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RESEARCH ARTICLE

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The miRNA-mRNA interactome of murine induced pluripotent stem cell-derived chondrocytes in response to inflammatory cytokines

Alison K. Ross[1,2,3,4] | **Rodrigo Coutinho de Almeida**[5] | **Yolande F. M. Ramos**[5] | **Jiehan Li**[1,2,3,4] | **Ingrid Meulenbelt**[5] | **Farshid Guilak**[1,2,3,4]

[1]Department of Orthopaedic Surgery, Washington University, St. Louis, MO, USA

[2]Department of Biomedical Engineering, Washington University, St. Louis, MO, USA

[3]Shriners Hospitals for Children, St. Louis, MO, USA

[4]Center of Regenerative Medicine, Washington University, St. Louis, MO, USA

[5]Department of Biomedical Data Sciences, Section Molecular Epidemiology, Leiden University Medical Center, Leiden, the Netherlands

Correspondence
Farshid Guilak, Department of Orthopaedic Surgery, Washington University, Couch Biomedical Research Bldg., Room 3121, Campus Box 8233, Saint Louis, MO 63110, USA.
Email: guilak@wustl.edu

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Abstract

Osteoarthritis (OA) is a degenerative joint disease, and inflammation within an arthritic joint plays a critical role in disease progression. Pro-inflammatory cytokines, specifically IL-1 and TNF-α, induce aberrant expression of catabolic and degradative enzymes and inflammatory cytokines in OA and result in a challenging environment for cartilage repair and regeneration. MicroRNAs (miRNAS) are small noncoding RNAs and are important regulatory molecules that act by binding to target messenger RNAs (mRNAs) to reduce protein synthesis and have been implicated in many diseases, including OA. The goal of this study was to understand the mechanisms of miRNA regulation of the transcriptome of tissue-engineered cartilage in response to IL-1β and TNF-α using an in vitro murine induced pluripotent stem cell (miPSC) model system. We performed miRNA and mRNA sequencing to determine the temporal and dynamic responses of genes to specific inflammatory cytokines as well as miRNAs that are differentially expressed (DE) in response to both cytokines or exclusively to IL-1β or TNF-α. Through integration of mRNA and miRNA sequencing data, we created networks of miRNA-mRNA interactions which may be controlling the response to inflammatory cytokines. Within the networks, hub miRNAs, miR-29b-3p, miR-17-5p, and miR-20a-5p, were identified. As validation of these findings, we found that delivery of miR-17-5p and miR-20a-5p mimics significantly decreased degradative enzyme activity levels while also decreasing expression of inflammation-related genes in cytokine-treated cells. This study utilized an integrative approach to determine the miRNA interactome controlling the response to inflammatory cytokines and novel mediators of inflammation-driven degradation in tissue-engineered cartilage.

KEYWORDS
induced pluripotent stem cell, inflammation, microRNA, RNA-sequencing, tissue engineering

Abbreviations: DE, differentially expressed; IL-1, interleukin-1; iPSC, induced pluripotent stem cell; miPSC, murine induced pluripotent stem cell; miRNA, miR:microRNA; MMP, matrix metalloproteinase; mRNA, messenger RNA; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OA, osteoarthritis; PDiPSCs, pre-differentiated iPSCs; RNA, ribonucleic acid; RNA-seq, RNA-sequencing; sGAG, sulfated glycosaminoglycan; TNF-α, tumor necrosis factor-alpha.

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1 | INTRODUCTION

Osteoarthritis (OA) is a debilitating joint disease that affects over 32 million adults in the United States alone.[1] OA is characterized not only by the degeneration of articular cartilage, but also by subchondral bone thickening, osteophyte formation, and modification of synovial fluid composition, which leads to joint pain, inflammation, and stiffness.[2] These processes are mediated in part by pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). These cytokines are elevated in the synovial fluid, synovial membrane, subchondral bone, and cartilage of patients with OA, and act by both suppressing synthesis of cartilage matrix components and inducing expression of catabolic and degradative enzymes and inflammatory cytokines.[3-5] The presence of inflammation within an OA joint provides a challenging environment for articular cartilage repair, as pro-inflammatory cytokines will cause degeneration of native and tissue-engineered cartilage, as well as prevent chondrogenesis of stem cells.[6,7]

Thus, a number of approaches have targeted the IL-1 or TNF-α pathways as a therapy for OA[8-11] or as a means of inhibiting inflammatory effects on tissue-engineered cartilage.[12-14] Although these studies show promise for targeted anti-inflammatory therapies, clinical studies have not shown consistent and clear effects on OA.[15] Furthermore, although IL-1 and TNF-α use many shared signaling pathways and mediators, it has been shown that they have distinct roles in OA. IL-1 plays a role in sustained cell infiltration and cartilage destruction, while TNF-α is important for early joint swelling and driving the inflammatory cascade.[3,16] Because there are no effective disease modifying treatments available that address both the symptoms and structural changes of OA,[17,18] a further understanding of the molecular mechanisms regulating cartilage response to inflammation is needed for the development of alternative therapeutic approaches.

In this regard, multiple studies suggest that interaction between microRNAs (miRNAs) and messenger RNAs (mRNAs) may play a critical role in the regulation of chondrocyte biology [reviewed by Swingler et al[19] and by Asahara[20]]. miRNAs are small, endogenous, noncoding RNAs that play an important role in the regulation of many biological processes, such as proliferation, differentiation, and diseases including OA. They act by binding to target mRNAs which results in suppressed protein synthesis by translational inhibition, or, primarily, mRNA degradation.[21-23] miRNAs have unique expression profiles in different tissues, cell types, developmental stages, and diseases states,[24] and are attractive therapeutic targets because of their ability to regulate multiple genes and target pathways in complex diseases. miRNAs have been shown to participate in chondrogenesis, chondrocyte homeostasis, and OA.[25-28] A number of experiments have shown differentially expressed (DE) miRNAs from OA affected tissues compared to normal cartilage,[29-31] and some have explored the effects of IL-1β treatment on miRNA changes. However, these studies have not determined the specific roles of miRNAs in regulating chondrocyte response to IL-1 or TNF-α while controlling for factors such as stage of disease, body mass index, or location of tissue harvest.[26] The deregulation of miRNAs is an important factor in the pathogenesis of OA and understanding their specific roles and regulatory networks in response to inflammatory cytokines is important to guide miRNA-based therapies.

In a recent study, an integrative analysis of miRNA and mRNA sequencing data comparing preserved (eg, undamaged) and lesioned human osteoarthritic cartilage revealed the miRNA interactome that potentially regulates the chondrocyte transcriptome in healthy or pathologic cartilage.[32] Integration of RNA-sequencing (RNA-seq) data allows for an unbiased and comprehensive study of the miRNA-mRNA interactions, which could reveal previously unknown pathways or mediators in complex disease processes. Application of this technique to chondrogenically differentiated stem cells and tissue-engineered cartilage would provide a better understanding of how tissues designed to replace damaged cartilage are going to respond within a diseased joint. Furthermore, induced pluripotent stem cells (iPSCs) provide a source of large numbers of genetically defined cells that are capable of chondrogenic differentiation for cartilage repair or in vitro OA disease modeling.[33-35]

The goal of this study was to determine the mechanisms of miRNA regulation of tissue-engineered cartilage in response to inflammatory cytokines using an in vitro murine induced pluripotent stem cell (miPSC) model system previously developed in our lab.[33,34] This three-dimensional culture system also allows for the study of precise experimental factors while maintaining miPSC-derived chondrocytes in a rounded shape within the cartilaginous extracellular matrix, which is required to maintain their phenotype and as well as the cell-matrix interactions that can significantly influence the response to exogenous stimuli.[33,34,36] Furthermore, this model system allows for the control of many parameters that have confounded other miRNA-based studies,[37] leading to inconsistencies among different studies on key miRNAs and mechanisms of action. We performed an integrative analysis of miRNA and mRNA sequencing data to functionally characterize the miRNA regulatory networks driving IL-1β and TNF-α mediated responses of tissue-engineered cartilage (Figure 1A). We identify key miRNA mediators driving novel mechanisms in the inflammatory response of articular chondrocytes and explore their ability to attenuate inflammation in a miPSC model system of OA. This study provides a thorough description of the miRNA-mRNA network following cytokine exposure, which can serve as the basis for designing new therapies to prevent inflammation-driven degradation. Furthermore, these findings can be applied to tissue

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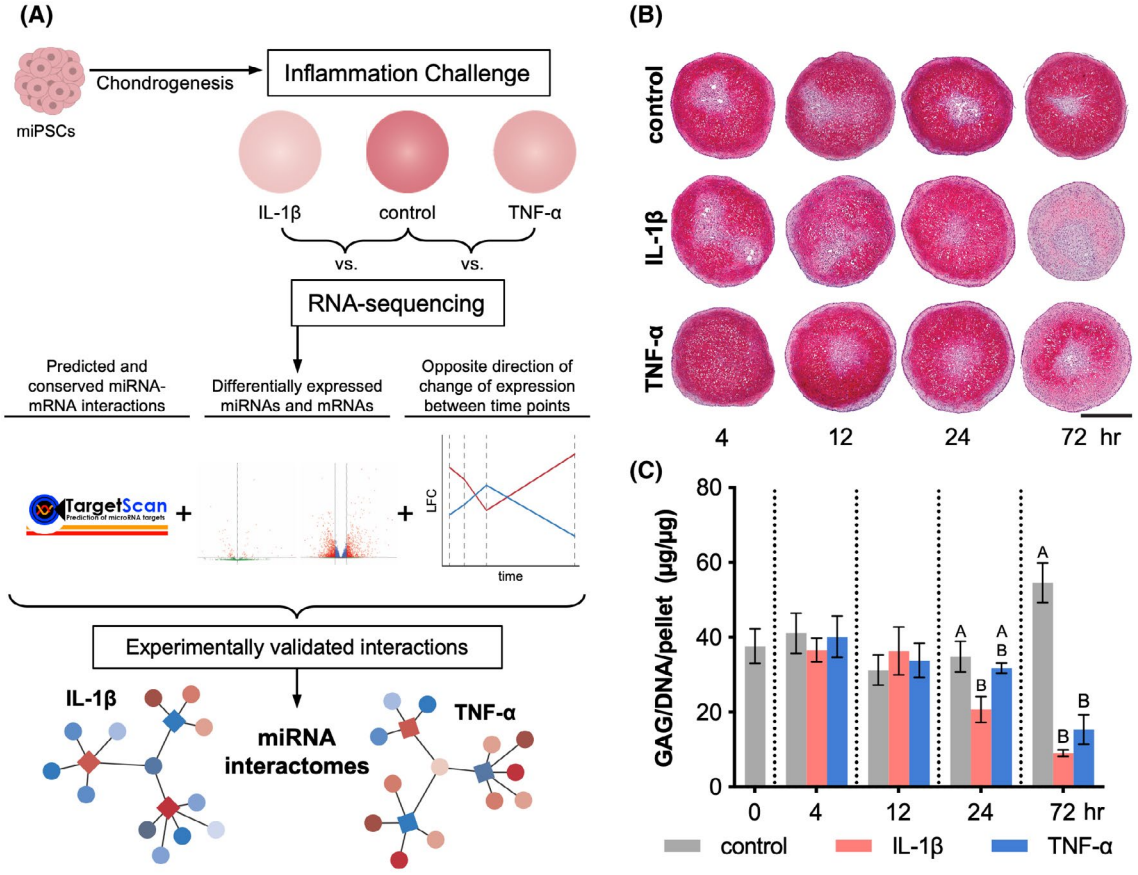

FIGURE 1 Overview of experimental and analytical approach to evaluate tissue level responses of miPSC-derived cartilage to inflammation. A, miPSCs were chondrogenically differentiated, and then, exposed to inflammatory cytokines, IL-1β or TNF-α, for 4, 12, 24, and 72 hours, followed by RNA-sequencing. Predicted and conserved miRNA-mRNA interactions in which the miRNA and mRNA were differentially expressed and had opposite direction of change of expression level from one time point to the next were selected. MiRNA-mRNA pairs were further filtered for those that have been experimentally validated, and miRNA interactomes were constructed. At each time point. B, pellet sections were stained for safranin-O/hematoxylin/fast green (scale bar = 500 μm) and (C) sGAG content was measured, normalized to DNA content per pellet (mean ± SEM; n = 3). At each time point, differences among groups with different letters are statistically significant ($P < .05$)

engineering strategies for the treatment of OA, providing a better understanding of the response of this tissue to the inflammatory environment of an OA joint, such that therapies can be designed to improve replacement of damaged tissues and prevent disease progression.

2 | MATERIALS AND METHODS

2.1 | Cell culture and differentiation

Murine iPSCs (miPSCs), derived from mouse primary fibroblasts from adult C57BL/6 mice using a non-integrating system, were purchased from the Gates Center for Regenerative medicine (University of Colorado, Denver). iPSCs were cultured in KnockOut Dulbecco's Modified Eagles Medium (DMEM) (Gibco), 20% lot-selected FBS (Atlanta Biologicals), 100 nM MEM nonessential amino acids (NEAA, Gibco), 55 μM 2-mercaptoethanol (2-me, Gibco), 2 mM Glutamax (Gibco), 1% penicillin/streptomycin (P/S, Gibco), and maintained on mitomycin C-treated mouse embryonic fibroblasts (Millipore).

miPSCs were differentiated toward a mesenchymal state using a high-density micromass culture.[33] Differentiation medium contained DMEM-high glucose (DMEM-HG, Gibco), 1% culture medium supplement containing recombinant human insulin, human transferrin, and sodium selenite (ITS+, BD), 100 nM MEM NEAA, 55 μM 2-me, 24 ng/mL gentamicin, 50 μg/mL L-ascorbic acid, and 40 μg/mL L-proline. On days 3-5, this medium was supplemented with 100 nM of dexamethasone and 50 ng/mL of bone morphogenetic protein 4 (BMP-4, R&D Systems). After 15 days of culture, the micromasses were dissociated with pronase (Millipore Sigma) and collagenase type II (Worthington Biochemical) and the pre-differentiated iPSCs (PDiPSCs) were plated on gelatin-coated dishes in expansion medium containing DMEM-HG, 10% lot-selected FBS, 1% ITS+, MEM NEAA, 55 μM 2-me, 1% P/S, 50 μg/mL L-ascorbic acid,

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40 μg/mL L-proline, and 4 ng/mL of basic fibroblast growth factor (bFGF, R&D Systems). Passage 2 PDiPSCs were pelleted by centrifugation of 250-300 k cells and cultured for 21 days in chondrogenic medium consisting of DMEM-HG, 1% ITS+, 100 nM MEM NEAA, 55 μM 2-me, 1% P/S, 50 μg/mL L-ascorbic acid, 40 μg/mL L-proline, 100 nM dexamethasone, and 10 ng/mL TGF-β3 (R&D Systems).

2.2 | Inflammatory challenge

After 21 days of chondrogenic culture, pellets underwent inflammatory challenge for 72 hours, which involved culture in chondrogenic media with 1 ng/mL IL-1β, 20 ng/mL TNF-α, or control media without cytokines. TGF-β and dexamethasone were removed during this period. To capture the short- and long-term responses of cells to inflammation, pellets were collected after 4, 12, 24, and 72 hours, rinsed in PBS, and stored at −80ºC for RNA sequencing or at −20ºC for biochemical analysis.

2.3 | RNA-sequencing

To extract RNA from tissue-engineered cartilage, frozen pellets were homogenized with a miniature bead beater. Total cellular RNA was isolated using the mirVana miRNA Isolation Kit (Invitrogen) for small RNA sequencing or Total RNA Purification Plus Kit (Norgen) for mRNA sequencing. Three pellets were pooled per sample and each sample was sequenced on an Illumina HiSeq3000 (n = 3). RNA-seq reads were trimmed (Trimmomatic,[38] Cutadapt[39]), aligned (STAR aligner[40] to map small RNA-seq reads to a custom murine miRNA database based on miRBase v22[41] and RNA-seq reads to mm10 from Gencode vM15), counted (featureCounts[42]), and only protein coding genes were included in downstream mRNA sequencing analysis.

2.4 | Differential expression analysis and miRNA-mRNA network construction

The R package, DESeq2,[43] was used to determine DE miRNAs and genes through pairwise comparisons at each time point. Networks of miRNA-mRNA interactions were constructed through the following selection criteria: 1. conserved and predicted miRNA-mRNA target pairs (TargetScan Mouse release 7.2[44]); 2. differential expression (DE) at any time point (miRNA: $P_{adj} < .05$; mRNA: $P_{adj} < .05$, $|\log_2(\text{FoldChange})\ (\text{LFC})| > 1$; P_{adj} is the false discovery rate computed by the Benjamini-Hochberg multiple testing correction); 3. opposite direction of change of expression level between time points for miRNA and mRNA based on the assumption that miRNA targeting of an mRNA will lead to mRNA degradation[22,23,32]; 4. miRNA-mRNA interaction is experimentally validated (miRTarBase v7.0[45] and TarBase v7[46] databases). K-means clustering was applied to the normalized counts (RPKM, reads per kilobase per million mapped reads) of DE mRNAs at any time point in RStudio. The number of clusters, 5, was determined by the sum of squared error method. Enrichr[47,48] was used for KEGG pathway analysis. Interactive networks were visualized on a web application with the R package, Shiny[49] (https://guilaklab.shinyapps.io/validatednetwork/).

2.5 | In vitro experiments

Day 21 pellets were digested with collagenase type II and chondrocyte-like cells were plated in monolayer in media containing DMEM-HG, 10% FBS, 1% ITS+, 100 nM MEM NEAA, 55 μM 2-me, 1% P/S. After 4-5 days, cells were transfected with mirVana miRNA mimics and antagomirs (Invitrogen): controls [mimic (M), mimic Negative Control #1; inhibitor (I), inhibitor Negative Control #1]; miR-29b-5p [M, MC10103; I, MH10103]; miR-17-5p [M, MC12412; I, MH12412]; miR-20a-5p [M, MC10057; I, MH10057]. Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) was used according to manufacturer's protocol and FBS and P/S were removed from media prior to transfection. Twenty-four hours after transfection, cells were subject to an inflammatory challenge of 0.5 ng/mL IL-1β, 20 ng/mL TNF-α, or control media without cytokines. Cell culture media was collected and stored at −20°C and cells were lysed with buffer RL and stored at −80°C for analysis at 24 and 48 hours.

2.6 | Histological and biochemical analysis of pellet cultures

After 72 hours of inflammatory challenge, pellets were washed with PBS and fixed in 10% of neutral buffered formalin for 24 hours, paraffin embedded, and sectioned at 8 μm thickness. Slides were stained for Safranin-O/hematoxylin/fast green.[50]

Pellets were digested overnight in 125 μg/mL papain at 65°C for biochemical analysis. DNA content was measured with PicoGreen assay (Thermo Fisher) and total sulfated glycosaminoglycan (sGAG) content was measured using a 1,9-dimethylmethylene blue assay at 525 nm wavelength.[51]

2.7 | Gene expression validation with quantitative real time PCR

For mRNA gene expression analysis, isolated, total cellular RNA was reverse transcribed using Superscript VILO

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cDNA master mix (Invitrogen). Quantitative, reverse transcription polymerase chain reaction (qRT-PCR) was performed using Fast SYBR master mix (Applied Biosystems) following the manufacturer's protocol. Primer pairs were synthesized by Integrated DNA Technologies, Inc: *Ccl2* [forward (F), 5′-GGCTCAGCCAGATGCAGTTAA-3′; reverse (R), 5′-CCTACTCATTGGGATCATCTTGCT-3′[; *Mmp13* [F, 5′-GGGCTCTGAATGGTTATGACATTC-3′; R, 5′-AGCGCTCAGTCTCTTCACCTCTT-3′]; *r18s* [F, 5′-CGGCTACCACATCCAAGGAA-3′; R, 5′-GGGCCT CGAAAGAGTCCTGT-3′]. Data are reported as fold changes and were calculated using the $\Delta\Delta C_T$ method and are shown relative to the non-transfected control media group at each time point and ribosomal 18s is used as the reference gene.

2.8 | MMP activity assay

Matrix metalloproteinase (MMP) activity in culture media was measured by detecting the quenching of a fluorogenic substrate Dab-Gly-Pro-Leu-Gly-Met-Arg-Gly-Lys-Flu (Sigma-Aldrich).[52] Latent MMP's were activated using p-aminophenylmercuric acetate, and total MMP activity was measured as the difference between a broad-spectrum MMP inhibitor, GM6001 (EMD Biosciences Inc) and a scrambled negative control peptide (EMD Biosciences Inc). Fluorescence was measured at 485 nm excitation and 535 nm emission and is reported as relative fluorescence units (RFU).

2.9 | Statistical analysis

Statistical analysis was performed with the JMP Pro software package. A two-way ANOVA with Tukey's HSD post hoc test was used to analyze all biochemistry, qRT-PCR, and MMP activity assay data ($\alpha = 0.05$).

3 | RESULTS

3.1 | Histological and biochemical response of tissue-engineered cartilage to inflammation

After 21 days of culture in chondrogenic media, the resulting cartilaginous pellets showed a spherical shape, and pellets in control media without cytokines exhibited robust safranin-O staining (Figure 1B). However, when treated with inflammatory cytokines, either 1 ng/mL of IL-1β or 20 ng/mL of TNF-α, pellets exhibited reduced safranin-O staining by 72 hours, indicating a decrease in the primary cartilage extracellular matrix component, aggrecan (Figure 1B). Biochemical analysis by DMMB assay, normalized to dsDNA content, showed a corresponding decrease in sGAGs at 24 and 72 hours in groups receiving IL-1β or TNF-α, as compared to the pellets in control media (Figure 1C).

3.2 | Differentially expressed miRNAs and genes in response to inflammatory cytokines

Day 21 pellets were subject to IL-1β and TNF-α inflammatory challenge and collected for RNA-seq at 4, 12, 24, and 72 hours. The IL-1β or TNF-α stimulated pellets were compared pairwise to control at all time points in order to determine DE miRNAs and genes (mRNAs) at each time point. Over time, there were increasing numbers of DE miRNAs and genes. From 4 to 72 hours following treatment, the number of significantly DE miRNAs increased from 4 (4 upregulated, 0 downregulated) to 86 (37 upregulated, 49 downregulated) in IL-1β-treated pellets and 4 (4 upregulated, 0 downregulated) to 168 (100 upregulated, 68 downregulated) in TNF-α-treated pellets (Figure 2A). Similarly, the number of DE mRNAs from 4 to 72 hours increased from 772 (481 upregulated, 291 downregulated) to 1781 (849 upregulated, 932 downregulated) in IL-1β-treated pellets and 612 (516 upregulated, 96 downregulated) to 1147 (635 upregulated, 512 downregulated) in TNF-α-treated pellets.

While IL-1β and TNF-α groups shared the same DE miRNAs at 4 hours, over time there was an increasing number of both shared miRNAs as well as miRNAs that were uniquely up- or downregulated with each cytokine. However, with respect to the mRNA transcriptome, we identified many DE genes that were specific to each cytokine at early time points. At later times, there were increasing numbers of shared and unique DE genes in response to either IL-1β or TNF-α (Figure 2B). There were 348 shared, 424 IL-1β specific, and 264 TNF-α-specific DE genes at 4 hours, which increased to 560 shared, 1221 IL-1β specific, and 587 TNF-α-specific DE genes at 72 hours.

Analysis of the DE miRNAs over time showed that most of the miRNAs that are expressed at early time points (4 and 12 hours) are also expressed at later time points (24 and 72 hours) for both IL-1β and TNF-α-treated groups (Figure 2C). On the contrary, there were more DE genes unique to each time point in addition to the genes that were DE at multiple time points (Figure 2C). Cluster analysis revealed five unique expression profiles of genes over time for both IL-1β and TNF-α pellets (Figure 2D), and KEGG pathway analysis shows that each of these clusters are enriched for different pathways (Tables S1 and S2). These results show that there are unique miRNA and mRNA profiles with some overlapping genes and miRNAs, and others that are dependent on IL-1β or TNF-α.

Differential expression analysis reveals miRNAs that are DE compared to controls at each time point (Figure S1).

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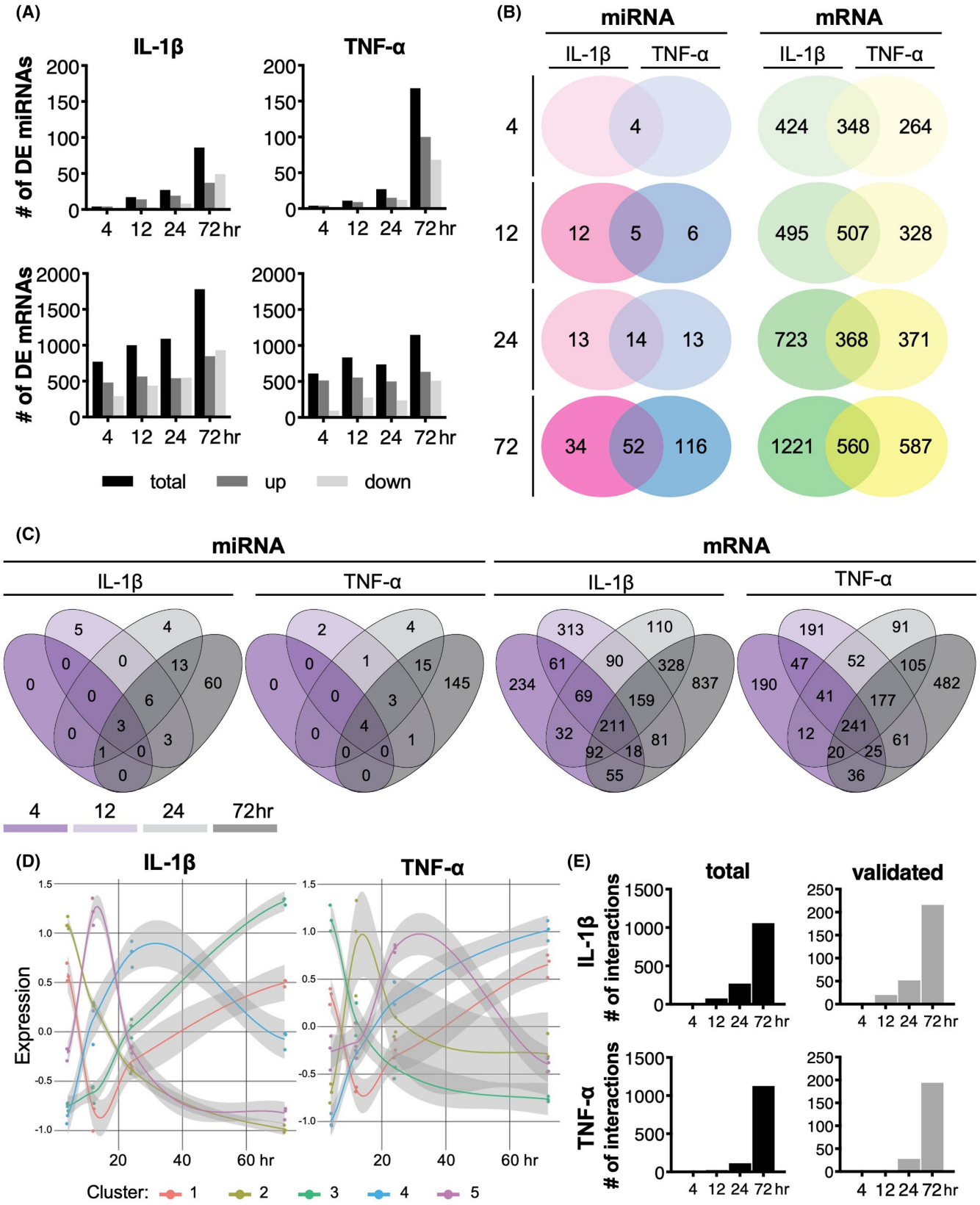

FIGURE 2 Differential expression (DE) analysis of miRNAs and mRNAs. A, The number of up and downregulated miRNAs and mRNAs in response to IL-1β or TNF-α over time. Distribution of shared or unique miRNAs or mRNAs (B) with each inflammatory cytokine over time or (C) at each time point. D, Expression patterns of clusters of differentially expressed miRNAs. Points on graph represent the scaled reads per kilobase per million mapped reads (RPKM) values for the centers of each sample in the cluster (n = 3). E, The number of total and validated miRNA-mRNA interactions for IL-1β and TNF-α following RNA- and small RNA-sequencing data integration

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At 4 hours, miR-222-5p (IL-1β, TNF-α: LFC (P_{adj}), 1.27 (1.6×10^{-18}), 1.57 (8.3×10^{-30})) and −3p (IL-1β, TNF-α: LFC (P_{adj}), 0.84 (1.5×10^{-12}), 0.72 (6.2×10^{-9})), miR-92a-1-5p (IL-1β, TNF-α: LFC (P_{adj}), 1.43 (1.2×10^{-7}), 1.02 (2.5×10^{-3})), and miR-155-5p (IL-1β, TNF-α: LFC (P_{adj}), 0.52 (2.3×10^{-3}), 0.67 (2.2×10^{-6})) were upregulated in response to both IL-1β and TNF-α. At 72 hours, among the most highly upregulated miRNAs in both IL-1β and TNF-α-treated pellets were miR-146a-5p (IL-1β, TNF-α: LFC (P_{adj}), 3.05 (2.0×10^{-15}), 4.10 (3.0×10^{-28})), miR-7a-5p (IL-1β, TNF-α: LFC (P_{adj}), 3.79 (7.6×10^{-3}), 4.86 (1.1×10^{-4})), and miR-155-5p (IL-1β, TNF-α: LFC (P_{adj}), 1.57 (1.4×10^{-40}), 1.43 (1.6×10^{-33})); those most highly downregulated with both cytokines include: miR-341-5p (IL-1β, TNF-α: LFC (P_{adj}), −2.53 (4.0×10^{-27}), −1.85 (1.7×10^{-16})), miR-410-5p (IL-1β, TNF-α: LFC (P_{adj}), −1.81 (1.1×10^{-2}), −1.82 (1.0×10^{-2})), and miR-665-3p (IL-1β, TNF-α: LFC (P_{adj}), −1.61 (8.0×10^{-11}), −1.11 (1.5×10^{-5})). Several miRNAs were found to be regulated exclusively by IL-1β, such as miR-138-1-3p and miR-154-3p (Figure S3A), or by TNF-α, such as miR-7678-3p, miR-6972-3p, miR-147-3p, miR-210-5p, and miR-421-5p (Figure S3B). Similarly, DE genes were visualized with volcano plots (Figure S2), and KEGG pathway analysis was conducted on significant DE mRNAs at each time point to determine enriched pathways over time for both IL-1β (Table S3) and TNF-α (Table S4) groups. The DE mRNAs in response to both cytokines were significantly enriched for genes involved in *TNF signaling pathway*, *cytokine-cytokine receptor interaction*, and *IL-17 signaling pathways* and characterized by genes such as *Ptgs2*, *Nfkb1* and *2*, MMP's, and chemokine CC and CXC subfamily genes. Additional pathways of interest that were involved at various time points were *rheumatoid arthritis*, *protein digestion and absorption*, and *PI3K-Akt signaling* pathways, characterized by genes such as *Tgfb3*, MMP's, *Ccl2*, and *Col2a1* and other collagens.

3.3 | miRNA interactomes in response to inflammatory cytokines

To determine the functional roles that miRNAs have on predicted target genes, RNA and small RNA-seq data sets were integrated such that networks could be constructed to visualize relationships between miRNA-mRNA predicted target pairs that are conserved (TargetScan database), contain a miRNA and mRNA that are each DE, and whose levels of expression are changing in opposite directions from one time point to the next (Figure S4; Figures 3 and 4). The total number of interactions increased over time in both the IL-1β and TNF-α groups from 15 to 1059 and 1 to 1128, respectively. There were 550 genes and 49 miRNAs in the IL-1β network and 445 genes and 78 miRNAs in the TNF-α network at 72 hours. Upon further filtering for only interactions that have been previously experimentally validated from the miRTarBase or TarBase databases, the number of interactions within networks reduced to 216 (153 genes and 28 miRNAs) in the IL-1β and 194 (128 genes and 24 miRNAs) in the TNF-α network at 72 hours (Figure 2E).

To determine which miRNAs within the networks regulate the inflammatory responses, KEGG pathway analysis was conducted on the genes within the networks including all interactions (Tables 1 and 2). Genes in inflammation and OA-related pathways (*PI3K-AKt signaling pathway, ECM-receptor interaction, cytokine-cytokine receptor interaction, MAPK signaling, protein digestion and absorption, TNF signaling pathway JAK-STAT signaling pathway*, and *IL-17 signaling pathway*; Tables 1-3) were overlaid onto the network and quantified the number of connections from each miRNA. Hub miRNAs, miR-17-5p and miR-20a-5p and miR-29b-3p, were identified in both IL-1β and TNF-α networks and are potentially controlling the miRNA response to these cytokines. miR-17-5p was connected to 52 genes (29 validated interactions) in the IL-1β network and 36 genes (21 validated interactions) in the TNF-α network at 72 hours. miR-20a-5p was connected to 52 genes (23 validated interactions) in the IL-1β network and 36 genes (17 validated interactions) in the TNF-α network at 72 hours. miR-29b-3p was connected to 79 genes (30 validated interactions) in the IL-1β network and 54 genes (23 validated interactions) in the TNF-α network at 72 hours. Inflammation and cartilage-related interactions with these hub miRNAs are listed in Table 3.

3.4 | In vitro validation of miRNA-mRNA interactome targets

To probe specific miRNA-mRNA interactions and to determine the therapeutic potential of hub miRNAs within the networks, miRNA mimics or inhibitors were delivered to monolayer chondrocyte-like cells prior to challenge with inflammatory cytokines. The miR-29b-3p hub had the most connections to inflammation and cartilage/OA-related genes in both the IL-1β and TNF-α networks, and RNA-seq analysis shows that miR-29b-3p expression increased over time (Figure 5A). miR-29b-3p mimics and inhibitors alone had no significant effect on the overall inflammatory status of the cells in response to either cytokine. Expression levels of *Ccl2* and *Mmp13*, markers of inflammation and matrix degradation in OA, were not significantly decreased with miR-29b-3p mimic or inhibitor delivery 24 hours after inflammatory cytokines were given to cells, as compared to their respective controls (Figure 5B). Furthermore, miR-29b-3p mimics and had no effect on the MMP activity levels at 24 and 48 hours in TNF-α groups. Likewise, there were no significant differences in MMP activity levels after 24 hours in IL-1β exposed cells; however, at 48 hours, miR-29b inhibitor resulted

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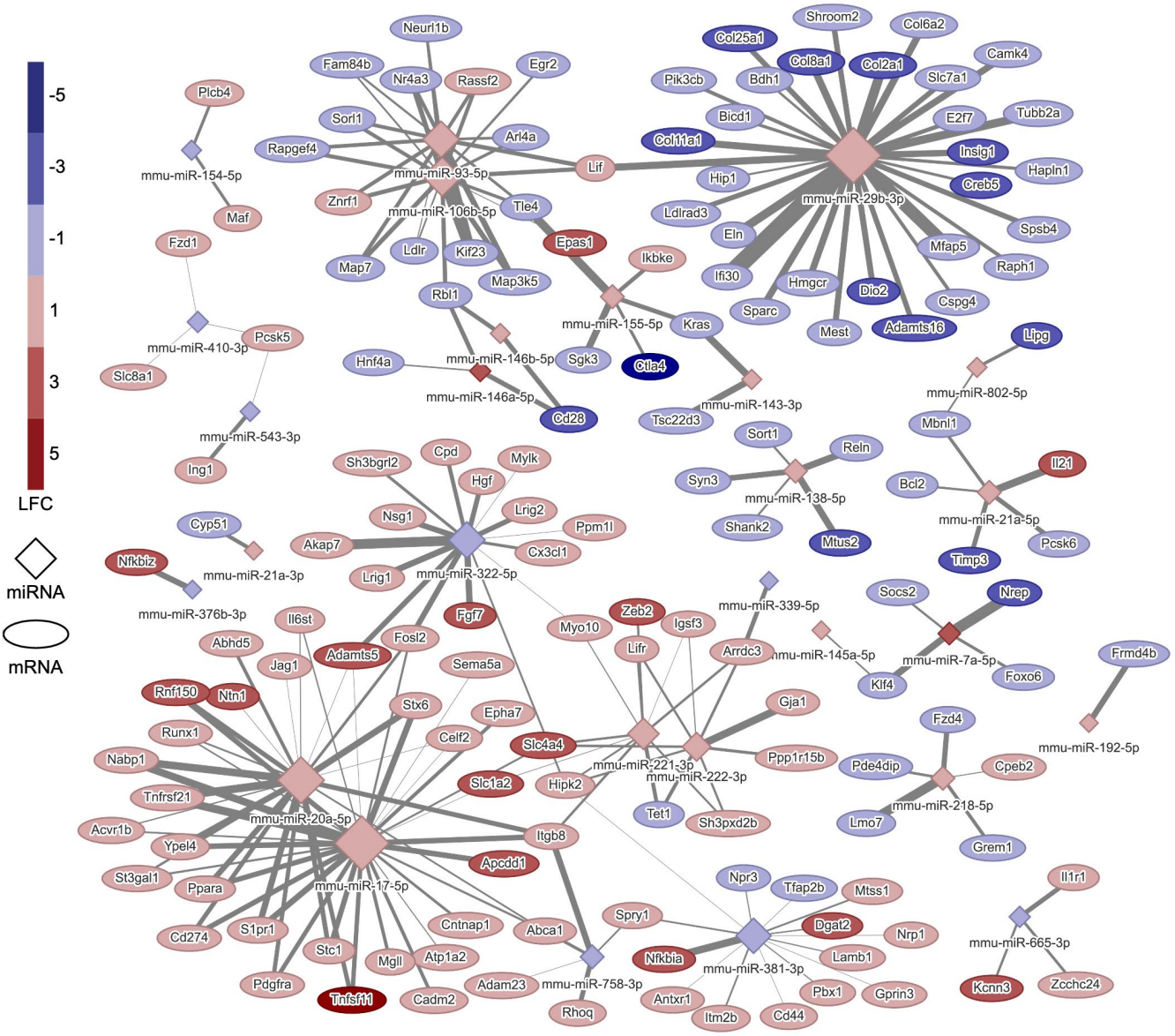

FIGURE 3 IL-1β miRNA-mRNA interactome at 72 hours: network of conserved and predicted miRNA-mRNA target pairs that are differentially expressed, are changing expression levels in opposite directions from 24 to 72 hours, and have been experimentally validated in response to 1 ng/mL IL-1β. The color of the node indicates the LFC of the gene or miRNA, and the size of the node is proportional to the number of connections. The thickness of the line corresponds to the context++ score from the TargetScan database. LFC, $\log_2$(FoldChange)

in significantly lower MMP activity levels as compared to the inhibitor control, but this effect was not significant when compared to the non-transfected control (Figure 5C).

In addition to the miRNA-29b-3p hub, network analysis revealed that miR-20a-5p and miR-17-5p, which are miRNAs expressed from the miR-17 ~ 92 cluster, may also be controlling the inflammatory response of tissue-engineered cartilage to inflammation. RNA-seq results show that both miR-20a-5p and miR-17-5p increase from 4 to 24 hours, but slightly decrease from 24 to 72 hours in both IL-1β and TNF-α stimulated pellets (Figure 5A). With the addition of miRNA-20a & 17-5p mimics, there is a significant decrease in *Mmp13* and *Ccl2* expression at 24 hours after IL-1β and TNF-α delivery (Figure 5D). Additionally, there is a significant decrease in MMP activity in culture media at both 24 and 48 hours when miRNA-20a & 17-5p mimics are delivered to IL-1β and TNF-α stimulated pellets. The addition of miR-29b-3p mimics further decreases MMP activity levels in cell culture supernatant at 24 hours in both IL-1β and TNF-α groups as compared to miRNA-20a & 17-5p mimics alone, while the addition of miR-29b-3p inhibitor did not show significantly lower levels of MMP activity with either cytokine or at any time point.

4 | DISCUSSION

In this study, we quantified the transcriptomic changes that occur over time in tissue-engineered cartilage in response

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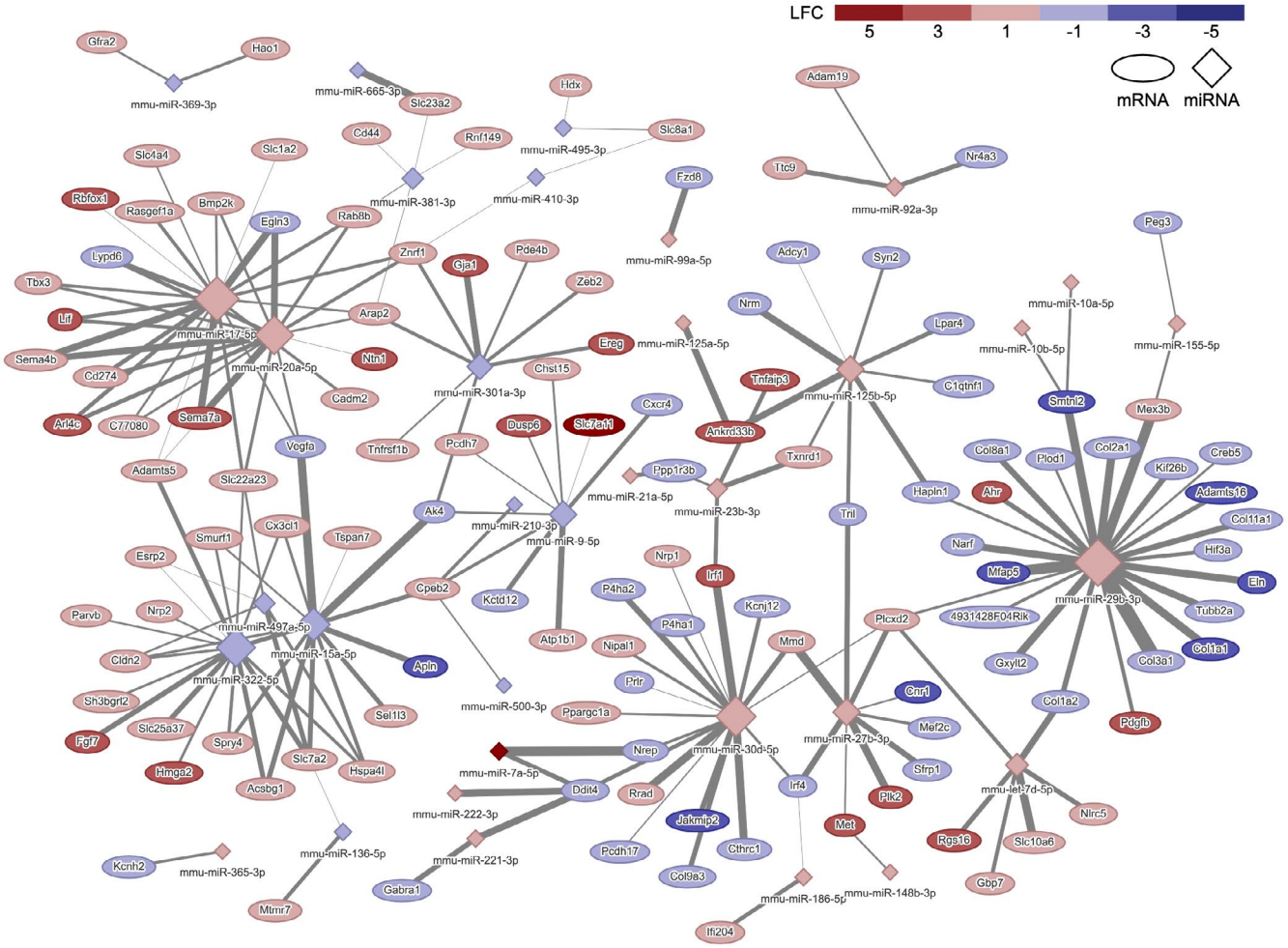

FIGURE 4 TNF-α miRNA-mRNA interactome at 72 hours: network of conserved and predicted miRNA-mRNA target pairs that are differentially expressed, are changing expression levels in opposite directions from 24 to 72 hours, and have been experimentally validated in response to 20 ng/mL TNF-α. The color of the node indicates the LFC of the gene or miRNA, and the size of the node is proportional to the number of connections. The thickness of the line corresponds to the context++ score from the TargetScan database. LFC, $\log_2$(FoldChange)

to inflammatory cytokines IL-1β and TNF-α. Integration of mRNA and miRNA RNA-seq data sets in response to these cytokines revealed regulatory networks, in which the mechanisms of action for previously studied and novel hub miRNAs can be explored as well as their ability to modulate inflammation. Our results show the presence of distinct and common miRNA signatures in response to these cytokines, resulting in complex changes in the miRNA-mRNA interactome over time. These findings provide further data in support of the use of iPSCs-derived cartilage as a system for in vitro disease modeling and have important implications for the development of new approaches to target the chondrocyte inflammatory response.

Through RNA-seq analysis, we determined the temporal and dynamic response of genes to specific inflammatory cytokines. There have been a number of previous studies exploring the DE genes in OA tissues as compared to controls,[53-55] and a few that explore the time course of OA.[56-58] However, these studies are on the scale of days-weeks, where previous studies have shown specific, dynamic patterns of gene expression in response to inflammatory stimuli within hours.[59] Here, we found that different genes were up- and downregulated at various time points, with genes expressed at each time point exclusively and expressed across multiple time points (Figure 2A,C). Over time, DE genes in response to either IL-1β and TNF-α were found to be involved in similar inflammation and matrix degradation signaling pathways (Tables 2 and 3). Furthermore, cluster analysis revealed the patterns of temporal expression of genes in response to inflammatory cytokines (Figure 2D). These findings provide a comprehensive exploration of the transcriptomic changes of tissue-engineered cartilage, as well as time course of these responses, both of which are important for guiding therapies to inhibit both chronic inflammation and flares that occur within a diseased joint.

Similarly, we characterized the temporal response of miRNAs to IL-1β and TNF-α and found that most of the DE miRNAs were up- and down-regulated at the 24 and 72 hours time points. There were a number of shared

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TABLE 1 Enriched pathways of genes within the IL-1β network at 72 hours

Pathway	*P* value	Genes
PI3K-Akt signaling pathway	1.3×10^{-9}	*Lama3; Lamc2; Il6ra; Pik3cb; Lpar4; Thbs2; Fgf1; Egfr; Gys1; Fgf7; Reln; Gng2; Ibsp; Erbb4; Itgb8; Pdgfra; Hgf; Lamb1; Igf1; Ngf; Pgf; Ereg; Col2a1; Kitl; Col6a2; Fgf18; Col9a1; Bcl2; Sgk3; Kras; Fgfr3; Creb5; Fgf10*
Rap1 signaling pathway	3.5×10^{-7}	*Pdgfra; Prkcb; Hgf; Pik3cb; Lpar4; Igf1; Adcy1; Ngf; Fgf1; Egfr; Pgf; Fgf7; Plcb4; Cnr1; Kitl; Fgf18; Kras; Rapgef5; Fgfr3; Rapgef4; Fgf10*
Focal adhesion	2.7×10^{-6}	*Pdgfra; Prkcb; Hgf; Lama3; Lamc2; Lamb1; Pik3cb; Igf1; Thbs2; Egfr; Pgf; Mylk; Reln; Col2a1; Ibsp; Col6a2; Col9a1; Bcl2; Itgb8*
ECM-receptor interaction	2.7×10^{-6}	*Reln; Sv2c; Col2a1; Ibsp; Col6a2; Lama3; Col9a1; Itgb8; Lamc2; Lamb1; Thbs2; Cd44*
Pathways in cancer	5.8×10^{-6}	*Epas1; Il23r; Lama3; Lamc2; Il6ra; Pik3cb; Lpar4; Adcy1; Fgf1; Egfr; Fgf7; Gng2; Ednrb; Il13ra1; Fzd1; Pdgfra; Jag1; Prkcb; Fzd4; Hgf; Lamb1; Igf1; Pgf; Runx1; Nfkbia; Bmp2; Plcb4; Kitl; Fgf18; Bcl2; Kras; Il6st; Fgfr3; Fgf10*
Protein digestion and absorption	6.5×10^{-6}	*Col17a1; Col15a1; Col27a1; Col2a1; Mme; Col11a1; Eln; Fxyd2; Col6a2; Col9a1; Atp1a2; Slc8a1*
Cytokine-cytokine receptor interaction	2.1×10^{-5}	*Il21; Gdf10; Mstn; Il1r1; Il23r; Il34; Lif; Lifr; Il6ra; Tnfrsf11b; Ngf; Acvr1b; Cxcl2; Cx3cl1; Cxcl10; Bmp3; Bmp2; Il18rap; Tnfsf11; Il6st; Tnfrsf21; Il13ra1*
cGMP-PKG signaling pathway	2.3×10^{-5}	*Oprd1; Mef2c; Kcnj8; Atp1a2; Adcy1; Adra2c; Slc8a1; Adra2a; Mylk; Nppc; Plcb4; Ednrb; Fxyd2; Adora1; Pde3a; Creb5*
Insulin secretion	2.3×10^{-5}	*Rims2; Adcyap1; Plcb4; Prkcb; Fxyd2; Atp1a2; Cck; Kcnn3; Adcy1; Rapgef4; Creb5*
MAPK signaling pathway	2.3×10^{-5}	*Pdgfra; Mef2c; Prkcb; Il1r1; Hgf; Igf1; Ngf; Dusp8; Fgf1; Egfr; Pgf; Ereg; Cacna1g; Cacna1i; Fgf7; Erbb4; Kitl; Fgf18; Kras; Fgfr3; Map3k5; Fgf10*

miRNAs, such as miR-155-3p and miR-146a-5p, which have previously been implicated in inflammatory responses of various cells,[60-63] and have significantly increased expression in response to both cytokines in these findings. Interestingly, miR-155-5p and miR-222-3p were miRNAs that were significantly upregulated at 4 hours. In bovine articular cartilage, miR-222-3p has been shown to have higher expression in weight-bearing regions of articular cartilage condyles, and therefore, is thought to play a role in mechanotransduction.[64] Superficial zone cartilage shows a higher expression of miR-222 compared to middle zone chondrocytes, and during dedifferentiation and subsequent redifferentiation, expression of miR-222 increased, and then decreased, respectively.[65] Furthermore, in developing human cartilage, miR-222 was found to be more highly expressed in hypertrophic compared to precursor chondrocytes.[28] These findings implicate a role for miR-222-3p in chondrogenesis. Our results suggest that this miRNA is also rapidly responsive to inflammatory cytokines.

We also identified several miRNAs that showed specific responses to IL-1β or TNF-α. For example, miR-210-5p expression was significantly decreased in response to TNF-α but not IL-1β. Previous studies of animal models of OA have shown that delivery of miR-210 can reduce inflammation in the joint space by inhibiting NF-κB.[66] Another example is miR-140, which is highly expressed in articular cartilage and a critical regulator of skeletal development and cartilage homeostasis.[67,68] Some studies, however, show that miR-140 is increased, while others show decreased expression in OA cartilage.[69] In our study, miR-140-3p was the third most abundant miRNA across all samples and was significantly decreased only in response to TNF-α at 72 hours. This finding could explain the confounding results for miR-140 in OA studies as this miRNA appears to be both stimulus- and time-dependent. miRNAs that have not been previously implicated in healthy or diseased cartilage were also discovered through this analysis, such as miR-341-5p and miR-665-3p which were significantly decreased with both inflammatory cytokines, miR-154-3p which was

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TABLE 2 Enriched pathways of genes within each cluster in response to TNF-α. KEGG pathway analysis of the top 5 enriched pathways, ranked by p-value, and their associated genes

Pathway	*P*-value	Genes
Protein digestion and absorption	1.1×10^{-12}	*Col18a1; Col15a1; Col27a1; Col11a1; Eln; Col22a1; Col11a2; Atp1b1; Slc8a1; Col1a1; Col3a1; Col1a2; Col2a1; Col5a1; Slc7a8; Col4a4; Col9a1; Col9a3*
ECM-receptor interaction	3.9×10^{-10}	*Itga2; Lamc2; Thbs2; Col1a1; Reln; Col1a2; Col2a1; Ibsp; Col4a4; Itga11; Col9a1; Col9a3; Itgb7; Cd44; Itga9*
PI3K-Akt signaling pathway	2.2×10^{-9}	*Csf1r; Csf1; Pdgfb; Lamc2; Lpar4; Thbs2; Fgf7; Reln; Ibsp; Itgb7; Itga2; Vegfc; Ngf; Prlr; Ereg; Vegfa; Col1a1; Col1a2; Col2a1; Ppp2r2c; Col4a4; Itga11; Ddit4; Col9a1; Col9a3; Fgfr3; Met; Creb5; Itga9*
Focal adhesion	4.0×10^{-9}	*Cav3; Itga2; Pdgfb; Vegfc; Lamc2; Parvb; Thbs2; Vegfa; Mapk10; Col1a1; Reln; Col1a2; Col2a1; Ibsp; Col4a4; Itga11; Col9a1; Col9a3; Itgb7; Met; Itga9*
TNF signaling pathway	1.6×10^{-7}	*Cebpb; Edn1; Csf1; Ccl20; Lif; Tnfaip3; Traf1; Tnfrsf1b; Cxcl2; Cx3cl1; Mapk10; Cxcl10; Irf1; Creb5*
Human papillomavirus infection	5.8×10^{-7}	*Notch3; H2-q7; Lamc2; Thbs2; Prkcz; Reln; Ibsp; Itgb7; Ikbke; Fzd2; Fzd4; Itga2; Fzd8; Vegfa; Col1a1; Col1a2; Col2a1; Ppp2r2c; Col4a4; Irf1; Itga11; Col9a1; Col9a3; Creb5; Itga9*
Transcriptional misregulation in cancer	7.6×10^{-7}	*Csf1r; Arnt2; Cebpb; Mef2c; Gadd45a; Igfbp3; Hmga2; Etv1; Traf1; Etv5; Dusp6; Nr4a3; Tspan7; Bmp2k; Itgb7; Erg; Met*
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	4.2×10^{-6}	*Cacng8; Gja1; Cacnb3; Des; Itga2; Itga11; Dmd; Itgb7; Slc8a1; Itga9*
Hypertrophic cardiomyopathy (HCM)	2.1×10^{-5}	*Cacng8; Edn1; Cacnb3; Des; Itga2; Itga11; Dmd; Itgb7; Slc8a1; Itga9*
Pathways in cancer	2.8×10^{-5}	*Camk2b; Csf1r; Notch3; Pdgfb; Cxcr4; Lamc2; Lpar4; Adcy1; Fgf7; Egln1; Arnt2; Egln3; Edn1; Fzd2; Gadd45a; Fzd4; Txnrd1; Itga2; Fzd8; Vegfc; Traf1; Vegfa; Mapk10; Bmp2; Traf4; Col4a4; Fgfr3; Met*

TABLE 3 Inflammation and cartilage related genes[a] connected to hub miRNAs in the IL-1β and TNF-α networks at 72 hours

	IL-1β	TNF-α
miR-29b-3p	***Adamts16*** *;* ***Col11a1*** *; Col15a1;* ***Col25a1*** *; Col27a1;* ***Col2a1*** *;* ***Col6a2*** *;* ***Col8a1*** *; Col9a1;* ***Creb5*** *;* ***Dio2*** *;* ***Eln*** *; Ibsp;* ***Lif*** *; Mstn; Mybl2;* ***Pik3cb***	*Adam19;* ***Adamts16*** *; Adamts20;* ***Col11a1*** *; Col15a1; Col19a1;* ***Col1a1*** *;* ***Col1a2*** *; Col22a1; Col27a1;* ***Col2a1*** *;* ***Col3a1*** *; Col5a1;* ***Col8a1*** *; Col9a1;* ***Creb5*** *;* ***Eln*** *;* ***Hif3a*** *; Ibsp; Itga11; Mstn;* ***Pdgfb***
miR-17-5p	***Acvr1b*** *;* ***Adamts5*** *;* ***Atp1a2*** *; Bmp2;* ***Il6st*** *;* ***Itgb8*** *; Jag1;* ***Pdgfra*** *;* ***Ppara*** *;* ***Runx1*** *;* ***Tnfrsf21*** *;* ***Tnfsf11***	***Adamts5*** *; Bmp2;* ***Bmp2k*** *; Col4a4; Ereg; Itgb7;* ***Lif*** *;* ***Vegfa***
miR-20a-5p	***Acvr1b*** *;* ***Adamts5*** *; Atp1a2; Bmp2;* ***Il6st*** *;* ***Itgb8*** *;* ***Jag1*** *;* ***Pdgfra*** *;* ***Ppara*** *;* ***Runx1*** *;* ***Tnfrsf21*** *;* ***Tnfsf11***	***Adamts5*** *; Bmp2;* ***Bmp2k*** *; Col4a4; Ereg; Itgb7;* ***Lif*** *;* ***Vegfa***

[a]All interactions are listed, and interactions that have been experimentally validated are bolded.

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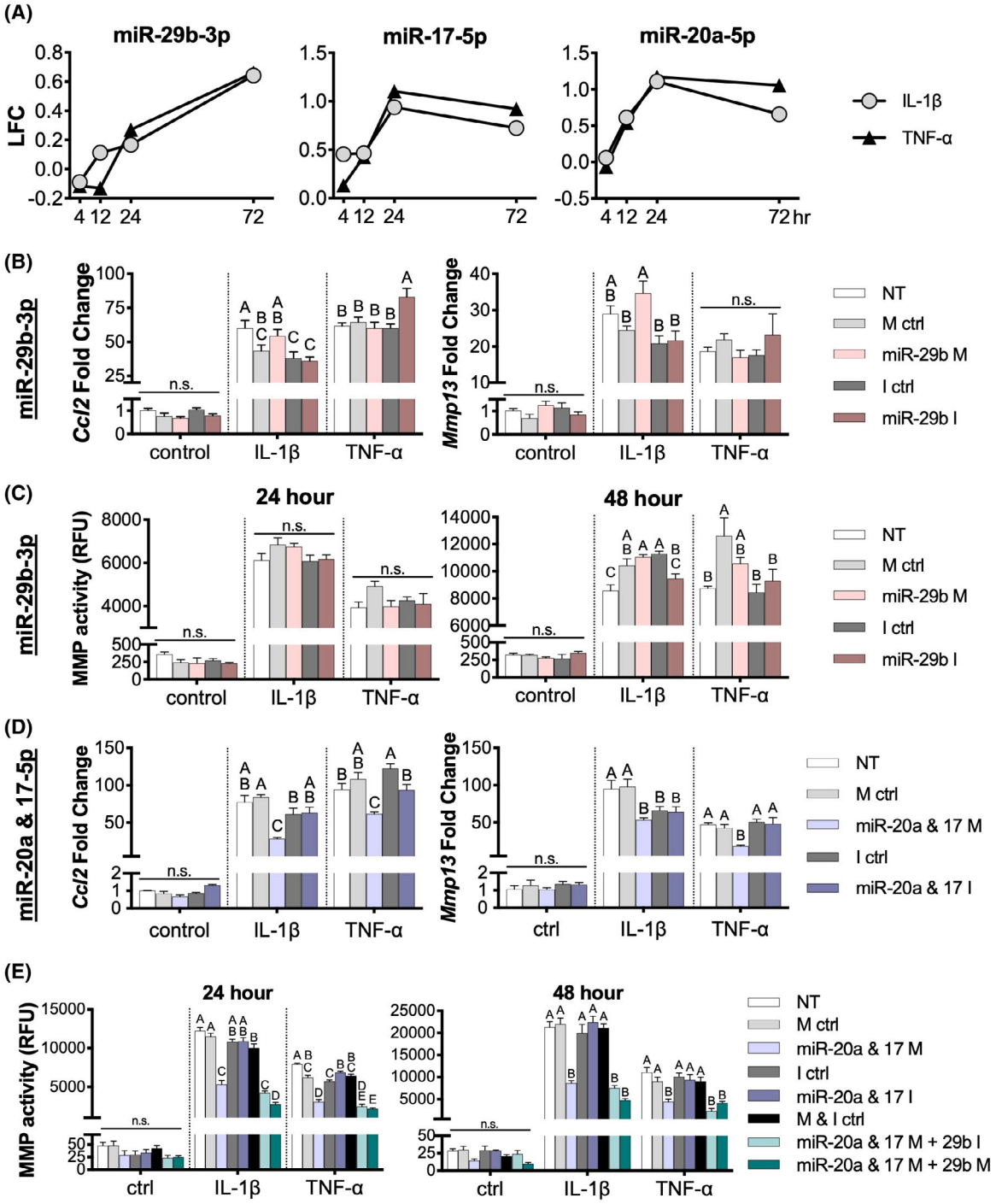

FIGURE 5 In vitro evaluation of hub miRNA mimic and inhibitor delivery. A, RNA-sequencing analysis showed expression of hub miRNAs miR-29b-3p, miR-17-5p, and miR-20a-5p in response to inflammatory cytokines, 0.5 ng/mL IL-1β or 20 ng/mL TNF-α. LFC, $\log_2$(FoldChange). miR-29b-3p mimics (M) and inhibitors (I) were delivered and cells were challenged with 0.5 ng/mL IL-1β or 20 ng/mL TNF-α to evaluate (B) *Ccl2* and *Mmp13* gene expression after 24 hours as measured by qRT-PCR and (C) MMP activity in cell culture media after 24 and 48 hours (n = 4; mean ± SEM). miR-17-5p and miR-20a-5p mimics (M) and inhibitors (I) were delivered in combination and cells were challenged with 0.5 ng/mL IL-1β and 20 ng/mL TNF-α to evaluate (D) *Ccl2* and *Mmp13* gene expression after 24 hours as measured by qRT-PCR and (E) MMP activity levels in cell culture media, determined with and without the further addition of miR-29b-3p mimics and inhibitors. (n = 4; mean ± SEM). Fold changes were determined relative to NT (non-transfected) control group. For each cytokine, groups with different letters are statistically significant ($P < .05$): n.s., not significant

significantly downregulated with IL-1β treatment only, and miR-421-5p, miR-7678-3p, miR-6972-3p, and miR-147-3p which were TNF-α specific.

To obtain a more complete understanding of the specific molecular mediators and networks regulating OA, studies are now using integrative-omics approaches (genomics,

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transcriptomics, proteomics, metabolomics, lipidomics, and epigenomics).[70] However, few studies have investigated the miRNA interactome by correlating miRNA expression levels to protein or mRNA changes in human OA tissues.[29,32] Here, we sought to provide an in-depth analysis of the specific contributions of IL-1β and TNF-α, as well as determine the response of tissue-engineered cartilage to the inflammatory environment of an OA joint. Thus, we created networks of miRNA-mRNA interactions which may be controlling the response to inflammatory cytokines IL-1β and TNF-α (Figures 3 and 4) through integration of mRNA and miRNA sequencing data. Using this approach, we were able to identify key miRNAs of interest, miR-29b-3p, miR-17-5p, and miR-20a-5p. miR-29b has been reported to be increased in both joint tissues in a murine model of OA and human OA cartilage tissues and negatively regulates Smad, NF-κB, and canonical Wnt signaling pathways.[71] In this study, miR-29b-3p was significantly increased in response to both IL-1β and TNF-α and has the most connections to inflammation-related genes in both validated networks (Figures 3 and 4). However, miR-29b-3p mimics or inhibitors alone were not able to attenuate inflammation in cells (Figure 5B,C).

The other hub miRNAs identified in this study were miR-20a-5p and miR-17-5p, which are members of the miR-17 ~ 92 cluster. The miR-17 ~ 92 family has been extensively studied as an oncogene, however, its role in other processes such as proliferation, development, and differentiation have also been explored.[72] While the role of this miRNA cluster in OA has not been reported, miR-17 and miR-20a showed significantly increased expression in hypertrophic chondrocytes as compared to precursor and differentiated chondrocytes in developing human cartilage.[28] Additionally, in humans with mutations in *MIR17HG* (the gene encoding the miR-17 ~ 92 family), display features of Feingold syndrome, including skeletal abnormalities[73] and mice with hemizygous deletion of the miR-17 ~ 92 cluster replicate the features seen in humans. Furthermore, this cluster has recently been implicated in the response of fibroblast-like synoviocytes, the resident cells of the synovial membrane, to inflammation in rheumatoid arthritis.[74,75] Here, we found a novel role for the miR-17 ~ 92 cluster, specifically miR-17-5p and miR-20a-5p, in the ability to modulate the inflammatory response of tissue-engineered cartilage. We show that delivery of miR-20a-5p and miR-17-5p mimics significantly decreases the MMP activity level, while also decreasing *Mmp13* and *Ccl2* expression levels in IL-1β and TNF-α-treated cells (Figure 5D). Furthermore, the addition of miR-29b-3p further decreased MMP activity levels, showing that targeting multiple hubs within the networks can increase the efficacy of miRNA targeted therapies (Figure 5E). Connected genes which may be direct targets and controlling this response are *Adamts5*, TGF-β signaling pathway members (*Bmp2*, *Acvr1b*), and inflammation pathway-related genes (*Tnfsf11*, *Il6st*, *Acvr1b*, *Tnfrsf21*, *Pdgfra*, *Lif*, *Vegfa*).

In comparison to a previously reported OA miRNA-mRNA interactome,[32] hub miRNAs from this study were also significantly DE in lesioned compared to preserved OA cartilage. Additionally, genes connected to miR-20a-5p and miR-17-5p, such as *Adamts5*, *Bmp2*, *Vegfa*, and *Lif*, and genes connected to miR-29b-3p, interestingly, many collagen genes (*Col2a1*, *Col6a2*, *Col8a1*, *Col9a1*, *Col11a1*, *Col15a1*, *Col25a1*, and *Col27a1*), were also DE in lesioned compared to preserved OA cartilage. However, this study did not find strong inverse correlations for any of these interactions, which could be due to the different experimental systems.

In summary, this study utilized an integrative approach to determine the miRNA-mRNA interactome controlling the response of tissue-engineered cartilage to inflammatory cytokines. The miRNA hubs identified through this analysis, miR-17-5p and miR-20a-5p, were further validated by showing that delivery of mimics for these miRNAs significantly decreased degradative enzyme production, as well as the expression of inflammation-related genes. Furthermore, we found that targeting multiple miRNAs further decreased inflammation in cells, highlighting the importance of comprehensively assessing the entire miRNA interactome. Importantly, several of the hub miRNAs and connected genes identified in this study are consistent with those previously shown to be DE in human OA tissues, providing further support for the use of this iPSC-based system as an in vitro model of human disease. This integrative analysis allowed us to discover novel mediators and mechanisms of inflammation in tissue-engineered cartilage and provides information to guide future studies seeking novel miRNA targeted therapies for arthritic diseases.

GSE ACCESSION NUMBER

The RNA-sequencing data in this manuscript can be retrieved from GEO (GSE147833).

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

A.K. Ross and F. Guilak designed the research; A.K. Ross, F. Guilak, I. Meulenbelt, R. Coutinho de Almeida, and Y.F.M. Ramos designed the analysis approach; A.K. Ross and J. Li performed the research; A.K. Ross, R. Coutinho de Almeida, and J. Li analyzed the data; A.K. Ross wrote the paper; all authors edited the paper.

ORCID

Alison K. Ross https://orcid.org/0000-0003-2563-220X
Rodrigo Coutinho de Almeida https://orcid.org/0000-0001-7966-056X
Yolande F. M. Ramos https://orcid.org/0000-0003-1459-413X
Jiehan Li https://orcid.org/0000-0002-0528-5303
Ingrid Meulenbelt https://orcid.org/0000-0001-7786-7081
Farshid Guilak https://orcid.org/0000-0001-7380-0330

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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