Multi-pathway Protective Effects of MicroRNAs on Human Chondrocytes in an In Vitro Model of Osteoarthritis

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Osteoarthritis (OA) is the most common degenerative joint disease. One of the main pathogenic factors of OA is thought to be inflammation. Other factors associated with OA are dysregulation of microRNAs, reduced autophagic activity, oxidative stress, and altered metabolism. MicroRNAs are small non-coding RNAs that are powerful regulators of gene expression. miR-140-5p is considered a cartilage-specific microRNA, is necessary for in vitro chondrogenesis, has anti-inflammatory properties, and is downregulated in osteoarthritic cartilage. Its passenger strand, miR-140-3p, is the most highly expressed microRNA in healthy cartilage and increases during in vitro chondrogenesis. miR-146a is a well-known anti-inflammatory microRNA. Several studies have illustrated its role in OA and autoimmune diseases. We show that, when human chondrocytes were transplanted individually with miR-140-5p, miR-140-3p, or miR-146a prior to stimulation with interleukin-1 beta and tumor factor necrosis-alpha as an inflammatory model of OA, each of these microRNAs exhibited similar protective effects. Mass spectrometry analysis provided an insight to the altered proteome. All three microRNAs downregulated important inflammatory mediators. In addition, they affected different proteins belonging to the same biological processes, suggesting an overall inhibition of inflammation and oxidative stress, enhancement of autophagy, and restoration of other homeostatic cellular mechanisms, including metabolism.

INTRODUCTION

Osteoarthritis (OA) is the most common degenerative joint disease, affecting 10%–13% of adults in western countries.1,2 There is yet no disease modifying treatment available. Patients with OA suffer pain, limited mobility, and reduced quality of life and often end up having joint replacement surgery. The exact causes of OA are unknown, but several risk factors have been identified, such as age, trauma, obesity, genetics, and other joint pathologies.3 Inflammation is considered a major factor associated with the risk of cartilage loss and OA perpetuation.4,5 At the molecular level, cartilage destruction occurs through the combined activities of cartilage degradation enzymes and inflammatory mediators. Increased levels of the inflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in the joint fluid have therefore been associated with the development of OA.6,7 Autophagy is an essential mechanism that ensures cellular homeostasis by degrading and recycling cellular components. Autophagy regulates expression of inflammatory cytokines, is compromised in aging cartilage,8,9 is defective in human OA chondrocytes and animal OA models, and can be regulated by microRNAs (miRNAs).10–13

miRNAs are small double-stranded non-coding RNAs that regulate gene expression in a sequence-based manner.14 The 5‘ strand is known as the leading strand and the 3‘ strand is called the passenger strand. Usually the leading strand is the functional strand, but sometimes both strands can regulate gene expression.15 Emerging evidence shows that one miRNA can target up to 100 genes, and one gene can be regulated by several miRNAs.16,17 miRNAs are thus potent post-transcriptional regulators of gene expression and are implicated in several human diseases, including OA and other arthritic diseases.18–20 miRNAs are therefore highly relevant as therapeutic molecules. miR-140 has been considered a cartilage-specific miRNA because it is predominantly expressed in cartilaginous tissue during development.21 Knockout studies showed miR-140 to be protective against OA development.22 There is ample evidence to suggest that both the 5‘ strand (miR-140-5p) and the 3‘ strand (miR-140-3p) are important for chondrogenesis and the biogenesis of OA. Both strands are highly expressed in healthy cartilage, miR-140-3p higher than miR-140-5p,23 and downregulated in OA cartilage and synovial fluid.24–26 Both strands are highly upregulated during in vitro chondrogenesis.26,27 Previously, we showed that miR-140-5p was essential for SOX9 expression, and thus for chondrogenesis, and identified RALA as a direct target.26 Additionally, we demonstrated that miR-140-5p has anti-inflammatory properties by targeting several proteins in the nuclear factor κB

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miR-140-5p also promotes autophagy in human chondrocytes and other cell types. miR-140-3p is known to inhibit TNF-α-induced inflammation in human smooth muscle cells and NF-κB activity in hepatocytes. miR-146a is one of the most studied miRNAs and has been shown to play a central role in immune responses by targeting IRAK1 and TRAF6, two important proteins in the NF-κB cascade. miR-146a is upregulated in early OA, possibly to counteract inflammation, and downregulated in late OA. Moreover, miR-146a reduced aging-associated and trauma-induced OA by inhibiting Notch 1, IL-1β, and IL-6. Like miR-140-5p, miR-146 promotes autophagy in chondrocytes. All this taken into consideration, these three miRNAs are potential candidates for OA gene therapy. Therefore, the current study aimed to further unravel the function of miR-140-5p, miR-140-3p, and miR-146a in an IL-1β and TNF-α induced in vitro model of OA. Here we show how single transfection of each miRNA regulated different proteins, often associated with the same biological pathways. miR-140-5p, miR-140-3p, and miR-146a all inhibited inflammation and altered various proteins involved in autophagy, proteasomal and lysosomal degradation pathway, metabolism, and regulation of reactive oxygen species (ROS). miR-140-5p and miR-140-3p also promoted expression of chondrogenic proteins. This supports our hypothesis that these miRNAs are promising candidates in miRNA-based therapy of OA.

RESULTS
Simulating OA in vitro using the inflammatory cytokines IL-1β and TNF-α strongly induces the expression of the inflammatory interleukins IL6, IL8, and IL1β and the matrix degrading enzyme MMP13. Figure 1A shows basal mRNA levels of IL6, IL8, IL1β, MMP13, and ADAMTS5 in non-treated cells and in response to stimulation by IL-1β and TNF-α in chondrocytes from three OA donors. Error bars represent a 95% confidence interval from technical triplicates. Figure 1B shows the induced protein expression of IL-6 and IL-8 proteins in non-treated and IL-1β and TNF-α-stimulated conditions in the same donors. β-actin (ACTB) was used as loading control.

Figure 1. Basal and Induced Gene Expression of Relevant OA Genes in Response to IL-1β and TNF-α
(A) qRT-PCR analysis of IL6, IL8, IL1β, MMP13, and ADAMTS5 mRNA levels in non-treated cells and in response to stimulation by IL-1β and TNF-α in chondrocytes from three OA donors. Error bars represent a 95% confidence interval from technical triplicates. (B) Western blot analysis of IL-6 and IL-8 protein levels in non-treated and IL-1β and TNF-α-stimulated conditions in the same donors. β-actin (ACTB) was used as loading control.

Each miRNA was transfected separately in independent reactions. Successful delivery of the miRNAs was validated with qRT-PCR in all three donors. The miRNAs were detected at much higher levels compared with cells transfected with a negative control sequence (Figure 2A). Figure 2B shows downregulation of RALA and TRAF6 proteins after transfection of miR-140-5p and miR-146a, respectively. RALA and TRAF6 are validated targets of these two miRNAs and showed that the transfected miRNAs were functionally active, although there were varying degrees of knockdown in the different donors. There is no validated target for miR-140-3p in chondrocytes yet.

miR-140-5p, miR-140-3p, and miR-146a Strongly Inhibited IL-1β- and TNF-α-Induced Inflammation
Since all three miRNAs have been shown to inhibit inflammation in different in vitro settings, we decided to see if this was also true for our established in vitro model of OA. Four days after miRNA transfection, the cells were stimulated with IL-1β and TNF-α. The following day, the cells were harvested. Each of the miRNAs seemed to counteract the inflammatory-mediated expression of IL6, IL8, and IL1β on the mRNA level (Figure 3A). Additionally, each miRNA also
downregulated IL-6 and IL-8 on the protein level but with varying degrees, depending on donor and miRNA. miR-140-3p and miR-146a exhibited the most potent protective effects, followed by miR-140-5p, with the best effect seen in donor 2 (Figure 3B). miR-140-3p and miR-146a strongly and consistently downregulated MMP13 in all three donors, while ADAMTS5 mRNA was downregulated by all three miRNAs in donor 1 cells only (Figure 3A). For technical reasons, donor 3 cells were transfected with miR-140-5p and miR-140-3p one passage after the transfection of miR-146a, necessitating different control samples and thus additional controls in both the qRT-PCR data (Figure 3A) and western blot images (Figure 3B).

Proteome Alteration following Overexpression of the miRNAs under OA-Simulating Conditions

To unravel other important effects these miRNAs might exhibit under the OA-simulated milieu, mass spectrometry proteomics was performed on cell lysates from all three donors. miR-140-5p, miR-140-3p, and miR-146a significantly altered the expression of 40, 36, and 37 proteins, respectively. However, many of the proteins belonged to the same biological pathways (Tables 1, 2, and 3), and some proteins were shared between the miRNAs (Table S1). 36% of the downregulated proteins were predicted to be targeted by miR-140-5p, 12% by miR-140-3p, and 26% by miR-146a (Tables 1, 2, and 3). In addition to inflammation and immune response, proteins involved in autophagy, ER-Golgi transport, the ubiquitin-proteasomal degradation pathway, ROS regulation, oxidative stress, and metabolism were altered. Cytoskeleton, mRNA/DNA processing, nuclear, and cell cycle control proteins were also altered (Tables 1, 2, and 3). Some selected proteins from the tables are pointed out in the following text. OAS2, IRF9, M4K4, IKIP, and STAT3 were upregulated by miR-140-5p. These proteins are known to be involved in immune responses and regulate inflammation. OAS2 and IRF9 are induced by interferons and inhibit viral replication, while M4K4 is a MAP kinase known to regulate inflammation. IKIP, inhibitor of the NF-κB kinase, is involved in NF-κB regulation, while STAT3 is known to have anti-inflammatory effects. STAT3 is also important in skeletal development and chondrogenesis. WNT5A, a transcriptional factor that plays an essential role in chondrocyte differentiation during development through induction of expression of SOX9, was also upregulated by miR-140-5p together
with several proteins involved in intracellular trafficking, histone modification, and other nuclear proteins (Table 1). miR-140-5p downregulated proteins involved in immune responses and some proteins that have undesirable effects on chondrogenesis and OA development, such as STA5A, CIR, and STAM2. STA5A has also been associated with chondrocyte hypertrophy and chondrocyte growth arrest in cartilage of dwarf children. CIR, on the other hand, has recently been shown to be upregulated in the synovial fluid of OA patients and OA porcine models. miR-140-5p also downregulated NUP93 and MEP50. The latter has been shown to regulate PRMT5, which is important in maintaining chondro-progenitor cells in mice limb buds. Metabolic enzymes and mitochondrial proteins like ACSL4, APLP2, and PGM2 were also downregulated. In addition, several nuclear proteins were downregulated, including HCFC1 and WDR5. The former is involved in cell cycle control, activation, and repression of transcription and has been shown to be involved in craniofacial development in zebrafish, while the latter is involved in histone modifications and is required for osteoblast differentiation. As expected, the direct target of miR-140-5p, RALA, was also downregulated. This is consistent with the western blot results in Figure 2B.

miR-140-3p, like miR-140-5p, upregulated STAT3 and downregulated STA5A, CIR, NUP93, and MEP50 (Tables 2 and S1). In addition, the NF-κB inhibitor IASPP, PDCD4, a tumor suppressor that is downregulated by inflammation, PCOC1, an important enzyme for collagen fibril formation, and DDAH1, an enzyme that reduces oxidative stress, were upregulated. Other upregulated proteins were mainly involved in mitochondria, protein degradation, trafficking, and gene expression processes. LTOR5, an activator of the potent autophagy inhibitor mTORC1, was downregulated by miR-140-3p together with other proteins involved in mitochondria, mRNA processing, and membrane bending.

miR-146a upregulated several proteins involved in the ubiquitination degradation pathway, mitochondrial metabolism, enzymatic cleavage
| Protein        | Protein Name                                                                 | Fold Change | Biological Process                                                                 | Predicted Targets                                                                 |
|---------------|------------------------------------------------------------------------------|-------------|--------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Uregulated by | miR-140-5p                                                                    |             |                                                                                      |                                                                                  |
| OAS2          | 2'-5'–oligoadenylate synthase 2                                               | 5.0         | inflammation, immune responses, apoptosis                                           |                                                                                  |
| IRF9          | interferon regulatory factor 9                                                | 3.0         |                                                                                      |                                                                                  |
| M4K4          | mitogen-activated protein kinase kinase kinase kinase 4                       | 2.6         |                                                                                      |                                                                                  |
| IKIP          | inhibitor of nuclear factor kappa B kinase                                    | 2.3         |                                                                                      |                                                                                  |
| STAT3         | signal transducer and activator of transcription 3                           | 2.3         |                                                                                      |                                                                                  |
| GBRAP         | gamma-aminobutyric acid receptor-associated protein                           | 5.0         |                                                                                      |                                                                                  |
| DHC24         | delta(24)-sterol reductase                                                    | 7.0         |                                                                                      | metabolism, oxidative stress protection                                          |
| MVD1          | diposphomevalonate decarboxylase                                              | 5.0         |                                                                                      |                                                                                  |
| PGM2          | phosphoglucosomutase-2                                                        | 2.5         |                                                                                      |                                                                                  |
| COG5          | conserved oligomeric Golgi complex subunit 5                                 | INFα        | Golgi apparatus, intracellular vesicle trafficking, cytoskeleton, chaperone          |                                                                                  |
| CKAP5         | cytoskeletal-associated protein 5                                             | 3.3         |                                                                                      |                                                                                  |
| RB3GP         | Rab3 GDP-activating protein catalytic subunit                                 | 2.8         |                                                                                      |                                                                                  |
| PFD6          | prefoldin subunit 6                                                           | 2.8         |                                                                                      |                                                                                  |
| TM9S2         | transmembrane 9 superfamily member 2                                          | 2.2         |                                                                                      |                                                                                  |
| NUP93         | nuclear pore complex protein                                                  | 7.0         | nuclear proteins, mRNA processing, spliceosome                                       |                                                                                  |
| MEF50         | methylosome protein 5                                                         | 6.0         |                                                                                      |                                                                                  |
| THOC5         | THO complex subunit 5 homolog                                                 | INFα        |                                                                                      |                                                                                  |
| RFXO1         | RNA binding protein fox-1 homolog 1                                          | 3.0         |                                                                                      |                                                                                  |
| TXN4A         | thioredoxin-like protein 4A                                                   | INFα        |                                                                                      |                                                                                  |
| WNT5A         | protein Wnt-5a                                                                | 6.0         | chondrogenesis                                                                        |                                                                                  |
| STRN          | Striatin                                                                     | INFα        | estrogen and IP3 signaling                                                            |                                                                                  |
| Downregulated by | miR-140-5p                                                                  |             |                                                                                      |                                                                                  |
| STA5A         | signal transducer and activator of transcription 5A                          | −INFβ       | inflammation                                                                          |                                                                                  |
| C1R           | complement C1r                                                               | −3.0        |                                                                                      |                                                                                  |
| STAM2         | signal transducing adaptor molecule                                          | −INFβ       |                                                                                      |                                                                                  |
| TIM9          | mitochondrial import inner membrane translocase subunit Tim9                 | −3.5        | metabolism                                                                           |                                                                                  |
| ACSL4         | long-chain-fatty-acid-CoA ligase 4                                            | −2.5        |                                                                                      |                                                                                  |
| MMSA          | methylmalonate-semialdehyde dehydrogenase                                     | −2.1        |                                                                                      | MMSAα                                                                            |
| MTDC          | bifunctional methylene tetrahydrofolate dehydrogenase                        | −2.0        |                                                                                      | MTDCα                                                                            |
| DCTN3         | dynactin subunit 3                                                           | −8.0        | ER-Golgi transport, intracellular and membrane trafficking, cytoskeleton             |                                                                                  |
| ANFY1         | Rabankyrin-5                                                                 | −4.6        |                                                                                      |                                                                                  |
| STX4          | Syntaxin-4                                                                   | −INFβ       |                                                                                      |                                                                                  |
| RALA          | Ras-related protein Ral-A                                                     | −3.3        |                                                                                      |                                                                                  |
| BAG2          | BAG family molecular chaperone regulator 2                                   | −2.8        |                                                                                      |                                                                                  |
| E41L2         | Band 4.1-like protein 2                                                        | −2.3        |                                                                                      |                                                                                  |
| HCFC1         | host cell factor 1                                                            | −6.0        | nuclear proteins, histone modifications, cell cycle control                          |                                                                                  |
| PCNP          | PEST proteolytic signal-containing nuclear protein                            | −INFβ       |                                                                                      |                                                                                  |
| WDR5          | WD repeat-containing protein 5                                                | −INFβ       |                                                                                      |                                                                                  |
| LEMD2         | LEM domain-containing protein 2                                               | −2.3        |                                                                                      |                                                                                  |

(Continued on next page)
downregulated in all donors, while translated in two of the donors (Figure 4D). Direct target of miR-140-5p, showed consistent downregulation in mRNA levels in all three donors (Figure 4D). DHCR24, the gene coding for DHC24, showed upregulation on western blot, were not detected by the mass spectrometry analysis and like the pro-apoptotic QORX. IL-6, IL-8, and TRAF6, all detected by also led to downregulation of proteins involved in ROS generation, inhibition of lysosomal degradation using Baflomycin A1 resulted in accumulation of lipidated GBRAP (GBRAP) (Figure 4C), which would have been degraded by autophagy. The accumulation of GBRAPII provides an estimate of the autophagic activity. CIR mRNA were downregulated in all donors, while STAT5A mRNA was downregulated in two of the donors (Figure 4D). RALA mRNA, a validated direct target of miR-140-5p, showed consistent downregulation in all three donors (Figure 4D) and by western blot (Figure 2B). DHC24, the gene coding for DHC24, showed upregulation on mRNA levels in all three donors (Figure 4D).

miR-140-3p Transfected Cells
Due to limited material from the miR-140-3p transfected cells, the western blot validation experiment was repeated in a fourth donor. The proteins LTOR5 and PDCD4 showed the same expression pattern as the proteomics for all three donors (Figure 4E). LAMTOR5 mRNA, coding for LTOR5, on the other hand, was downregulated in donor 1 only (Figure 4F). CIR and STAT5A were also targeted by miR-140-3p and were downregulated at mRNA levels, except for CIR in donor 3 (Figure 4G). BROX, a predicted target of miR-140-3p, was downregulated at the mRNA levels in two donors, while mRNA for DDAH1 showed upregulation on the mRNA levels in two donors (Figure 4G).

miR-146a Transfected Cells
RIPK2 was downregulated at both the protein and mRNA levels in all three donors (Figures 4H and 4I). STAT2 mRNA was downregulated in one donor, while TP53I3 mRNA, the gene coding for QORX, was downregulated in two donors (Figure 4J).

**DISCUSSION**
The exact causes of OA are unknown, but at the cellular level, inflammation, reduced autophagy, increased production of ROS, increased mitochondrial DNA damage, and altered metabolism are all hallmark of OA chondrocytes. All these processes are linked together in a complex network. Inflammation is thought to be a perpetuating force driving disease progression by upregulating enzymes that break down the articular cartilage. Autophagy has been shown to control inflammation by degrading IL-1B and inhibiting its secretion, while inhibition of autophagy enhanced processing and secretion of IL-1β. Moreover, this effect was suggested to be reduced by ROS inhibition. ROS itself can lead to mitochondrial DNA damage and altered metabolism. Thus, changes in one of the processes will likely affect the other processes.

| Protein | Protein Name | Fold Change | Biological Process | Predicted Targets |
|---------|--------------|-------------|--------------------|------------------|
| CSTF2   | cleavage stimulation factor subunit 2 | –INF⁷⁻⁸ | RNA polymerase activity, mRNA splicing |      |
| RFAC1   | DNA-directed RNA polymerases I and III subunit | –INF⁹⁻¹⁰ | |      |

INF, infinity.

⁷Only detected in miRNA transfected cells
⁸Only detected in control transfected cells
⁹Predicted targets according to TargetScan
¹⁰Predicted targets according to miRDB
¹¹Predicted target according to miRwalk: prediction based on coding region
¹²Predicted target according to miRwalk prediction based on 3’UTR

Table 1. Continued

miRNAs are known to affect many genes belonging to the same biological process. miRNA-based gene therapy strategies are therefore promising candidates for the treatment of OA by fine-tuning or ensuring homeostatic control of some of the cellular processes that are altered in OA. miR-140-5p, miR-140-3p, and miR-146a are all involved in cartilage and OA biology and are potential candidates for miRNA-based therapy of OA. However, there is much to learn about their functional roles in cartilage and OA. Here we show how these miRNAs act on several cellular pathways associated with OA in an IL-1β and TNF-α induced in vitro model of OA, including inflammation, autophagy, ROS regulation, and metabolism. Other proteins involved in the ubiquitin degradation pathway, ER-Golgi trafficking, mRNA processing, and cell cycle control, were also altered by these miRNAs.

Our initial results showed upregulation of OA genes and proteins in chondrocytes in response to IL-1β and TNF-α. To address our hypothesis about the protective effects of the three miRs under these conditions, we first validated their successful overexpression in all donors, yet with a variation that is likely to be caused by natural donor variation. The miRNAs led to downregulation of IL6, IL8, and IL1β on the mRNA level as well as on the protein level, with variation in the degree of potency. Overall miR-140-3p and miR-146a gave better
| Protein          | Protein Name                                      | Fold Change | Biological Process                                      | Predicted Targets                                                                 |
|------------------|--------------------------------------------------|-------------|---------------------------------------------------------|------------------------------------------------------------------------------------|
| **Upregulated by miR-140-3p** |                                                  |             |                                                         |                                                                                    |
| ISAPP            | RelA-associated inhibitor                        | INF<sup>a</sup> | inflammation, immune response, cell growth, apoptosis |                                                                                    |
| GILT             | gamma-interferon-inducible lysosomal thiol reductase | INF<sup>a</sup> |                                                         |                                                                                    |
| CNPY4            | protein canopy homolog 4                         | 2.8         |                                                         |                                                                                    |
| STAT3            | signal transducer and activator of transcription 3 | 2.1         |                                                         |                                                                                    |
| DDAH1            | (N(G),N(G))-dimethylarginine dimethylaminohydrolase 1 | INF<sup>a</sup> | mitochondrial respiratory machinery, NOS/ROS regulation |                                                                                    |
| NDUS7            | NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial | 3.1         |                                                         |                                                                                    |
| PPII3            | peptidyl-prolyl cis-trans isomerase-like 3       | 5.2         | proteasome, immunoproteasome, chaperones                |                                                                                    |
| DDS1             | 26S proteasome complex subunit DDS1              | INF<sup>a</sup> |                                                         |                                                                                    |
| PSMG2            | proteasome assembly chaperone 2                  | INF<sup>a</sup> |                                                         |                                                                                    |
| PSM9D            | 26S proteasome non-ATPase regulatory subunit 9   | INF<sup>a</sup> |                                                         |                                                                                    |
| ZFPL1            | zinc finger protein-like 1                       | INF<sup>a</sup> | ER-protein, Golgi, vesicle trafficking                  |                                                                                    |
| COG3             | conserved oligomeric Golgi complex subunit 3     | INF<sup>a</sup> |                                                         |                                                                                    |
| VP37C            | vacuolar protein sorting-associated protein 37C   | 2.8         |                                                         |                                                                                    |
| ERLEC            | endoplasmic reticulum lectin 1                  | 2.0         |                                                         |                                                                                    |
| PDCD4            | programmed cell death protein 4                  | 10.0        | tumor suppressor, apoptosis                             |                                                                                    |
| IBF4             | insulin-like growth factor-binding protein 4     | INF<sup>a</sup> |                                                         |                                                                                    |
| PP4R1            | serine/threonine-protein phosphatase 4 regulatory subunit 1 | INF<sup>a</sup> | chromatin, histone modifications, nuclear, mRNA export from the nucleus |                                                                                    |
| NUP93            | nuclear pore complex protein Nup93               | 7.2         |                                                         |                                                                                    |
| MEF50            | methylspermine 50                                | 5.1         | splicesome, transcription regulation, mRNA/DNA processing |                                                                                    |
| RNH2A            | ribonuclease H2 subunit A                        | INF<sup>a</sup> |                                                         |                                                                                    |
| T2AG             | transcription initiation factor IIA subunit 2    | INF<sup>a</sup> |                                                         |                                                                                    |
| MYOY2            | myeloma-overexpressed gene 2 protein             | 2.0         |                                                         |                                                                                    |
| PINN             | Pinin                                            | 2.0         |                                                         |                                                                                    |
| PP12C            | protein phosphatase 1 regulatory subunit 12C     | INF<sup>a</sup> | scaffold protein, actin cytoskeleton                    |                                                                                    |
| NHRF1            | Na(+)/H(+) exchange regulatory cofactor NHE-RF1   | 2.0         |                                                         |                                                                                    |
| FSTL1            | follistatin-related protein 1                    | 2.2         | skeletal development                                    |                                                                                    |
| PCOC1            | procollagen C-endopeptidase enhancer 1           | 2.1         | glycoprotein that binds and drives enzymatic cleavage of type I procollagen |                                                                                    |
| **Downregulated by miR-140-3p** |                                                  |             |                                                         |                                                                                    |
| STS5A            | signal transducer and activator of transcription 5A | −5.0        | inflammation, innate immunity                           |                                                                                    |
| CIR              | complement C1r subcomponent                      | −2.0        |                                                         |                                                                                    |
| LTR5R            | regulator complex protein LAMTOR5                | −2.3        | autophagy                                               |                                                                                    |
| MIC27            | MICOS complex subunit MIC27                      | −5.0        | mitochondrial proteins and chaperones                   |                                                                                    |
| RT35             | 28S ribosomal protein S35, mitochondrial          | −INF<sup>a</sup> |                                                         |                                                                                    |
| GPD2             | glycerol-3-phosphate dehydrogenase, mitochondrial | −2.1        |                                                         |                                                                                    |
| CSTF1            | cleavage stimulation factor subunit 1            | −3.0        | mRNA processing                                         |                                                                                    |
| BROX             | BRO1 domain-containing protein                    | −3.0        | membrane bending                                        | BROX<sup>a</sup>                                                                  |

INF<sup>a</sup>, infinity
<sup>a</sup>Only detected in miRNA transfected cells
<sup>b</sup>Only detected in control transfected cells
<sup>c</sup>Predicted target according to miRwalk: prediction based on 3’UTR
| Protein          | Protein Name                          | Fold Change | Biological Process                                      | Predicted Targets                      |
|------------------|---------------------------------------|-------------|---------------------------------------------------------|----------------------------------------|
| **Upregulated by miR-146a** |                                       |             |                                                         |                                        |
| CUL1             | Cullin-1                              | 7.0         | ubiquitination and degradation, lysosomal                |                                        |
| NEUR1            | Sialidase-1                           | 6.0         | mitochondrial metabolism, phospholipid metabolism        |                                        |
| PPiF             | peptidyl-prolyl cis-trans isomerase F, mitochondrial | 3.8         | mitochondrial metabolism, phospholipid metabolism        |                                        |
| PCAT1            | lysophosphatidylcholine acyltransferase | 2.6         |                                                         |                                        |
| PCOC1            | procollagen C-endopeptidase enhancer 1 | 2.0         | enzymatic cleavage of type I procollagen                |                                        |
| GIT2             | ARF GTPase-activating protein GIT2     | 2.3         | GTPase-activating protein (GAP) activity                 |                                        |
| **Downregulated by miR-146a** |                                       |             |                                                         |                                        |
| TAP1             | antigen peptide transporter 1         | –INF⁴       | inflammation, innate/adaptive immune responses           |                                        |
| SEP10            | Septin-10                             | –7.0        |                                                         |                                        |
| STAT2            | signal transducer and activator of transcription 2 | –4.4        |                                                         |                                        |
| RIPK2            | receptor-interacting serine/threonine-protein kinase 2 | –INF⁴       |                                                         |                                        |
| ABCF1            | ATP-binding cassette sub-family F member 1 | –3.3        |                                                         |                                        |
| SHPK             | sedoheptulokinase OS                  | –INF⁴       |                                                         |                                        |
| QORX             | quinone oxidoreductase PIG3           | –4.5        | oxidative stress/ROS                                    |                                        |
| MTIE             | Metallothionein-1E                    | –3.0        |                                                         |                                        |
| LAMP1            | lysosome-associated membrane glycoprotein 1 (CD107a) | –3.0        | lysosomal, chaperone/ proteinfolding                     |                                        |
| DNJA1            | DnaJ homolog subfamily A member 1     | –2.8        |                                                         |                                        |
| HYOU1            | hypoxia upregulated protein 1         | –2.3        |                                                         |                                        |
| NDU2             | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 | –INF⁴       | mitochondrial respiratory, metabolism                  |                                        |
| TOMD4            | mitochondrial import receptor subunit | –5.0        |                                                         |                                        |
| F120B            | constitutive coactivator of peroxisome proliferator-activated receptor gamma | –INF⁴       |                                                         |                                        |
| PNPO             | pyridoxine-5'-phosphate oxidase       | –2.7        | transcription and cell cycle control, histone, chromatin factors, and nuclear proteins |                                        |
| HCFC1            | host cell factor 1                    | –6.0        |                                                         |                                        |
| SP1H             | FACT complex subunit SPT16            | –6.0        |                                                         |                                        |
| PELO             | protein pelota homolog                | –INF⁴       |                                                         |                                        |
| DDX21            | nuclear RNA helicase 2                | –2.2        |                                                         |                                        |
| AAAS             | Aladin                                | –2.0        |                                                         |                                        |
| GTPB1            | GTP-binding protein 1                 | –INF⁴       | degradation of target mRNA, circadian mRNA stability    |                                        |
| MEDI8            | mediator of RNA polymerase II transcriptionsubunit 18 | –INF⁴       | coactivator of transcription of all RNA pol II genes    |                                        |
| TM169            | transmembrane protein 109             | –INF⁴       | DNA-damage response/DNA repair                           |                                        |
| TRIPC            | E3 ubiquitin-protein ligase           | –INF⁴       |                                                         |                                        |
| ADPPT            | L-aminoacidoplate-semialdehyde dehydogenase-phosphopantetheinyl transferase | –INF⁴       | post-translational modification                          |                                        |
| ATAD1            | ATPase family AAA domain-containing protein 1 | –INF⁴       | regulation of cell surface expression of AMPA receptors |                                        |
| FMNL3            | formin-like domain-containing protein 1 | –INF⁴       | cytoskeletal organization and adherens juctions         |                                        |
| GEPI             | Gephyrin                              | –INF⁴       |                                                         |                                        |
| VEZA             | Veratin                               | –INF⁴       |                                                         |                                        |

(Continued on next page)
reduction of IL6 and IL8 compared with miR-140-5p. miR-140-3p and miR-146a also exhibited a strong downregulatory effect on MMP13. ADAMTS5 mRNA was downregulated by all three miRNAs in one donor and by miR-140-3p in another donor.

Proteomic analysis revealed a broader picture of how these miRNAs might work. The three miRNAs downregulated different pro-inflammatory mediators. miR-140-5p and miR-3p both downregulated STAS5A and C1R, two proteins that have been associated with inflammation. STAS5A was detected as an inflammatory response biomarker and has been associated with chondrocyte hypertrophy in mice and dwarfism in humans. STA5A has also been shown to have a binding site in the ADAMTS5 promoter. C1R is the first component of the complement system and has been shown to be upregulated in the synovial fluid of OA patients. C1R activates C1S, which has been implicated in matrix degradation of articular cartilage in rheumatoid arthritis (RA). Additionally miR-140-5p and miR-140-3p upregulated the anti-inflammatory mediator STAT3, which is also important for SOX9 expression and cartilage formation during development. ISAPP, a potent inhibitor of NF-kB, was upregulated by miR-140-3p, miR-146, on the other hand, downregulated inflammation and immune-related proteins: TAP1, SEP10, STAT2, ABCF1, and RIPK2. TAP1 is an antigen peptide transporter upregulated by TNF-α in chondrocytes and is known to play an important role in immune responses, with a polymorphism that has recently been shown to be associated with the inflammatory joint disease ankylosing spondylitis. SEP10 is induced by TNF-α. STAT2 was detected in OA chondrocytes but not in healthy controls, suggesting a role in OA. ABCF1 is thought to be a regulator of the translation of inflammatory cytokine pathways, and it has been shown to regulate and be regulated by TNF-α. A hyperactive RIPK2 allele is involved in onset of early OA. RIPK2 has a distinct expression profile, together with cartilage destruction markers in chondrocytes stimulated with synovial fibroblasts from RA patients. RIPK2 downregulation also inhibited catabolic genes induced by cartilage damaging toxin T-2. Thus, our results support the in vivo findings that miR-146a inhibited OA development. In summary, all three miRNAs have an overall inhibitory effect on inflammation, which is likely to be beneficial for the prevention of OA.

The role of miR-146a as an anti-inflammatory miRNA has been extensively elucidated in the literature, and its roles as age and OA-attenuating, cartilage protecting, and autophagy enhancing are emerging. Yet one study published last year by Zhang et al. claimed a contradicting role to miR-146a in mice. In this study, the authors show that miR-146a KO mice have less cartilage degeneration compared to WT mice in spontaneous and instability induced OA models. They also show that miR-146a aggravates pro-inflammatory cytokines and suppresses the expression of COL2A and SOX9. Whether that could be explained by how the KO mice were generated or other factors is yet to be understood.

Autophagy is an essential mechanism that ensures cellular homeostasis by degrading old and damaged cellular components and recycling of macromolecules. The consequence of reduced autophagy and other degradation pathways is production of ROS, which may lead to DNA damage and ultimately cell death. Reduced autophagy, accumulation of dysfunctional organelles and/or proteins, and increased ROS production has been reported in OA chondrocytes in several studies while enhancing autophagy was shown to be chondro-protective in a mouse model of OA. This suggests that altered autophagy is involved in OA development. Our data showed a pro-autophagy tendency for all three miRNAs. GBRAP, an autophagy marker and a member of the Autophagy-related protein8 (ATG8) family, which is crucial for autophagosome formation and degradation of cytosolic cargo, was upregulated by miR-140-5p. We detected autophagic flux by western blot, evident by the conversion of GBRAP from the type I to type II form involved in autophagosome biogenesis. miR-140-5p led to more accumulation of GBRAPII compared to control. However, when stimulated with cytokines, the effect was reduced. A pro-autophagic role of miR-140-5p is consistent with previous findings where miR-140-5p promoted autophagy in human chondrocytes. A pro-autophagic effect of miR-140-5p has also been demonstrated in other cell types.

miR-140-3p, on the other hand, downregulated the autophagy inhibitory protein LTORS (encoded by the gene LAMTOR5). Autophagy is closely linked with the ER-Golgi and proteosomal...
degradation systems. miR-140-5p, miR-140-3p, and miR-146a led to both upregulation and downregulation of several proteins involved in these processes, perhaps suggesting a regulatory role to establish homeostatic control. Also, ROS and oxidative stress proteins were affected by the three miRNAs. miR-140-5p upregulated DHC24, an enzyme that protects cells against oxidative stress and apoptosis by reducing caspase 3 activity. Interestingly, this protein is also important in cartilage and skeletal development, as mutations within this gene lead to severe developmental abnormalities, including short limbs. Mirza et al. showed that bones from DHC24 KO mice lacked proliferating chondrocytes in the growth plate and showed abnormal hypertrophy of prehypertrophic chondrocytes. In addition, H2O2-induced hypertrophy was prevented by lentiviral delivery of DHC24. Thus, miR-140-5p might protect the cells from ROS through upregulation of DHC24. DHC24 was validated by qRT-PCR to be upregulated in all three donors. miR-140-3p may protect against oxidative stress by upregulating the enzyme DDAH1. Shi et al. demonstrated that DDAH1 deficiency increased oxidative stress and led to increased kidney fibrosis in mice. DDAH1 mRNA upregulation was validated by qRT-PCR in two donors. QORX, on the other hand, was downregulated by miR-146a. Porte et al. showed that overexpression of QORX accumulated ROS both in vitro and in vivo. Its downregulation in our data might suggest that miR146a protects chondrocytes from excessive ROS formation. QORX (TP5313) mRNA showed downregulation in two donors.
Figure 5 illustrates some of the proteomics findings and shows the many possible platforms that can be altered to prevent or perhaps even treat OA by delivery of miR-140-5p, miR-140-3p, and miR-146a. SOX9, ACAN, and IkB, three important proteins that we have shown previously to be positively regulated by miR-140-5p, miR-140-3p, and miR-146a regulated expression of key components of inflammation, autophagy, and other degradation pathways. A proposed model of how this might promote cartilage integrity and protection under adverse inflammatory conditions is shown. Arrows and green boxes represent positive regulation, while the perpendicular lines and red boxes represent inhibition.

**Figure 5. A Proposed Model of the Three miRNAs’ Mode of Action**

miR140-5p, miR-140-3p, and miR-146a regulated expression of key components of inflammation, autophagy, and other degradation pathways. A proposed model of how this might promote cartilage integrity and protection under adverse inflammatory conditions is shown. Arrows and green boxes represent positive regulation, while the perpendicular lines and red boxes represent inhibition.

**MATERIALS AND METHODS**

**Isolation and Culture of Human Articular Chondrocytes (ACs)**

ACs were isolated from discarded OA cartilage tissue after total knee replacement surgery and cultured as previously described. Only tissue with no macroscopic signs of OA was used. All donors provided written informed consent. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway. Briefly, the cartilage was cut into tiny pieces and subsequently digested with Collagenase type XI (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 90–120 min. Chondrocytes were washed three times and resuspended in culture medium consisting of DMEM/F12 (GIBCO/ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% human plasma (Octaplasma AB, Oslo Blood Bank, Norway) supplemented with platelet lysate (corresponding to 10⁹ platelets/mL plasma) (PLP), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. PLP was prepared as previously described. The culture medium was changed every 3–4 days. After the first passage, amphotericin B was removed. At 70%–80% confluence, cells were detached with trypsin-EDTA (Sigma-Aldrich) and seeded into new culture flasks.

**miRNA Mimics, Transfection, and Stimulation with IL-1β and TNF-α**

The Amaxa nucleofector system and the Amaxa Human Chondrocyte Nucleofector Kit were used for electroporation following the protocols from the manufacturer (Lonza, Walkersville, MD, USA). Briefly, each reaction contained 1.0 × 10⁶ cells, 5µM of pre-miR mimics (Table S2) in a total volume of 100 µL nucleofection solution. The cells were seeded in 20% PLP without antibiotics and left to recover overnight. The following day (day 1), the medium was changed to 10% PLP with 1% penicillin/streptomycin. On day 4, ACs were stimulated with 0.1 ng/mL recombinant IL-1β (rIL-1β) or 10 ng/mL rTNF-α (R&D Systems, Minneapolis, MN) for 24 h before harvesting for analysis.

**Autphagic Flux**

On day 5, 2 h prior to harvesting the cells, ACs were treated with 100 nM Bafilomycin A1 (Sigma-Aldrich).

**Isolation of miRNA, cDNA Synthesis, and qRT-PCR**

Total RNA containing miRNAs was isolated using the miREasy mini kit according to the manufacturer’s protocol (QIAGEN, Germantown, MD, USA). cDNA synthesis and qRT-PCR were performed following protocols from the manufacturer using the Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). 10 ng miRNA in a total volume of 15 µL was reverse transcribed into cDNA. All samples were run in technical triplicates. Each replicate contained 1.33 µL cDNA in a total volume of 15 µL. The thermocycling parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. U18 was used as endogenous control. qRT-PCR results are shown as relative fold changes using mean values from technical triplicates with a 95% confidence interval. All donors are shown separately in the figures.

**Western Blotting**

Cell lysates corresponding to 200,000 cells were loaded onto a 4%–20% gradient or 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Proteins were separated by gel electrophoresis, transferred
to polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes and incubated with appropriate antibodies before visualizing the bands using the myECL imager (Thermo Fisher Scientific).

**In-Solution Digestion**

400 μL ice cold acetone (Sigma-Aldrich, Oslo, Norway) was added to the samples, vortexed, and precipitated at −20°C overnight. Samples were centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was discarded. Proteins were re-dissolved in 50 μL 6 M urea and 100 mM ammonium bicarbonate (Sigma-Aldrich), pH 7.8. For reduction and alkylation of cysteines, 2.5 μL 200 mM dithiothreitol (DTT; Sigma-Aldrich) was added, and the samples were incubated at 37°C for 1 h followed by addition of 7.5 μL 200 mM iodoacetamide (Sigma-Aldrich) for 1 h at room temperature in the dark. The alkylation reaction was quenched by adding 10 μL 100 mM DTT. The proteins were digested with trypsin gold (Promega, Madison, WI, USA) in a final volume of 250 μL for 16 h at 37°C. The digestion was stopped by adding 20 μL 1% formic acid (Sigma-Aldrich), and the generated peptides were purified using ZipTips (Millipore, Billerica, MA, USA) and dried using a Speed Vac concentrator (Eppendorf, Hamburg, Germany).

**Nano LC-QExactive Orbitrap Mass Spectrometry**

Peptides were analyzed using an Ultimate 3000 nano-ultra-high-performance liquid chromatography (UHPLC) system (Dionex, Sunnyvale, CA, USA) connected to a Q Exactive mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nano electrospray ion source. For liquid chromatography separation, an Acclaim PepMap 100 column (C18, 3 μm beads, 100 Å, 75 μm inner diameter) (Dionex, Sunnyvale CA, USA) capillary of 50 cm bed length was used. A flow rate of 300 nL/min was employed with a solvent gradient starting with 97% solvent A and 3% solvent B (A is always 100% B) to 35% B for 97 min, and to 50% B for 20 min, and then to 80% B for 2 min. Solvent A was 0.1% formic acid (in water) and solvent B was 0.1% formic acid, 90% acetonitrile, and 9.9% water. The mass spectrometer was operated in the data-dependent mode to automatically switch between mass spectrometry (MS) and tandem MS (MS/MS) acquisition. Survey full scan MS spectra (from m/z 400 to 1,700) were acquired with the resolution R = 70,000 at m/z 200 after accumulation to a target of 1e6. The maximum allowed ion accumulation times were 100 ms. The method used allowed sequential isolation of up to the ten-most intense ions, depending on signal intensity (intensity threshold 1.7e4), for fragmentation using higher collision induced dissociation (HCD) at a target value of 10,000 charges and a resolution R = 17,500. Target ions already selected for MS/MS were dynamically excluded for 30 s. The isolation window was m/z = 2 without offset. The maximum allowed ion accumulation for the MS/MS spectrum was 60 ms. For accurate mass measurements, the lock mass option was enabled in MS mode, and the polydimethylcyclosiloxane ions generated in the electrospray process from ambient air were used for internal recalibration during the analysis.

**Data Analysis**

Data were acquired using Xcalibur v2.5.5 and raw files were processed to generate peak list in Mascot generic format (*.mgf) using ProteoWizard release version 3.0.7230. Database searches were performed using Mascot in-house version 2.4.0 to search the SwissProt database (human, 21.01.2016, 20187 proteins) assuming the digestion enzyme trypsin, at maximum one missed cleavage site, fragment ion mass tolerance of 0.05 Da, parent ion tolerance of 10 ppm, and oxidation of methionines, and acetylation of the protein N terminus as variable modifications. Scaffold (version Scaffold_4.4.3, Proteome Software, Portland, OR) was used to validate MS2-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold local false discovery rate (FDR) algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability. A threshold of 2-fold and multiple testing corrections (p < 0.05, Benjamini Hochberg) were used for analysis of differently expressed proteins using the Scaffold software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.07.011.

**AUTHOR CONTRIBUTIONS**

Conceptualization, R.N.A, T.A.K., and J.E.B.; Methodology, R.N.A.; Investigation, R.N.A, T.A.K., and J.E.B.; Writing – Original Draft, R.N.A.; Writing – Review & Editing, T.A.K. and J.E.B.; Funding Acquisition, J.E.B.; Resources, J.E.B.; Supervision, T.A.K. and J.E.B.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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