as the percentage of higher, positive correlations (r > 0.5) was considerably larger between IncRNAs and the differentially expressed genes that lie within a 100-kb window (44%) than with all differentially expressed genes (11%) (Figure 5A).

Furthermore, it is known that antisense IncRNAs can regulate their overlapping sense genes in cis (14), which has not previously been investigated in OA. We found an enrichment for higher positive correlations between antisense differentially expressed IncRNAs and their sense genes (r > 0.5 in 61%) compared to correlations between antisense differentially expressed IncRNAs and all differentially expressed genes (r > 0.5 in 10%), suggesting that indeed antisense IncRNAs often regulate their sense genes in cis (Figure 5B). Therefore, to completely understand the transcriptional regulation of IncRNAs in the OA process, the total IncRNA transcriptome should be considered, not solely the IncRNAs. Of importance is the notion that these correlations are not yet proof of a (direct) downstream effect of IncRNAs on the mRNAs.
Given these observations, we selected the antisense IncRNA P3H2-AS1 as proof of principle to establish whether it regulates its sense gene. Down-regulation of P3H2-AS1 resulted in a significant down-regulation of P3H2 expression levels (Figure 6), thereby confirming that P3H2-AS1 regulates its sense gene in cis. P3H2 encodes an enzyme that catalyzes posttranslational 3-hydroxylation of proline residues and plays a critical role in collagen chain assembly, stability, and crosslinking and was recently found to be highly up-regulated in lesioned OA cartilage, and therefore likely involved in the OA process (4). Antisense IncRNAs can affect biogenesis or mobilization of target RNA on multiple levels, such as transcription, splicing, and translation (14). To elucidate the exact mechanism of P3H2-AS1 regulating P3H2 and investigate whether P3H2-AS1 can be used as a potential preclinical target by modulating P3H2 expression levels via P3H2-AS1, complementary functional studies employing clustered regularly interspaced short palindromic repeat/Cas9, RNA fluorescence in situ hybridization, or crosslinked immunoprecipitation are necessary (33).

In conclusion, our improved detection strategy resulted in the characterization of IncRNAs robustly expressed in OA cartilage. Our data signify that intergenic as well as antisense IncRNAs play an important role in regulating the pathophysiology of OA. Moreover, we observed that in addition to a previous finding that intergenic IncRNAs function in cis, antisense IncRNAs can exert their function in cis, which we confirmed in vitro. Future studies regarding IncRNAs and OA should be complemented by functional validation, e.g., by modulating IncRNA expression levels using antisense LNA GapmeRs, in order to confirm whether a correlation signifies a biologic relationship between IncRNA and mRNA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Ms van Hoolwerff had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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