microRNA-140 Targets RALA and Regulates Chondrogenic Differentiation of Human Mesenchymal Stem Cells by Translational Enhancement of SOX9 and ACAN

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Abstract

Lesions of articular cartilage do not heal spontaneously. One treatment strategy would be to make cartilage in the laboratory by directed chondrogenic differentiation of mesenchymal stem cells (MSCs). To promote our understanding of the molecular control of chondrogenesis, we have compared the changes in microRNAs (miRNAs) during in vitro chondrogenesis of MSCs with those observed in uncultured and dedifferentiated articular chondrocytes (ACs). Several miRNAs showed a reciprocal relationship during the differentiation of MSCs and dedifferentiation of ACs. miR-140-5p and miR-140-3p changed the most during in vitro chondrogenesis, they were the miRNAs most highly expressed in tissue-engineered chondrocytes, and they were also among the miRNAs most highly expressed in uncultured ACs. There was a 57% overlap for the 100 most highly expressed miRNAs in differentiated MSCs and uncultured ACs, but for other miRNAs, the expression pattern was quite different. We transiently and stably inhibited and overexpressed miR-140-5p and miR-140-3p in differentiating MSCs and dedifferentiating ACs, respectively, to describe global effects and identify and validate new targets. Surprisingly, SOX9 and aggrecan proteins were found to be downregulated in anti-miR-140 transduced differentiating MSCs despite unchanged mRNA levels. This suggests that miR-140 stimulates in vitro chondrogenesis by the upregulation of these molecules at the protein level. RALA, a small GTPase, was identified as a miR-140 target and knockdown experiments showed that RALA regulated SOX9 at the protein level. These observations shed new light on the effect of miR-140 for chondrogenesis in vitro and in vivo.

Introduction

Articular cartilage has very limited regeneration capacity, and small injuries may lead to severe pain and joint disability due to secondary osteoarthritis (OA) [1,2]. Treatment strategies aim to establish repair tissue with properties identical to native cartilage that integrates with the tissue surrounding the lesion. Many surgical techniques have been developed, but so far, none fulfill these requirements. Currently, autologous chondrocyte implantation (ACI) is the most widely used cell-based procedure for cartilage repair [3]. ACI involves the isolation of chondrocytes from a piece of cartilage followed by monolayer expansion before implantation as a cell suspension back into the defect. Monolayer expansion leads to dedifferentiation of the chondrocytes, which means that the extracellular matrix (ECM) molecules type II collagen (COL2) and aggrecan (ACAN) specific for hyaline cartilage are replaced by type I collagen (COL1) and versican, typical of fibrocartilage [4,5]. ACI-derived repair tissue consists mainly of fibrocartilage and has inferior properties compared to hyaline cartilage [6,7]. An alternative strategy would be to make articular cartilage in the laboratory based on cells and biomaterials. If the engineered tissue could be made with the thickness, size, and shape of the cartilage lost from the lesion, it could be implanted to fill the lesion with the ready-made articular cartilage. For this strategy, autologous bone marrow-derived mesenchymal stem cells (BM-MSCs) are a very attractive source of cells. They may be obtained from the BM with minimal discomfort and no residual morbidity and are thought to be very similar
to the mesenchymal precursors of embryonal chondrocytes [8]. To achieve this goal, it is essential to understand the molecular mechanisms involved in the chondrogenic differentiation of MSCs. As the dedifferentiation of articular chondrocytes (ACs) may move them in the direction of MSCs [9], one strategy would be to compare the MSC differentiation and AC dedifferentiation programs.

In the present study, we focused on microRNAs (miRNAs), which are small endogenous RNA molecules that regulate gene expression in a sequence-dependent manner and are thought to be involved in most biological processes, including cartilage development [10–12].

miRNAs assert their effect through the RNA-induced silencing complex, where they interact by complementary binding of the miRNA seed sequence (nucleotides 2–7 from the 5’-end) to mRNA molecules, primarily in their 3’ untranslated region (UTR), resulting in either degradation of the mRNA or translational repression [13–15]. Specifically, we compared changes in miRNA expression during chondrogenic differentiation of MSCs with those occurring during dedifferentiation of ACs. We identified miR-140-5p and miR-140-3p to be the miRNAs most highly expressed in chondrogenically differentiated MSCs and uncultured ACs. We transiently and stably inhibited and overexpressed miR-140-5p and miR-140-3p in differentiating MSCs and dedifferentiating ACs to describe global effects and identify and validate new targets. Surprisingly, we found that the inhibition of miR-140 led to the downregulation of key chondrogenic markers SRY (sex determining region Y)-box 9 (SOX9) and ACAN at the protein level. We validated RALA, a small GTPase not previously known to be involved in chondrogenesis, as a new direct target of miR-140-5p and showed that a knockdown of RALA during early chondrogenesis led to a significant upregulation of SOX9 protein expression.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Information about Taqman assays, pre-miR miRNA precursor mimics, lentiviral vectors, and antibodies are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Isolation and culture of cells

BM was obtained from healthy voluntary donors. Cartilage used for miRNA profiling was obtained from legs amputated for reasons other than cartilage disease as previously published [16]. For all other experiments, cartilage was selected from the healthy looking part of tissue removed during total knee replacement surgery. The Regional Committee for Medical Research Ethics, Southern Norway, approved the study, and all donors gave written consent. BM-MSCs and ACs were isolated and cultured as described previously [16,17] and in Supplementary Material.

Total RNA/miRNA isolation and RT-qPCR

Total RNA and miRNA were purified using the miRNeasy mini kit following protocols from the manufacturer (Qiagen). cDNA synthesis and RT-qPCR was performed following protocols from the manufacturer using the High Capacity cDNA Reverse Transcripton Kit (Applied Biosystems) [17]. Subsequently, RT-qPCR was performed using the Taqman Gene or MicroRNA Expression assays and Taqman Universal PCR master mix with the 7300 Real-Time RT PCR system (Applied Biosystems). For details, see Supplementary Material.

Global miRNA profiling

BM-MSCs were differentiated in alginate in chondrogenic medium as described in Supplementary Material. All sample preparation and hybridization was performed according to the manufacturer’s instructions (NanoString Technologies). Hundred nanograms of total RNA was used as input for the nCounter human v1 miRNA kit containing 734 unique probes based on miRbase release 14.0, (cat.no.GXA-MIR-24) and a custom-made chondrogenic gene set (Supplementary Table S2). All hybridization reactions were incubated at 65°C for a minimum of 12 h, purified, and counted on the nCounter Prep Station and Digital Analyzer (NanoString). Normalization for lane-to-lane variation, positive spike-in-control series, and endogenous controls were performed according to the manufacturer.

miRNA profiling of AC dedifferentiation was obtained previously using the miRNA microarray service from LC Sciences [16]. Reanalysis of the normalized AC microarray data [16] and primary analysis of the BM-MSC Nanostring data were performed using J-express PRO (www.molmine.com) and included log(2) transformation and hierarchical clustering. Differential expression between samples was determined using the Rank product false discovery rate method [18].

Transient transfection

The Amaxa Nucleofection system was used according to the Human Chondrocyte Nucleofector Kit protocol from the manufacturer (Lonza). For details, see Supplementary Material.

Lentiviral transduction

For details on the preparation of transduction competent lentiviral particles, see Supplementary Material. BM-MSCs were transduced at passage 2, whereas ACs were transduced after 2 days in culture. A multiplicity of infection of 1 was used to transduce with miRZip anti-microRNA-140-5p, miRZip control, Lentii-miR microRNA-140-5p, and Lentii-miR control vectors. Cells were cultured with lentiviral particles overnight, in medium containing 10% FBS and 1 µg/mL of Polybrene (Santa Cruz Biotechnology), then washed several times with PBS before adding fresh growth medium containing 10% human platelet lysate plasma (hPLP) and antibiotics. All lentiviral constructs contained copGFP, but only the miRZip vectors contained a Puromycin-resistant gene for the selection of transduced cells. Therefore, BM-MSCs cultures were treated with Puromycin at a concentration (1 µg/mL) sufficient to kill >95% of untransduced cells in pilot experiments after 48 h before embedding the cells in 1% alginate for chondrogenic differentiation. ACs were cultured for 14 days in monolayer and sorted for GFP-positivity on a
FACS-Aria cell sorter (Becton, Dickinson) to achieve >95% positive cells before isolation of total RNA.

**Microarray analysis**

Microarray analyses of transduction and transfection experiments were performed at the Norwegian Microarray Consortium, Oslo University Hospital. For details, see Supplementary Material.

**Bioinformatics analysis**

Gene lists were compared using freely available software (Whitehead Institute for Biomedical Research, http://jura.wi.mit.edu/bioc/tools/). Gene Ontology (GO) overrepresentation analysis was performed using the DAVID Bioinformatics Resource Database v6.7 [19]. Evaluation of predicted targets for miRNAs was performed using the mirWalk-search engine (www.unm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) [20] supplemented with searches in TargetScan 6.2 (http://targetscan.org) and microRNA.org (www.microrna.org).

Analysis of possible base-pairing between miR-140-5p and the 5’UTRs of SOX9 and ACAN was performed using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/mnahybrid/) with and without helix constraint for nucleotides 2–7 of the miRNA seed sequence.

**Western blotting**

The alginate discs were crushed with a pestle in liquid nitrogen and suspended in Laemmli loading buffer. Cell lysates corresponding to 100,000 BM-MSCs were loaded onto an 8% or 10% polyacrylamide gel. For transiently transfected ACs in monolayer cultures, cell lysates corresponding to 200,000 cells were used. Proteins were transferred to PVDF membranes and blocked for 1 h in 5% milk. The membrane was incubated overnight with appropriate primary antibodies followed by washing and incubation with horseradish peroxidase-conjugated secondary antibodies. Pierce ECL western blotting substrate solution (Thermo Scientific) was used for development, and pictures were captured using the Carestream Image Station 4000 R Pro and the Carestream MI software (Carestream). To determine molecular weights, the Precision Plus Protein All Blue Standards was used (Biorad). β-actin (ACTB) was used as loading control.

**Glycosaminoglycan secretion**

Sulfated glycosaminoglycan (GAG) secretion was quantified using the Blyscan Sulfated GAG Assay kit following protocols from the manufacturer (Biorcolor) and read on an automated plate reader (FluoStar OPTIMA, BMG Labtech).

**miRNA-luciferase reporter assay**

Luciferase reporter assays (GoClone Reporter, SwitchGear Genomics) for 3’UTRs from selected possible targets and appropriate controls were performed in a 96-well format (50 ng of reporter/well) using Lenti-X 293T cells and the Lightswitch Luciferase Assay System according to the manufacturers protocol and read on an automated plate reader (FluoStar OPTIMA).

**Fluorescence immunohistochemistry**

Fixation, embedding, sectioning, and fluorescence staining were performed as previously described [21]. For details, see Supplementary Material.

**Statistics**

Individual donors are presented with no statistics applied, aggregates are presented with mean and standard error. Array data were analyzed as described above, otherwise results were analyzed and graphed using Graphpad Prism 6 (Graphpad Software). When aggregates are presented, statistical testing was performed using repeated-measures one-way ANOVA with Dunnett’s multiple comparisons test on log-transformed values to account for nonnormal distribution. Significance was assumed with corrected \( P \) values <0.05. Correlations were calculated using the Spearman nonparametric method.

**Results**

**Comparison of miRNA profiles between differentiated MSCs and uncultured ACs**

MSCs were validated (Supplementary Fig. S1) and differentiated using a common chondrogenic strategy with expression of standard chondrogenic markers at the mRNA and protein level (Fig. 1A). The characteristics of the dedifferentiating ACs have been described previously [16]. Several miRNAs changed significantly during differentiation and dedifferentiation (Fig. 1B, C, Table 1 and Supplementary Table S3), with more transcripts changing at later time points. RT-qPCR validation of the global miRNA profiling was performed for six miRs (Supplementary Fig. S2). Several miRNAs showed a reciprocal relationship during the differentiation of MSCs and dedifferentiation of ACs, including miR-140-5p and miR-140-3p (Fig. 1B, C). These miRNAs also changed the most during culture (Table 1 and Supplementary Table S4). miR-140-5p and miR-140-3p are known to be predominantly expressed in articular cartilage [12,16,22]. Similarly, miR-630 has previously been labeled a chondromiRNA and was the most highly upregulated miRNA on day 3 and day 7 in the differentiated MSCs [23].

Chondrocytes differentiated from MSCs should be as similar as possible to native ACs. This should presumably include also expressing an identical miRNA profile. To evaluate this, we performed a comparison between the miRNA expression in chondrogenically differentiated MSCs with that found in uncultured ACs (Fig. 1D). There was a 57% overlap for the 100 most highly expressed miRNAs in differentiated MSCs and uncultured ACs (Spearman’s \( r=0.3261, P<0.0001 \)). miR-140-5p and miR-140-3p did not only change the most during the culture of MSCs and ACs but were also among the most highly expressed miRNAs in the differentiated MSCs and the uncultured ACs (Fig. 1D and Supplementary Table S4). However, the nonoverlapping miRNAs highly expressed in differentiated MSCs were either not expressed or barely detectable in uncultured ACs, and the nonoverlapping miRNAs highly expressed in uncultured ACs were either not expressed or barely detectable in differentiated MSCs (data not shown), suggesting a
considerable difference at the level of miRNA expression between the two cell populations.

Overexpression and inhibition of miR-140-5p and miR-140-3p reveals changes in the transcriptome and possible miRNA targets

With previously published data [12,16], the results presented here strongly suggest a role for miRNA-140 in chondrogenesis. However, the mechanism by which miR-140 impacts on cartilage formation has not been fully described. To elucidate the miR-140 functionality in chondrogenic differentiation and dedifferentiation, we performed transduction experiments to permanently inhibit the upregulation of miR-140-5p in differentiating MSCs or permanently inhibit the downregulation of miR-140-5p and miR-140-3p in dedifferentiating ACs (Fig. 2A, C). We also performed transient transfection assays to evaluate changes in gene expression soon after the introduction of exogenous pre-miR-140 (Fig. 2E). Transduction efficiencies of MSCs were confirmed before embedding using flow cytometry (65%–95% GFP-positive, mean 79%, with similar levels of positivity within donors) and by fluorescence microscopy during and at termination of differentiation (Supplementary Fig. S3). Expression changes in miR-140 and the effect on BMP2, a previously validated mRNA target of miR-140 [24], verified the functional efficiency of the transductions and transfections (Fig. 2B, D, F). Both miR-140-5p and miR-140-3p were downregulated after transduction of anti-miR-140-5p (Fig. 2B).

RNA was obtained at time points indicated in Fig. 2, and microarray analysis was performed. First, we carried out a GO term analysis on the transcriptome data (Supplementary Table S5) with input lists of statistically significant up- or downregulated genes (Supplementary Table S6) after either stable miR-140-5p inhibition in differentiating MSCs or transient miR-140-5p and miR-140-3p overexpression in dedifferentiating ACs. Inhibition of miR-140-5p led to the upregulation of genes involved in cytoskeleton remodeling and cell division and downregulation of genes related to extracellular regions and ECM. These changes were mirrored in the transient overexpression of miR-140-5p and miR-140-3p where downregulated genes mapped to GO terms related
### Table 1. Significantly Up- and Downregulated miRNAs in Mesenchymal Stem Cells Undergoing Chondrogenic Differentiation

<table>
<thead>
<tr>
<th>Upregulated</th>
<th>Day 1</th>
<th>Fold change from control</th>
<th>Day 3</th>
<th>Fold change from control</th>
<th>Day 7</th>
<th>Fold change from control</th>
<th>Day 21</th>
<th>Fold change from control</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsamir-1974</td>
<td>249</td>
<td>8.1</td>
<td>hsamir-630</td>
<td>1577</td>
<td>15.3</td>
<td>hsamir-630</td>
<td>2171</td>
<td>21.0</td>
</tr>
<tr>
<td>hsamir-1975</td>
<td>158</td>
<td>5.5</td>
<td>hsamir-1974</td>
<td>244</td>
<td>11.1</td>
<td>hsamir-1975</td>
<td>416</td>
<td>14.3</td>
</tr>
<tr>
<td>hsamir-1290</td>
<td>74</td>
<td>5.2</td>
<td>hsamir-1975</td>
<td>320</td>
<td>8.0</td>
<td>hsamir-140-5p</td>
<td>3,285</td>
<td>13.7</td>
</tr>
</tbody>
</table>

| Downregulated | | None | | hsamir-193a-3p | 19  | 5.3 | hsamir-221 | 972  | 2.6 | hsamir-221 | 374  | 6.6  |
|               | |     | | hsamir-1246 | 270  | N/A | hsamir-181a | 2,310 | N/A | hsamir-181a | 2,310 | N/A  |
|               | |     | | hsamir-1274a | 666 | 1.8 | hsamir-335 | 29  | 4.6 | hsamir-100 | 1,222 | 3.1 |
|               | |     | |               |     |     | hsamir-47e | 132 | 2.8 | hsamir-216a | 254 | 2.2  |

Statistics for assuming significance; rank product was used to compute the false discovery rate, cut-off set at $<0.10$ for days 1, 3, and 7 and $<0.05$ for day 21.
to cytoskeleton and cell cycle and upregulated genes mapped to the extracellular region terms as well as terms relating to regulation of cell death (Supplementary Table S5).

For hypothesis generation, we produced lists of genes with a change in mean expression more than 1.5-fold without statistical testing. Comparing these lists revealed that no more than seven genes changed in all three experiments, even at this very permissive threshold (Fig. 3). To further increase the likelihood that these genes were direct targets of miR-140, we performed a bioinformatic search for genes with computationally predicted interaction with the miR-140 seed sequences in seven openly available databases. This provided additional support for five of the seven genes: CD248, LIPA, RALA, THBS2, and TMEM119. From scrutiny of lists of genes that changed in only two of the three conditions to look for interesting targets, we also identified CD44 and JAG1, which were downregulated in dedifferentiating ACs transiently transfected with miR-140 and upregulated in differentiating MSCs transduced with anti-miR-140. The characteristics for the computationally predicted binding sites in the 3'UTR are given in Table 2. Notably, we found some of the interactions to be broadly conserved between species.

Validation of RALA as a target of miR-140-5p

RT-qPCR was performed to confirm our findings from the microarray analysis (Fig. 4). RALA, CD248, TMEM119, LIPA, JAG1, THBS2, and CD44 mRNAs were all found to be upregulated in differentiated MSC transduced with a short hairpin anti-miR-140-5p construct (Fig. 4A). However,
following transient overexpression of pre-miRs-140 in ACs, we found the downregulation of only RALA and CD248 following transfection of pre-miR-140-5p and of TMEM119, JAG1, and THBS2 following transfection of pre-miR-140-3p. This is in line with the predicted conserved binding sites (Table 2), although JAG1 does have a broadly conserved binding site for both miR-140-5p and miR-140-3p. LIPA was not affected by transfection of any of the sequences (Fig. 4B).

Based on the level of species conservation and the RT-qPCR observations, we investigated the direct interaction between the 3’UTR and miR-140-5p and miR-140-3p on three of the genes: RALA, JAG1, and CD248. Plasmids with the 3’UTR of these genes cloned upstream of a luciferase reporter were co-transfected with pre-miR-140-5p and pre-miR-140-3p, respectively. We found that only with the RALA 3’UTR did we see a reduction in the luciferase signal (Fig. 4C). Interestingly, we found evidence of binding for both the miR-140-5p and the miR-140-3p strand, in contradiction to the RT-qPCR finding but in line with the bioinformatics, which predicted an interaction also with the -3p strand. We confirmed the interaction at the protein level in both MSCs and ACs, but only with mir-140-5p (Fig. 4D).

**The effect on chondrogenesis by inhibition of miR-140-5p expression**

To further study the global effect of inhibiting miR-140-5p during chondrogenesis, we first examined the temporal changes of miR-140-5p expression in the lentivirally anti-miR-140-5p transduced cells in three separate donors (Fig. 5A). Before commencing differentiation, the level in anti-140-5p treated cells was approximately 40% of the control cells. There was relatively little donor difference in the miR-140 levels in the control transduced cells, but there was a considerable difference in the degree of miR-140 inhibition between donors over 3 weeks of differentiation. This is likely due to the fact that the integrated anti-miR-140 is constitutively expressed under the CMV promotor, and with the induction of endogenous miR-140 during differentiation, the available anti-miR-140s are simply saturated. The donor variation was mirrored in the cumulative GAG assay where miR-140 inhibition lead to decreased GAG production (Fig. 5B).

Key chondrogenic genes such as SOX9 and ACAN were unchanged, whereas COL2A1 was downregulated in the microarray analysis of gene expression in the anti-miR-140 transduced cells on day 7 of MSC differentiation (Supplementary Table S6). Figure 5C shows RT-qPCR analyses for these genes in each donor at several time points. The mRNA levels for these genes showed little between-donor variations and increased particularly from day 0 to day 7. There was little difference in the gene expression between anti-miR-140 transduced and control cells for all time points except perhaps for COL2A1 mRNA for donors 1 and 3, which were reduced on day 7. For SOX9 and ACAN mRNA, the levels were similar or even slightly increased in the anti-miR-140 transduced cells, supporting the microarray analysis from day 7. We proceeded to investigate protein levels of these molecules on day 7 (Fig. 5D, COL2 not detected) and day 14 (Fig. 5E). Despite unchanged or even slightly higher levels of mRNAs in the anti-miR-140 transduced cells, the SOX9 and ACAN protein levels were greatly reduced in the cells from all three donors for both proteins. This was not the result of a general inhibition of protein synthesis, as the levels of the loading control, ACTB, was similar for the anti-miR-140 transduced and control cells. In line with the mir-140-5p expression, donor 2 showed the smallest difference in expression in treated cells. The effect of the anti-miR-140 transduction was similar on the SOX9 protein level on day 14 of differentiation, whereas ACAN levels were reduced at this time point only in donor 3 (Fig. 5E). At this time point, COL2 was detectable. Similar to GAG levels, the COL2 levels in donor 3 were very low and required prolonged exposure to
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Synonyms</th>
<th>mir-140</th>
<th>Predicted in databases (out of 7)</th>
<th>Conserved (no. of species)b</th>
<th>Pairing in 3’UTRc</th>
<th>Pairing alignmentd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD248</td>
<td>endosialin; TEMI</td>
<td>-5p</td>
<td>4</td>
<td>Poorly conserved (5)</td>
<td>131-138 (8mer)</td>
<td>3’ GAUGGUAUCCCAUUUUGUGAC 5’</td>
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<td></td>
<td>117:5’ CAGGGUCCUCUCUCAACCACUA 3’ CD248</td>
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<tr>
<td>LIPA</td>
<td>LAL; CESD</td>
<td>-5p</td>
<td>2</td>
<td>Poorly conserved (4)</td>
<td>803-809 (7mer-1A)</td>
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<td>788:5’ CCCUGUGGAGACUAUACCACUA 3’ LIPA</td>
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<td>RALA</td>
<td>RAL</td>
<td>-5p</td>
<td>6</td>
<td>Broadly conserved (19)</td>
<td>1708-1715 (8mer)</td>
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<td>112-118 (7mer-m8) 716-721 (6mer)</td>
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<td>1164:5’ CGUACAAUAUACUGAACCACUU 3’ JAG1</td>
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<td>142:5’ CAAUGGUGGU-UUCUGUGGUU 3’ JAG1</td>
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<td></td>
<td>2886:5’ UUGUGCUGUACAAUGACCAUG 3’ CD44</td>
</tr>
</tbody>
</table>

aUsing mirWalk searching in DIAAnMt, miRanda, mirDB, mirWalk, PicTar5, PITA, Targetscan.
bObtained from Targetscan6.2 (www.targetscan.org).
cObtained from Targetscan6.2 or miranda (www.microrna.org) (6mer: an exact match to positions 2-7, 7mer-1A: an exact match to positions 2-7 followed by an “A”, 7mer-m8: an exact match to positions 2-8, 8mer: an exact match to positions 2-8 followed by an “A”).
dShown for the best pairing/most conserved site.
be detected. Under these conditions, COL2 levels were reduced in donor 3 in the anti-miR-140 transduced cells. No clear difference could be seen for the COL2 levels in cells from donors 1 and 2. SOX9 is the primary transcription factor regulating COL2A1 expression [25]. Changes in the mRNA levels of COL2A1 reflecting the SOX9 protein expression could be seen on day 7 and day 14 in donor 3 and day 7 in donor 1.

For miR-140 to directly affect SOX9 and ACAN protein levels, even if the mRNA levels were grossly unchanged, one would expect to find sequence complementarities between miR-140 and SOX9 and ACAN mRNAs. However, the mirWalk search engine, which combines several commonly available miRNA target databases, retrieved no possible targets for the miR-140 seed sequence binding sites in the 3′UTR of SOX9 and ACAN. However, as most databases
FIG. 5. Differentiating MSCs transduced with anti-miR-140 show impaired chondrogenesis and reduced expression of SOX9 and ACAN proteins, but little or no change in corresponding mRNAs. (A) Kinetics of miR-140-5p expression in anti-miR-140 transduced (black circles) and anti-negative control transduced (black triangles) MSCs during chondrogenic differentiation. Table inserts represent relative inhibition at each time point. (B) Cumulative values of sGAGs obtained from supernatants at every medium change of transduced MSCs undergoing differentiation in alginate discs. (C) Gene expression values relative to GAPDH of SOX9, COL2A1, and ACAN in anti-miR-140 transduced (gray bars) and anti-negative control transduced (black bars) MSCs at day 0, 7, 14, and 21 of chondrogenic differentiation. Fold change values relative to control transduced MSCs for each time point are given above each pair of bars. Asterisks indicate not detected. (D) Western blots of SOX9 and ACAN in cell lysates from anti-miR-140-5p and control transduced MSCs at day 7 after chondrogenic differentiation. For donor 3, SOX9 and ACAN needed longer exposure to visualize bands, indicating lower expression. ACTB was exposed identically for all donors. (E) Western blots of SOX9, ACAN, and COL2 in cell lysates from anti-miR-140-5p and control transduced MSCs at day 14 after chondrogenic differentiation. For donor 3, SOX9, ACAN, and COL2 needed longer exposure to visualize bands indicating lower expression. ACTB was similarly expressed in all donors. D1, donor 1; D2, donor 2; D3, donor 3; sGAGs, sulfated glycosaminoglycans; CTRL, control. Donor markings on top of panel (A) apply to (A, B, C) and (D). Curve symbols in panel (A) apply to (A) and (B). Values on horizontal axis represent days after embedding into alginate.
have not been updated with 5’UTR sequences, we manually performed these comparisons. This revealed targets with the best binding thermodynamics (lowest calculated minimum free energy) ranging from −18.4 to −22.8 kcal/mol (Supplementary Fig. S4), well within the range of what has been found with validated 5’UTR targets [26].

Another explanation for the upregulation of SOX9 and ACAN proteins following anti-miR-140-5p treatment is that miR-140 regulates these proteins by targeting a gene responsible for the degradation or inhibition of their translation. ADAMTS5 is an enzyme that degrades ACAN and has been shown to be a target of miR-140. Therefore, we performed western blotting to see if ADAMTS5 protein expression changed after anti-miR-140 inhibition. In the control cells, there were two bands representing ADAMTS5 for donors 1 and 2 and one sharp band for donor 3 (Supplementary Fig. S5). In the anti-miR-140-treated cells, there was an additional band for all donors suggesting proteolytic cleavage of ADAMTS5. It is uncertain if this cleavage of ADAMTS5 is responsible for the reduced ACAN protein levels, but ADAMTS5 has been shown to be activated by proteolytic cleavage [27]. However, this would not explain the reduced SOX9 levels in the anti-miR-140-5p-treated cells. To see if RALA could regulate SOX9 protein expression, we transfected siRNA against RALA before differentiating the cells. Figure 6 shows data for two of the donors. RALA mRNA and protein was clearly reduced after siRNA treatment (Fig. 6A, B). SOX9 and ACAN mRNA were barely changed in one of the donors, whereas COL2A1 mRNA was slightly upregulated (less than 1.7- and 1.5-fold) in both donors (Fig. 6A). However, after 2 days of differentiation, SOX9 protein was clearly increased in the RALA siRNA-treated cells for both donors (Fig. 6C).

Discussion

miRNAs have emerged during the past decade as a novel class of regulators of gene expression. A unique set of miRNAs is present in pluripotent stem cells and are essential for their self-renewal and differentiation [28]. miRNAs are also important for multipotent stem cell differentiation and organogenesis [29]. The importance of miRNAs for cartilage development was illustrated by severe skeletal defects in mice lacking the Dicer1 gene in chondrocytes [30]. miR-140 has been identified as a cartilage-specific miRNA [31,32]. Mice deficient in miR-140 have an OA-like pathology and a shorter skeleton compared to wild-type mice, whereas transgenic mice overexpressing miR-140 were resistant to antigen-induced arthritis [12]. The matrix-degrading protease ADAMTS5 is a target of miR-140, which partly explains the protective role of miR-140 against OA progression [12]. Other published miR-140 targets are mRNAs SP1, PDGFRα, SMAD3, BMP2, CXCL12, and HDAC4 [31,33–37]. miR-140 has also been shown to be activated by SOX9 [38,39] and thus to correlate with SOX9 expression in embryonic and in vitro chondrogenesis [40,41]. Recently, miR-140 has also been proposed to play a role in early bone development [42].

Against this background, we show that miR-140-5p and miR-140-3p are the most highly upregulated miRNAs during chondrogenic differentiation of MSCs, and among the most highly expressed miRNAs in uncultured ACs, supporting previous findings that miR-140 is essential for chondrogenesis and cartilage homeostasis. Downregulation of the targets described above may be important for cartilage homeostasis, but perhaps not sufficient to explain the effect of miR-140 observed in knockout mice and in the results shown in this study. We, unexpectedly, observed that the inhibition of miR-140-5p led to a reduction of SOX9 and ACAN protein but not their corresponding mRNAs, indicating that miR-140 induces a post-transcriptional upregulation of these essential chondrogenic molecules. This would go to the core of AC functionality and might explain the extreme upregulation of

![FIG. 6. Knockdown of RALA expression by siRNA leads to an upregulation of SOX9 protein.](image_url)
miR-140 during in vitro chondrogenic differentiation of MSCs and the essential role previously described for miR-140 in vivo chondrogenesis.

However, this raises new questions: what is the mechanism for this observation, and how can this be reconciled with the rest of our data and existing literature? miRNA-induced upregulation of translation has been described in other model systems. miR-369-3p was found to direct the association between RISC-associated proteins and the AU-rich elements in TNFx mRNA. Upon cell cycle arrest, this changed from an inhibitory to a translation activation signal [43,44]. Another mechanism was seen with miRNA-10a and miRNA-122, which upon binding to their target sites in the 5′UTR of the mRNAs stimulated mRNA translation [26,45,46]. The binding of miR-10a to the 5′UTR of mRNAs of several ribosomal proteins was found to not depend on the seed sequence but to be mediated by several short sequences of complementarity between miR-10a and the target sequences. Using the full length of miR-140 as potential binding sequences, we found several possible binding motifs, both including and excluding the seed sequence, in the 5′UTRs of SOX9 and ACAN mRNAs. This may reveal a new mechanism for the interaction between miR-140 and chondrogenic molecules and also points to a large body of work that needs to be done to explain the molecular mechanisms for how this interaction subsequently leads to translational upregulation.

Another mechanism by which miRNAs may stimulate translation is through the inhibition of a translation repressive molecule. Like other canonical miRNA effects, this requires specific binding of the miRNA seed sequence to its mRNA target, followed by mRNA degradation or translational repression. For our experiment, if the mechanism was mRNA degradation, we should be able to recognize the effect molecule from the list of upregulated mRNAs in differentiating MSCs transduced with anti-miR-140. This list contains 80 differentially expressed genes (FDR < 0.05), none of which is known to repress SOX9 or ACAN translation. However, since RALA was shown to be a direct target of miR-140-5p, we performed RALA knockdown experiments using siRNA to determine if RALA could affect SOX9 mRNA or protein expression. This was found to be the case: knockdown of RALA during early chondrogenesis led to a substantial upregulation of SOX9 protein, but not mRNA. RALA has not previously been described to be important for chondrogenesis. RALA is a small GTPase involved in TGF-β/Activin signaling [47]. It contributes to internalization of Activin receptors, thus reducing the number of receptors available for ligand interaction, which in turn could inhibit signaling [47]. Activin A receptor was shown to be positively linked to chondrogenesis, with COL2 and sulfated GAGs being reduced when the Activin A receptor was knocked down [48]. Furthermore, RALA has been shown to inhibit exocytosis [49]. ACAN and its associated GAGs are transported through the endoplasmic reticulum–Golgi pathway before being secreted from the cell surface [50]. However, we show here that RALA inhibits SOX9 protein abundance without affecting SOX9 mRNA or actin protein. This suggests an effect of RALA on the translation of some, but not all genes. RALA has previously been linked to negative regulation of translation [51]. Our observation suggests an element of specificity to this interaction, but exactly how this occurs and how this affects in vitro chondrogenesis remains to be established.

SOX9 is considered to be the master regulator transcription factor for chondrogenesis [52]. Particularly, it has been shown to activate COL11A1, COL2A1, and ACAN. When SOX9 was eliminated from undifferentiated mesenchyme in limb buds, mature cartilage and bone failed to develop, suggesting that SOX9 was required for specification of osteochondroprogenitors [53]. We see the expected effect of SOX9 protein downregulation on COL2A1 mRNA in donor 3 at day 7 and day 14 and at day 14 on COL2 protein. It is, however, a puzzling observation that the substantial downregulation of SOX9 protein seen in donors 1 and 2 on day 7 and day 14 is not reflected consistently in COL2A1 mRNA. Surprisingly, we do not see changed ACAN mRNA levels when SOX9 is downregulated. However, the control of ACAN is not completely understood, and although SOX9 has been shown to enhance ACAN expression [54], it has also been described that other factors may independently regulate its expression [55].

One possible explanation for the decreased ACAN protein in the anti-miR-140 transduced cells could be through the effect of the aggrecanase ADAMTS5. ADAMTS5 was validated as a miR-140 target previously [12], both at the mRNA and protein level, but it was not upregulated in our anti-miR-140 microarray experiments. Still, if the canonical effect of miR-140 on ADAMTS5 in our experimental set-up is translational repression, then the reduced ACAN protein levels observed in the anti-miR-140 transduced cells could well be mediated by increased levels of ADAMTS5 protein. We did not see clear differences in absolute expression of ADAMTS5 protein, but we did see changes suggestive of cleavage and activation of ADAMTS5 protein in the anti-miR-140 transduced cells. Interestingly, we also see a reduction in secreted GAGs. These are predominantly chondroitin sulfate and keratan sulfate, which attach to ACAN in native articular cartilage. Reduction in ACAN in the anti-miR-140 transduced cells could possibly affect GAG release.

Using the gene lists obtained from microarray analysis, we searched in seven different databases to screen for predicted binding sites of the miR-140 seed sequence. This strategy identified five possible targets. In addition, JAG1 and CD44 were predicted to be targets and changed accordingly in two of the three conditions. miR-140 is highly conserved across species, suggesting that this miRNA occupies an important biological role [24]. Predicted target sites that are also broadly conserved across species are more likely to be important targets [11]. To further increase the likelihood of finding targets, we looked for conserved predictions. Only RALA and JAG1 were broadly conserved across species. Of the genes analyzed, only RALA was confirmed by RT-qPCR to be upregulated in anti-miR-140 transduced cells, downregulated by pre-miR-140-5p transduction, downregulated at the protein level by transient transfection of pre-miR-140-5p, and downregulated in the luciferase reporter assay. We did not identify all the previously validated miR-140 targets in our experiments. This may be due to different experimental strategies, but may also be due to the fact that the same miRNA does not necessarily target the same mRNA in different cells, or even in the same cells under different conditions [56].

Interestingly, we found that the GO terms most enriched with mRNAs downregulated following miR-140-5p
inhibition in differentiating MSCs included ECM or extracellular space molecules. The same GO terms were also enriched for upregulated mRNAs in dedifferentiating ACs transiently overexpressing pre-miR-140. This is difficult to reconcile with the idea of these ECM molecules being direct targets of miR-140 but could be explained by the miR-140-mediated post-transcriptional enhancement of SOX9, the master regulator of chondrogenesis. However, the effect could also partly be due to miR-140 targeting chondrogenic repressors. On the other hand, the GO terms best associated with upregulated mRNAs following miR-140 inhibition and with downregulated mRNAs in pre-miR-140-treated cells included molecules involved with the cytoskeleton and cell cycle. This corresponds with observations made for miR-140 in cancer cells, and most likely stems from canonical miR-140 inhibition effects [57].

At early time points, we saw only few miRNAs changing significantly. Interestingly, on day 3 and day 7 miR-630 was the most upregulated miRNA during in vitro chondrogenesis. This is in line with recent findings linking this miRNA to chondrogenesis [23]. On day 7 and day 21, the up- and downregulated miRNAs during in vitro chondrogenesis included several previously known to be linked to differentiation or chondrogenesis, that is, miR-21, miR-210, miR-181a, miR-221, and miR-335 [58–62]. MiR-21 has been shown to be the most upregulated miRNA during in vitro chondrogenesis [23]. On day 7 and day 21, the up- and downregulated miRNAs during in vitro chondrogenesis included several previously known to be linked to differentiation or chondrogenesis, that is, miR-21, miR-210, miR-181a, miR-221, and miR-335 [58–62]. MiR-21 has been shown to be upregulated by TGF-β and has in some experiments been associated with increased COL1 synthesis [63]. COL1A1 mRNA is highly expressed during in vitro chondrogenesis [21], and a change from COL2 to COL1 synthesis is a hallmark of dedifferentiating chondrocytes [64]. Consistent with this, miR-21 is also one of the upregulated miRNAs during dedifferentiation. MiR-210 is commonly labeled a hypoxic miRNA, being upregulated under hypoxic conditions [65]. In line with studies of adipose tissue-derived MSCs [66], we see in this study that miR-210 is gradually upregulated during chondrogenesis and downregulated during dedifferentiation. The upregulation would be consistent with increasingly hypoxic conditions in the 3D cultures, where matrix production and increased metabolism may lead to decreased oxygen diffusion into discs. We observed miR-335 to be downregulated during chondrogenesis. This is in line with already published findings that the downregulation of miR-335 is critical in MSC differentiation [62,67]. Recently, miR-574-3p was shown to inhibit chondrogenesis of MSCs [68]. Interestingly, we find this miRNA to be significantly upregulated at day 21 in differentiating MSC and the complementary miR-574-5p to be upregulated at day 7 in dedifferentiating AC indicating that this miRNA could be further explored in the setting of in vitro chondrogenesis.

Conclusion

In this study, we show that the miRNA profile of chondrocytes differentiated from MSCs partly resembles that of uncultured ACs. We also show that miR-140-5p and miR-140-3p are the most highly upregulated miRNAs during chondrogenic differentiation of BM-MSCs, and they are among the most highly expressed miRNA in uncultured chondrocytes. We identify RALA as a novel target of miR-140-5p, and inhibition of RALA during chondrogenesis led to an upregulation of SOX9 protein expression indicating a previously unknown role of RALA in chondrogenesis.

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Author Disclosure Statement

The authors declare that they have no potential conflicts of interest.

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