**Small RNA signatures of the anterior cruciate ligament from patients with knee joint osteoarthritis**

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Abstract

The anterior cruciate ligament (ACL) is susceptible to degeneration, resulting in joint pain, reduced mobility and osteoarthritis development. There is currently a paucity of knowledge on how anterior cruciate ligament degeneration and disease leads to osteoarthritis. Small non-coding RNAs (sncRNAs), such as microRNAs, and small nucleolar RNA, have diverse roles including regulation of gene expression. We profiled the sncRNAs of diseased osteoarthritic ACLs to provide novel insights into osteoarthritis development. Small RNA sequencing from the ACLs of non- or end-stage human osteoarthritic knee joints was performed. Significantly differentially expressed sncRNAs were defined and bioinformatic analysis was undertaken. A total of 184 sncRNAs were differentially expressed; 68 small nucleolar RNAs, 26 small nuclear RNAs and 90 microRNAs. We identified both novel and recognised (miR-206, -365 and -29b and -29c) osteoarthritis-related microRNAs and other sncRNAs (including SNORD72, SNORD113, SNORD114). Significant pathway enrichment of differentially expressed miRNAs, including differentiation of muscle, inflammation, proliferation of chondrocytes and fibrosis. Putative mRNAs of the microRNA target genes were associated with the canonical pathways ‘Hepatic Fibrosis Signalling’, and ‘Osteoarthritis’. The establishing sncRNA signatures of ACL disease during osteoarthritis could serve as novel biomarkers and potential therapeutic targets in ACL degeneration and osteoarthritis development.

# Introduction

Ligaments are resilient connective tissues essential for bone-to-bone connections within joints (1). The anterior cruciate ligament (ACL) is the most commonly damaged ligament (2) with an incidence of approximately 68.6 ACL ruptures per 100,000 people (3) resulting in considerable socialand economic costs (4, 5). In the USA alone, there are approximately 100,000-175,000 ACLs surgeries per year, with cost exceeding of 2 billion. dollars (6, 7). ACL injuries can also lead to significant functional impairment in athletes, muscle atrophy, weakness, joint instability, meniscal lesions, and are associated with development of osteoarthritis (OA) (8, 9). Greater than 50% of ACL injury patients eventually develop OA, with the degree and progression of disease being accelerated in these cases (10, 11). Moreover, reports demonstrate that there is an association between ACL degeneration and subsequent knee OA, suggesting the importance of ACL degradation in OA pathogenesis (12).

There is currently substantial interest in the area of epigenetic regulation in ageing, disease, and repair mechanisms in musculoskeletal tissues such as muscle (13, 14), cartilage(15), tendon (16, 17) and ligament (18, 19). Small non-coding RNAs (sncRNAs) are a class of epigenetic molecules which include microRNAs (miRNAs or miRs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs). Small noncoding RNAs are important regulators of gene expression and are in encoded in DNA and RNA, but are not translated into proteins. (20). Their aberrant expression profiles in musculoskeletal conditions such as ACL injury are expected to be associated with cellular dysfunction and disease development (21). We have previously identified changes in the sncRNA profiles in ageing and OA human (15) and equine cartilage (22, 23), ageing human and equine tendon (24, 25) and ageing and OA murine joints and serum (26). However, the pathogenesis and contribution of ACL degeneration to the development and acceleration of OA is currently unknown. Identifying sncRNAs associated with ACL degeneration and comprehending their role in OA could have an important impact on the understanding of OA pathogenesis and future management.

To date, there is little known about the sncRNA changes in human diseased ACL. We hypothesise that sncRNA expression is altered in ACLs derived from OA joints in comparison to healthy joint and that their identification may elucidate underlying mechanisms of ACL degeneration. In this study, we therefore undertook a non-biased approach; small RNA sequencing of ACLs from human OA knee joints and compared these to our control samples derived from human non-OA knee joints.

# Material and methods

## *Sample collection*

# ACLs from non-OA, healthy knee joints (control) *n*=4 (age (mean ± standard deviation); 47.3 ± 1.7years) were obtained from a commercial biobank (Articular Engineering) (<https://www.articular.com/>). Diseased OA ACLs were obtained from the knee joints of patients undergoing total knee arthroplasty for end-stage OA treatment *n*=4 (age (mean ± standard deviation); 74.8 ± 5.4 years). Control samples were scored according ICRS grading system (27). Samples were collected in RNA later and stored at -80°C until used. In addition to the above samples, RNA from non-OA ACLS *n* =4 (age (mean ± standard deviation); 51.2 ± 3.3 years) and diseased OA ACLs *n* = 4 (age (mean ± standard deviation); 77 ± 2.1 years) were used for validation.

## *RNA extraction*

# RNA was extracted from ACL tissues was pulverised into a powder with a dismembranator (Mikro-S, Sartorius, Melsungen, Germany) under liquid nitrogen. Total RNA was extracted using the miRNeasy kit (Qiagen, Manchester, UK) according to the manufacturer's instructions (15). The RNA samples were quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer (Agilent, Stockport, UK) using an RNA Pico chip (Agilent, Stockport, UK). 1000ng RNA per ACL sample was submitted for library preparation using NEBNext® Small RNA Library Prep Set for Illumina (New England Biosciences (NEB), Ipswich, USA) but with the addition of a Cap-Clip™ Acid Pyrophosphatase (Cell script, Madison, USA) step to remove any 5’ cap structures on some snoRNAs (26) and size selected using a range 120-300bp (including adapters). These steps enabled both miRNAs and snoRNAs to be identified using an unbiased approach. The pooled libraries were sequenced on an Illumina HiSeq4000 platform with version 1 chemistry to generate 2 x 150 bp paired-end reads.

# *2.3 Data processing*

Reads files in fastq format were generated from sequence data measured from 1 lane ofan Illumina HiSeq4000 through the steps which include basecalling and de-multiplexing of indexed reads using CASAVA version 1.8.2; adapter and quality trimming using Cutadapt version 1.2.1(28) and Sickle version 1.200. Small RNA expression values were obtained through aligning reads to Ensembl GRCH38.96 human genome reference sequences using Tophat (29) version 2.0.10 with option “–g 1”; counting aligned reads against gene features using HTseq-count version 0.6.1p1; filtering genes by biotype and keep the small RNA genes for further analysis.

For mature miRNA expression profiling, the human mature miRNA sequences from miRbase (30) were used as the reference. A Salmon (31) ) index was created from these sequences, and reads shorter than 28 nucleotides in length were aligned and quantified against this index using Salmon. The obtained pseudocounts were processed and used for differential expression analysis using the DESeq2 package performed in R environment. (32).

"The DESeq2 analysis initially involved estimating normalisation factors to account for variations in library size among the samples. Libraries were normalised using the median-of-ratios method implemented in DESeq2 (estimateSizeFactors function). Data variation across samples was modelled using negative binomial distributions. Subsequently, a generalised linear model was fitted to the data to capture the relationships between observed counts and OA status. From this model, log2 fold change values and p-values for the contrast were derived. The Benjamini-Hochberg (BH) method was employed to adjust p-values and control the false discovery rate.

Count per million (CPM) values were calculated by dividing each gene count by the total counts for that sample and subsequently multiplying by a million. Finally, small RNAs were deemed significantly differentially expressed if they met the criterion of an FDR-adjusted p-value of less than 5%(33) and a CPM of at least 10 to categorise a gene as significant.

Sequence data have been submitted to National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO); E-MTAB-5715. E-MTAB-9106.

***2.4 Pathway analysis of differentially expressed miRNAs and their predicted targets***

Potential biological associations of the DE miRNAs in OA ACLs were identified using Ingenuity Pathway Analysis (IPA), (IPA 2020, Qiagen Redwood City, USA) ‘Core Analysis’. Additionally, in order to identify putative miRNA targets, bioinformatic analysis was performed by uploading DE miRNA data into the MicroRNA Target Filter module within IPA. This analysis identifies experimentally validated miRNA-mRNA interactions from TarBase, miRecords, and the peer-reviewed biomedical literature, as well as predicted miRNA-mRNA interactions from TargetScan as well as creating biological networks describing functional associations. We applied a conservative filter at this point, using only experimentally validated and highly conserved predicted mRNA targets for each miRNA. Targets were then also filtered on the fibroblast cell type. Core analysis was then performed in IPA on the filtered mRNA target genes and their associated miRNAs. For each core analysis canonical pathways, novel networks, diseases and functions, and common upstream regulators were queried. IPA z-score (statistical measure of the match between expected relationship direction and observed gene expression) is computed to infer the activation state of a predicted biological function.

Additionally, TOPP Gene (34) was used for overrepresentation analysis of the mRNA targets from Target Filter using Fisher’s Exact test with FDR correction. This tests whether the input mRNAs associate significantly with specific pathways and generates a list of biological process gene ontology (GO) terms. Terms with FDR adjusted p < 0.05 were summarised using REViGO (35) with allowed similarity of 0.4 and visualised using Cytoscape (36).

***2.5 qRT-PCR validation***

Validation of a selected subset of small RNA sequencing results was undertaken in an independent cohort of human ACLs using real-time quantitative PCR (qRT-PCR). Total RNA was extracted as above. Small non-coding RNAs were chosen based on our current work, level of differential expression (p < 0.05 and logFC> 1.2) and following a literature review of differentially expressed genes (19, 37). PolyA cDNA was synthesized using 200 ng RNA and the miScript II RT Kit. A mastermix was prepared using the miScript SYBR Green PCR Kit (Qiagen, Crawley, UK) and the appropriate bespoke designed miScript Primer Assays (Qiagen, Crawley, UK) using 1 ng/μl cDNA according to the manufacturer's guidelines. Real-time PCR was undertaken using a LightCycler® 96 system (Roche). Assays for four genes – miR-99a, miR-30c, miR-222 and SNORA46- were selected as potential reference genes because their expression was unaltered in this study. Stability of this panel of genes was assessed by applying a gene stability tool, RefFinder (38). MiR-222 was identified by NormFinder as the most stable and relative expression levels were normalized to miR-222 and calculated using the 2^-DCT method (39).

***2.6 Statistical analysis***

The heatmap, volcano and principle component analysis (PCA) plots were made using MetaboAnalyst 3.5 (http://www.metaboanalyst.ca) which uses the R package of statistical computing software 30 (40).

3. Results

**3.1. Sample assessment**

The ages of the control group (non-OA, healthy knee joints) (age, mean ± standard deviation (48 ± 2.16)) and ACLs derived from OA joints (74.7 ± 5.42) were significantly different (Mann Whitney test, p<0.05), but age was not a determining factor to alter the outcome of this study (Supplementary Figure 1). Control samples had no clinical history of joint disease or osteoarthritis and were designated as control samples as they had an average International Cartilage Repair Society system (ICRS) score of 0 (27).

The summary of all donors’ information is provided in Supplementary Table 1.

**3.2. Small RNA sequencing data**

Quality metrics including the library depth and distribution of other small RNA have been provided in Supplementary Table 2, Supplementary Figures 2 and 3. We identified a total 590 miRNAs, 226 snoRNAs and 100 small nuclear (snRNAs) (with greater than 10 CPM in each sample).

There were 184 differentially expressed sncRNAs identified (false discovery rate (FDR<0.05)) and with at least 10 CPM in each sample. The categories of RNAs identified are in Figure 1A and included miRNAs, snoRNAs and snRNAs. Principle component analysis (PCA) revealed that the ACLs derived from non-OA joints (control) clustered together and could be clearly separated from the ACLs derived from OA knee joints (Figure 1B).

Of the 184 snRNAs there were 68 differentially expressed (DE) snoRNAs (64 reduced in OA and 4 increased in OA), 26 DE snRNAs (24 reduced in OA and 2 increased in OA) and 90 DE miRNAs (43 reduced in OA and 47 increased in OA) (FDR<0.05 and greater than 10 CPM in all samples) (Figure 1C, Supplementary Table 3). The most DE miRNAs are in Table 1, with snRNAs and snoRNAs in Table 2. We further generated a heatmap of the DE miRNAs (Figure 1D) and snRNAs and snoRNAs (Supplementary Figure 4).

**3.3. Pathway analysis of differentially expressed miRNAs**

To explore potential biological associations between the 90 DE miRNAs in ACLs derived from OA knee joints, we undertook an Ingenuity Pathway Analysis (IPA) ‘Core Analysis’. Network-eligible molecules were overlaid onto molecular networks based on information from Ingenuity Pathway Knowledge Database. Networks were then generated based on connectivity. Gene network inferred features were determined. Significant cellular functions deduced by the DE miRNAs included differentiation of muscle (P<0.001), inflammation (p<1.42E-10), proliferation of chondrocytes (p<0.03), fibrosis (p<0.001) and cell viability (p<0.03) (Figure 2A). The top scoring network identified was ‘Organismal Injury and Abnormalities’ (score 43) and included OA-related miRNAs such as miR-206, miR-101, let-7f, miR-455, miR-29b and miR-29c (Figure 2B).

**3.4 Pathway analysis of the differentially expressed miRNAs predicted target genes**

We undertook further pathway analysis to determine the mRNA targets of the DE miRNAs. 90 miRNAs that were DE in ACLs derived from OA knee joints compared to controls were initially input into MicroRNA Target Filter, resulting in 529 mRNAs as putative targets (Supplementary Table 4). These mRNAs were then input into IPA core analysis and all results were summarised in Supplementary Table 4. The top canonical pathways for target mRNAs of DE miRNAs in OA ACL are in Table 3. Two of the most significant of which were the osteoarthritis pathway (p<2.3E-23) and hepatic fibrosis (p<3.1E-32) (Figure 3). The most significant upstream regulators of these mRNAs included tumour necrosis factor (p<1.3E-101) and transforming growth factor β (TGFβ) (P<8.5E-83) (Table 4) and the top networks identified are in Supplementary Table 4. The network ‘cellular development, movement and genes expression’ (score 41) (Figure 4A) was overlaid with significant biological processes including apoptosis (p<1.7E-85), fibrosis (P<1.2E-79), inflammation (p<3.4E-88), and necrosis (p<7.2E-88). The network ‘inflammatory disease’ (score 35) (Figure 4B) shows pertinent significant biological processes including organisation of collagen fibrils (p<3.7E-07), fibrosis (p<2.6E-14), rheumatoid arthritis (p<3.6E-06), angiogenesis (p<8.9E-09), differentiation of bone (p<5E-06), inflammation of the joint (p<8.8E-07) and cartilage development (p<1.5E-07). To obtain an overview of pathways of putative target mRNAs, gene ontology (GO) tool TOPP Gene and the biological processes were summarised in REViGO and Cytoscape (Figure 5 and Supplementary Table 4).

**3.5 Validation of differential gene expression using qRT-PCR**

Taking into account the level of differential expression (P < 0.05 and logFC> 1.3) and following a literature search, we validated a subset of our DE miRs (Figure 6). Real-time quantitative PCR qRT-PCR analyses was undertaken using RNA from an independent cohort (n=6 for control and n=4 for OA ACL samples). In agreement with sequencing data miR-5100 and SNORD72 were higher expressed in OA ACL, whilst miR-206 and miR29c-3p had lower expression in OA ACL. For two miRs, miR-101 and let-7f qRT-PCR findings were not validated.

Discussion and conclusion

The global prevalence of knee OA is currently 5% and is projected to rise with an increase in the ageing population (41). It is proposed that there is an association between ACL degeneration and subsequent knee OA, suggesting the importance of ACL degradation in the mechanisms OA pathogenesis (42). One potential mechanism capable of regulating global alteration in a particular tissue is modification of sncRNA expression (43). To begin to elucidate the role that sncRNAs play in the global changes observed in the ACL during OA, and understand further the potential role of the ACL in OA, we undertook a non-biased approach; small RNA sequencing of ACLs from human OA knee joints and compared these to our control samples derived from human non-OA knee joints. In our previous study, we have identified changing miRNAs expression in ageing mice cruciate ligament, which has also been studied human osteoarthritic ACL (19, 44). However, this is the first time that, to our knowledge, small RNA sequencing has been used an unbiased manner to interrogate both snoRNAs and miRNAs, which can provide targets for future therapeutic approaches and novel markers for this ACL disease and OA dependent signatures.

There were 68 snoRNAs, 26 snRNAs and 90 miRNAs significantly different in ACLs derived from OA knee joints and the OA status of the donor accounted for the principal variability in the data. Additional bioinformatics was performed, to analyse the biological processes and pathways affected by the differentially expressed miRNAs and in addition, the putative mRNA targets of the differentially expressed miRNAs, enhancing our understanding of the roles of the dysregulated miRNAs in OA diseased ACL pathogenesis.

Several of the DE miRNAs found in this study, including miR-29b, miR -335, miR-424, and miR-941 were previously altered miRNAs in a study comparing ruptured ACLs to diseased OA ACLs (19). These miRNAs were found to be correlated with cartilage development and remodelling, extracellular matrix homeostasis and inflammatory response (19). We have found other miRNAs associated with OA including miR-206, miR-5100, and miR-29c, whose expression altered in ACLs derived from OA joints and correlated with the validated qPCR results in our current study (45-48).

Pathways identified by the DE miRNAs with known functions in OA in other tissues included inflammation (49), proliferation of chondrocytes (50), and fibrosis (51). Canonical pathways identified have roles in OA pathogenesis including senescence (52), fibrosis (51), TGFβ signaling (53), retinoic acid binding protein (RAR) activation (54) and peroxisome proliferator-activated receptor/retinoid X receptor (PPAR/RXR) activation (55, 56). These enriched top signaling pathways and canonical pathways give us further confidence that they may play an important role in the biological processes associated with ACL degeneration and OA development.

To address the roles of miRNAs in diseased OA ACLs, their mRNA target genes were also taken into consideration using IPA and gene ontology biological processes, which including extracellular matrix organisation, epigenetic regulation, cell signaling, cell growth and proliferation. In IPA, additional functions affected by these genes, known to have a role in OA pathogenesis and therefore with a potential role in OA ACLs were highlighted including apoptosis (57), fibrosis (51), inflammation of the joint (49), necrosis (58), organisation of collagen fibrils (12), angiogenesis (59), differentiation of bone (60) and cartilage development (61). Many canonical pathways enriched by the putative target genes were essential for OA pathogenesis, including the ‘osteoarthritis pathway’. Additionally downstream targets of these signaling pathways with known roles in OA pathogenesis were identified and included matrix metalloproteinase-3 (62), tissue inhibitor of metalloproteinase- 3 (63), and collagen X α1 (64). Hepatic fibrosis was the most significant canonical pathway identified from the putative mRNAs together with the DE miRNAs in our study. Synovial fibrosis is often found in OA (51) and fibrosis has previously been described in OA joints following ACL injury (65). Furthermore TGFβ, one of the most significant upstream regulator in our mRNA target gene analysis, is the master regulator of fibrosis (64). Many TGFβ-related genes including TGFβ2, TGFβ3, TGFβR1, TGFβR2 and TGFβR3 were predicted targets of the DE miRNAs including miR-98-5p, miR-128-5p, miR-136-3p, miR-17-5p; strongly implicating it in the fibrosis evident in the diseased ACLs in OA. These findings indicate the potential importance of these pathways in ACL degeneration associated with OA.

Another class of snRNA, snoRNAs, were altered in the OA ACLs in our study. This conserved class of non-coding RNAs are principally characterised as guiding site-specific post-transcriptional modifications in ribosomal RNA (66). Furthermore snoRNAs can modify and/or interact with additional classes of RNAs including other snoRNAs, transfer RNAs and mRNAs (67). A reliable modification site has been assigned to 83% of the canonical snoRNAs, with 76 snoRNAs described as orphan, meaning they act in an unknown or unique manner (68). Novel functions reported for snoRNAs include the modulation of alternative splicing (69), involvement in stress response pathways, (70) and the modulation of mRNA 3′end processing (71). Like miRNAs, snoRNAs are emerging as important regulators of cellular function and OA development (15, 20, 72, 73), in part due to their ability to fine-tune the ribosome to accommodate changing requirements for protein production during development, normal function and disease (74, 75) .

We have previously identified molecular mechanism for snoRNAs in cartilage ageing and OA (15) and their potential use as biomarkers for OA (26). Furthermore, other studies have identified that the snoRNAs, SNORD38 and SNORD48, are significantly elevated in the serum of patients developing cartilage damage a year following ACL injury and serum levels of SNORD38 were greatly elevated in patients who develop cartilage damage after ACL injury suggesting SNORD38 as a serum biomarker for early cartilage damage (76). In addition, we also found an upregulation of SNORD113 and SNORD114 in diseased OA ACLs. These snoRNAs are located in imprinted human loci and may play a role in the evolution and/or mechanism of epigenetic imprinting (68). They belong to the C/D box class of snoRNAs and most of the members of the box C/D family direct site-specific 2'-O-methylation of substrate RNAs. However, SNORD113 and SNORD114 differ from C/D box snoRNAs in their tissue specific expression profiles (including in fibroblasts, osteoblasts and chondrocytes (68) and the lack of complementarity to any RNA. As a result, they are not predicted to guide to 2'O-methylation but have novel, unknown roles (68). Additionally SNORD113-1 functions as a tumour suppressor in hepatic cell carcinoma by reducing cell growth and inactivating the phosphorylation of ERK1/2 and SMAD2/3 in MAPK/ERK and TGF-β pathways (77). We have previously identified that SNORD113-1 expression is also upregulated in OA human knee cartilage but downregulated in ageing human knee cartilage, whilst SNORD114 was upregulated in OA knee cartilage (78).

SNORD72 expression was upregulated in diseased OA ACLs in small RNA sequencing data set and validated with independent cohort using qRT-PCR. In hepatocellular carcinoma, the overexpression of SNORD72 was found to enhance cell proliferation, colony formation and invasion by stabilising inhibitor of differentiation (ID) genes which are a basic helix-loop-helix (bHLH) transcription factors (79). The ID family genes have been shown to play a role in cell proliferation and angiogenesis (80). The lack of a DNA binding domain results in inhibition of the binding of other transcription factors to DNA in a dominant negative fashion (81). The expression of some members of this family in rheumatoid arthritis synovium suggests they may have a role in human inflammatory disease (82). Whilst the downstream signaling of snoRNAs is principally unknown, snoRNAs regulate ribosome biogenesis (83). However a subclass of orphans do not have complimentary RNA sequences (84). Mao Chet et al., found that ribosome biogenesis was not affected following SNORD72 overexpression implying it exerts functionality in other ways (79). Therefore whilst some snoRNAs can regulate the expression of RNAs (85), others can reduce the gene stability (84) or directly activate or suppress enzymes (86). Together our snoRNA findings indicate that changes in ACL snoRNA expression could have important implications in knee OA through both canonical and non-canonical roles.

Our study has a number of limitations. Firstly, while we validated several differentially expressed sncRNAs from our small RNA sequencing data using the qPCR method, we observed a lack of agreement in several other sncRNAs that were also validated but differentially expressed through small RNA sequencing data, which may be due to differences in the unit of measurement between the two methods. The fold change should not be expected to be the same for two methods. This discrepancy may be attributed to the normalisation process in data analysis. Future studies will also consider the correlation between qPCR and sequencing results for the genes.

Secondly, our study was underpowered primarily due to the limited availability of human ACL tissue. Nevertheless, this is the first study of its kind to have used small RNA sequencing to interrogate both snoRNAs and miRNAs in an unbiased manner in healthy and OA diseased human ACLs. Our outcomes demonstrated good statistical power and confirmed the adequacy of the sample size. To ensure that the sample size was representative of the populations being sampled, additional samples were used to validate our results. Future studies in this field would benefit from analysing larger cohorts of normal and OA diseased human ACL samples, and our study will serve as a platform for sample size estimation for future RNA sequencing of human ACL tissue.

Thirdly, macroscopic grading of knee joint tissues was not performed due to limited images of diseased OA ACL samples, however images of control samples and according to ICRS scoring (27) demonstrated healthy knee joint cartilage with no signs of ACL degeneration.

There was also an imbalance between the sexes in the two groups, with most of the OA derived ACLs coming from males but all of the control group being sourced from females. In human tendon, we have previously demonstrated that males and females are transcriptionally different and gene expression in aged cells moves in opposite directions (25). Ligament degeneration has also been demonstrated to be influenced by lower concentrations of sex hormones in young female athletes (87). Finally, there were age discrepancies between the two groups and so we cannot discount an age effect on sncRNAs expression. Age and sex are non-modifiable systematic risk factors for ACL disease and the development of OA (88). However other contributing factors such as underlying disease, obesity and bone metabolism will also be taken into account in future studies. In addition, the future collection of ACLs from different ages and stages of OA will provide us with a time related insight into the ligament injury-related small non-coding RNA dysregulation in patients with OA.

In summary, ACL degeneration results in severe physical, social, economic consequences to the affected individual and leads to development of degenerative joint disease such as OA. our study revealed alterations in a number of classes of sncRNAs in ACL tissues derived from patients with knee OA compared to healthy ACLs from non-OA joints. Our functional bioinformatic analyses suggest that the dysregulated miRNAs may regulate cartilage development and remodeling, collagen biosynthesis and degradation, ECM homeostasis and pathology by interacting with their targets. Uniquely we also demonstrate that snoRNAs may also have a role in ACL degeneration. Collectively, our study provides novel insight into the ACL related sncRNA dysregulation in patients with OA that can be used a potential diagnostic markers and future therapeutic targets to treat ACL degeneration, facilitating prompt positive intervention in the associated development of OA.

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**Institutional Review Board Statement:** All experimental produces were conformed to Ethical Central University Research Ethics Committee, University of Liverpool. Ethical approval for the purchase human ACL tissue was granted by the Central University Research Ethics Committee C, University of Liverpool (RETH4721, 22/01/2019). Fully informed patient consent was given for the use of OA ACLs from patient undergoing total knee arthroplasty under the institutional ethical approval (Maastricht University Medical Centre approval IDs: MUMC 2017-0183).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Sequence data have been submitted to National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO); E-MTAB-9106.

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**Conflicts of Interest:** *The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

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**Table 1.** Differentially expressed miRs with the highest and lowest log2 fold-change when comparing control versus diseased OA anterior cruciate ligament

|  |  |  |
| --- | --- | --- |
| **miRNAs** | **Log2 fold-change** | **False discovery rate adjusted p-values** |
| *Upregulated miRs diseased OA ACLs* |  |  |
| hsa-miR-5100 | 3.75 | 6.2E-07 |
| hsa-miR-31-5p | 3.14 | 6.9E-15 |
| hsa-miR-129-5p | 2.42 | 4.0E-03 |
| hsa-miR-144-3p | 2.41 | 3.5E-04 |
| hsa-miR-486-5p | 2.33 | 3.2E-04 |
| hsa-miR-370-3p | 2.32 | 1.4E-06 |
| hsa-miR-543 | 2.20 | 6.3E-03 |
| hsa-miR-4521 | 2.19 | 5.1E-04 |
| hsa-miR-493-5p | 2.17 | 6.7E-04 |
| hsa-miR-411-3p | 2.16 | 3.9E-03 |
| *Downregulated miRs in diseased OA ACLs* |  |  |
| hsa-miR-206 | -6.13 | 1.9E-06 |
| hsa-miR-12136 | -4.35 | 3.3E-18 |
| hsa-miR-3182 | -3.20 | 3.8E-10 |
| hsa-miR-101-5p | -2.22 | 9.6E-03 |
| hsa-miR-338-3p | -2.08 | 1.5E-02 |
| hsa-miR-335-5p | -2.03 | 7.3E-03 |
| hsa-miR-190b-5p | -1.98 | 2.5E-03 |
| hsa-miR-29c-3p | -1.89 | 1.1E-02 |
| hsa-miR-103a-5p | -1.86 | 3.7E-06 |
| hsa-miR-30b-5p | -1.81 | 1.7E-02 |

**Table 2.** Small nucleolar RNAs (snoRNAs) and small nuclear RNA (sncRNA) identified as being differentially expressed between control and anterior cruciate ligaments derived from osteoarthritic joints

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Name | Family | Action | Target RNA and Site specific modification | Log2 fold-change | | False discovery rate adjusted p- values | | Up or down regulated | |
| SNORD114 | C/D BOX | Site-specific 2’-O-methylation | Unknown | 3.60 | | 4.7E-07 | | OA ACL | |
| SNORD113 | C/D BOX | Site-specific 2’-O-methylation | Unknown | 2.85 | | 9.8E-05 | | OA ACL | |
| RNU6 | Splicesome | Complex of snRNA and protein subunits that removes introns  from a transcribed pre-mRNA |  | 2.85 | | 4.8E-03 | | OA ACL | |
| SNORD72 | C/D BOX | Site-specific 2’-O-methylation | 28s rRNA  28S:U4590 | 1.83 | | 4.2E-02 | | OA ACL | |
| RNVU1-19 | Splicesome | Complex of snRNA and protein subunits that removes introns  from a transcribed pre-mRNA |  | 1.58 | | 4.8E-02 | | OA ACL | |
| RNU7-19P | Splicesome | Complex of snRNA and protein subunits that removes introns  from a transcribed pre-mRNA |  | -7.61 | | 4.0E-07 | | Control ACL | |
| RNU4-59P | Splicesome | Complex of snRNA and protein subunits that removes introns  from a transcribed pre-mRNA |  | -4.90 | | 1.4E-33 | | Control ACL | |
| SNORA36B | H/ACA box | H/ACA family of pseudouridylation guide snoRNAs | 18s rRNA  18S:U105 and U1244 | -4.25 | | 2.7E-06 | | Control ACL | |
| SNORA53 | H/ACA box | H/ACA family of pseudouridylation guide snoRNAs | Unknown | -3.68 | | 5.1E-15 | | Control ACL | |
| SNORA73B | H/ACA box | H/ACA family of pseudouridylation guide snoRNAs | Unknown |  | -3.61 | | 3.9E-05 | | Control ACL | |

**Table 3.** Top canonical pathways for target mRNAs of differentially expressed microRNAs in diseased osteoarthritic anterior cruciate ligaments

|  |  |  |
| --- | --- | --- |
| **Name** | **p-value** | **Overlap** |
| Hepatic Fibrosis Signalling Pathway | 1.62E-33 | 15.8 % |
| Hepatic Fibrosis/Hepatic Stellate Cell Activation | 3.06E-32 | 23.1 % |
| Cardiac Hypertrophy Signalling | 1.28E-28 | 12.3 % |
| Colorectal Cancer Metastasis Signalling | 1.97E-27 | 17.4 % |
| Role of Macrophages, Fibroblasts and Endothelial Cells | 2.32E-27 | 15.4 % |

**Table 4.** Top upstream regulators of differentially expressed s microRNAs in diseased osteoarthritic anterior cruciate ligaments

|  |  |
| --- | --- |
| **Name** | **p-value** |
| Tumour necrosis factor | 1.31E-101 |
| Hepatic Fibrosis/Hepatic Stellate Cell Activation | 8.50E-83 |
| Transforming growth factor B1 | 1.14E-81 |
| lipopolysaccharide | 1.45E-77 |
| tretinoin | 9.26E-77 |