miR-1247 Functions by Targeting Cartilage Transcription Factor SOX9

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Background: The function of miR-1247 was heretofore unknown.
Results: We show that miR-1247 directly targets SOX9, a transcription factor essential for cartilage formation and function.
Conclusion: miR-1247 may be an important regulator of cartilage function.
Significance: miR-1247 is a potential new target for joint repair.

microRNAs are a large and essential class of gene regulators that play key roles in development, homeostasis, and disease. They are necessary for normal skeletal development, and their expression is altered in arthritis. However, the specific role of individual microRNAs is only beginning to be unraveled. Using microRNA expression profiling in healthy human articular cartilage cells (chondrocytes), we identified miR-1247 expression as highly correlated with that of the differentiated cell phenotype. Transcribed from the DLK1-DIO3 locus, the function of miR-1247 is completely unknown. In mice its expression level was relatively high in cartilage tissue, and correlated with cartilage-associated microRNA miR-675 across a range of 15 different mouse tissues. To further probe miR-1247 function, overexpression and inhibition studies were performed in isolated human chondrocytes. Modulation of miR-1247 was found to exert profound phenotypic effects altering expression levels of cartilage master regulator transcription factor SOX9. SOX9 is essential for cartilage development and subsequent function throughout life, and mutations in this gene result in severe dwarfism. Putative miR-1247 binding sites were further investigated using luciferase reporter assays, which indicated binding of miR-1247 to a highly conserved region in the coding sequence of SOX9 but not in its 3’-UTR. Interestingly, depletion of SOX9 in human chondrocytes resulted in increased levels of the mature, processed microRNA, suggesting a negative feedback loop between miR-1247 and its target SOX9.

Significance:
- miR-1247 functions by targeting cartilage transcription factor SOX9.
- miR-1247 is a potential new target for joint repair.
- miR-1247 expression is altered in arthritis.
- miR-1247 may be an important regulator of cartilage function.

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EXPERIMENTAL PROCEDURES

Human Articular Chondrocyte Culture—Healthy human articular cartilage was harvested from the knee following amputation due to sarcomas not involving the joint space, after informed consent and following local ethics committee guidelines. HACs were isolated as detailed previously (14) and seeded at a density of 1.2 × 10^4 cells/cm^2 in DMEM with 10% FCS. Primary (passage 0; P0) cells were harvested after 6 days of culture or were subcultured for a further 5–7 days. Primary and first or second passage (P1–P2) cells were used in experiments.

miRNA Profiling—Total RNA, including small RNAs, was extracted with TRIzol (Invitrogen) from P0 or P2 HACs, according to the manufacturer’s instructions. For miRNA detection, 1 μg of RNA were reversed transcribed as indicated by the manufacturer, using the TaqMan miRNA RT kit (Invitrogen) and Megaplex RT Primers (pool A and pool B, independently) which contain specific stem-looped RT primers for, in total, 754 mature miRNAs, three endogenous control small RNAs, and one negative control. Reverse transcription product was combined with TaqMan Universal PCR Master Mix, No AmpErase uracil N-glycosylase (UNG) (Invitrogen) and loaded on to TaqMan Array Cards (Invitrogen) following the manufacturer’s instructions. Together, the TaqMan array cards (A and B) contain sequence-specific TaqMan primers to amplify all of the reverse-transcribed miRNAs and controls detailed above. We used the Applied Biosystems 7900 HT Real-time PCR system, and data were collected and processed using the Plate Utility and Automation Controller software (Applied Biosystems). For each miRNA, expression level was calculated as fold change in P2 versus P0 HACs, determined by ∆∆Ct analysis using RNU24 as calibrator.

Individual miRNA Detection—For detection of expression of individual miRNAs, 500 ng of total RNA (from P0 HACs cultured in 20% O2 tension and P2 HACs cultured in 20% or 1% O2 tension for 36 h) were reversed-transcribed with TaqMan miRNA reverse transcription kit as described above, but including the relevant miRNA-specific TaqMan primer (Invitrogen) in the reaction. Up to three primers were used simultaneously per reaction. Reverse Transcription was followed by quantitative PCR with TaqMan Universal PCR Master Mix, No AmpErase UNG, and the appropriate miRNA-specific TaqMan probe (Invitrogen). For each miRNA, relative expression level was determined by the standard curve method.

Samples of total RNA derived from mouse tissues were purchased from Zyagen (San Diego, CA). The RNA was extracted from a total of six male C57 BL/6 mice, 12–16 weeks old. miRNA expression was analyzed as detailed above from 1 μg of RNA from each tissue. For each miRNA, absolute quantification was performed by the standard curve method using known quantities of chemically synthesized miRNA mimics (Invitrogen).

Pri-miRNA and Pre-miRNA Detection—Pri-miR-1247 was reverse-transcribed using the high capacity cDNA reverse transcription kit, in the presence either random primers or oligo dT primers (Invitrogen). Pri-miR-1247 qPCR was performed with a SYBR Green PCR Master Mix (Invitrogen) including specific primers (miScript Precursor Assays, Qiagen).

Pre-miR-1247 was detected by Northern blot as described previously (15). Briefly, 15–20 μg of total RNA were resolved on a 15% acrylamide/7.5 m urea gel and transferred to Zeta-Probe membranes (Bio-Rad) that were cross-linked and heated at 80 °C during 1 h. Hybridization was performed at 50 °C using 5’-3’-labeled hsa-miR-1247-5p miRCURY LNA Power inhibitor (Exiqon), anti-hsa-miR-1247-5p and anti-hsa-miR-1247-3p inhibitors (Ambion) or an oligonucleotide complementary to RNU24 (5’-TCTTCACTAAGCAATTTCC-3’).

Messenger RNA Reverse Transcription and Real Time PCR—First, 200–500 ng of total RNA were reverse-transcribed using the high capacity cDNA reverse transcription kit (Invitrogen). Reverse transcription was followed by qPCR with a SYBR Green PCR Master Mix (Invitrogen). The specific primers used for the different miRNAs were as follows: COL2A1, 5’-GAGGAGTGGAGACTGTGGGTTCTCTA-3’ and 5’-TCCATGTTGACAAAGAACCTCTCCA-3’; SOX9, 5’-CGCCATCTCTCAAGGCAGCCTGTGC-3’ and 5’-CCTGGGATGCTGCCCGAGTTGC-3’; ADAM15, 5’-CCTGGATGACTGCGTCGAT-3’ and 5’-GGTCAGATGCACACTGT-3’; BMP1, 5’-GCAATGATGTGCGCAATCAG-3’ and 5’-GCCATGCACTTTGGAGTA-3’; DVL1, 5’-CAGGACTACTG-3’ and 5’-GTT-GCTGAGGCCCAGAT-3’; ELAC2, 5’-AACCGGCGCTTTGGACATCT-3’ and 5’-GGGCAACCACCCACGAAAG-3’; ETS2, 5’-AATGAGAAGAACAACACTCGAAGA-3’ and 5’-GGCGAGTCTGATGGAAATGGC-3’; FAM20C, 5’-TTAGAACAATGGAGAAGGCTTTTGG-3’ and 5’-GCTGTAGCCGGCACCAGAT-3’; RARA, 5’-CGTGGCCAAAATACACTACGAAACA-3’ and 5’-ACTGAATGGTTCGACAGGAGTCA-3’; RSPO1, 5’-CGCTGCTATCCACGTTTCG-3’ and 5’-TCACATTGGCGGAGCACACTG-3’; SULF1, 5’-CTCCGGAATCTGGCAGGATT-3’ and 5’-ACTGAAACTTCTGGAGAGTTCC-3’; and RPLP0, 5’-CCATTGAAATCCTGAGTGATGTG-3’ and 5’-CCCTGCGGTCCTCACCACATT-3’.

Transient Transfections—P0–P2 HACs were seeded at 1 × 10^4 cells/cm^2 the day preceding transfection. Transfection with 5 nm control or miR-1247 mimics (Invitrogen), 25 nm control or hsa-miR-1247-5p miRCURY LNA Power inhibitors (Exiqon) or 5–10 nm control or SOX9 siRNA (siMAX, MWG Biotechnology) and control or ADAM15 siRNAs (On-target Plus, Thermo Scientific) was carried out using Lipofectamine 2000 (Invitrogen) for 4 h in OptiMEM I (Invitrogen). After transfection, medium was replaced by pre-equilibrated DMEM (in 20 or 1% oxygen) containing 10% FCS and antibiotics. Cells were incubated in the appropriate oxygen tension for a further 44 h.

Plasmid DNA was transfected using the same procedure. After 24 h, cells were lysed, and luciferase activity was determined with the Dual-Glo luciferase assay system (Promega).

Plasmid Generation—pMiRGlO Dual-Luciferase miRNA Target Expression Vector was purchased from Promega. The sequence containing three consecutive perfect matches for hsa-miR-1247-5p (5’-TCCGGGGGACACCGGACGTGTTCCGGGGACGACCGGAGCAGCGAGGTC-3’) was excised from the previously produced...
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pSG5-Luc-3xmiR-1247 and subcloned between XhoI-XbaI restriction sites in pMirGlo to generate pMirGlo 3xmiR-1247. pMirGlo SOX9 3′-UTR was made by sub-cloning the SOX9 3′-UTR, which was excised from the pSGG_Luc SOX9 3′-UTR plasmid (SwitchGear Genomics) and introduced between XhoI-Nhel in pMirGlo. The putative SOX9 binding site (SOX9 BS, positions 426 to 614 of the CDS) was amplified by PCR using the oligonucleotides, 5′-TGGAGACTTCTGAACAGAGAG-3′ and 5′-CTTGAAGATGGCGTGGG-3′, and cloned into the pGEM-T Easy vector (Promega). SOX9 BS was excised from the pGEM-T Easy vector and introduced into pMiRGlo between XhoI-Nhel to generate pMiRGlo SOX9 BS, or between XhoI-XbaI to generate pMiRGlo SOX9 BS inverted.

ADAM15 3′-UTR was amplified by PCR using the oligonucleotides 5′-TGTCCTCGCTCTAATCTT-3′ and 5′-CTCCC-AAGATGTCAGTTT-3′ and inserted into the pCR-Blunt vector (Invitrogen). The predicted miR-1247 seed site was mutated using a Phusion® site-directed mutagenesis kit (New England Biolabs) with the following mutagenic primers: 5′-TGGAGACTTCTGAACAGAGAG-3′ and 5′-CTTGAAGATGGCGTGGG-3′. ADAM15 3′-UTR and ADAM15 3′-UTR (mut) were excised from pCR-Blunt and subcloned into pSG5-Luc (a kind gift of Dr Fatima Gebauer, Centre for Genomic Regulation, Barcelona, Spain). The vector pSG5- Renilla was also a gift from Dr. Gebauer. All of the constructs were verified by sequencing.

Western Blotting—For protein analysis, HACs were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid). Total protein extracts (10–30 μg) were separated by SDS-PAGE and transferred to PVDF membranes. Polyclonal anti-SOX9 (AB5809, Millipore), polyclonal anti-ADAM15 cytoplasmic domain (AB19036, Chemicon International), and monoclonal anti-tubulin (T9026, Sigma-Aldrich) antibodies were used, and proteins were visualized by ECL fluorography (GE Healthcare).

Statistical Analysis—Statistical analysis was performed using GraphPad Prism (version 6.0). Statistical significance was evaluated by two-tailed paired Student’s t test. Spearman’s correlation analysis was used for the correlation analysis of the data. All data are shown as means ± S.E. p values < 0.05 were considered statistically significant.

RESULTS

miRNA Expression Profile Is Altered During Dedifferentiation of Human Articular Chondrocytes—When subcultured in vitro, HACs undergo loss of the differentiated phenotype, (dedifferentiation), showing reduced SOX9 expression levels and reduced capacity for synthesizing the key cartilage matrix molecules (16, 17). Thus, to determine miRNAs potentially relevant for cartilage function, we assessed those whose expression was altered during HAC dedifferentiation. Total RNA was extracted from primary culture HACs (P0) isolated from healthy cartilage from three different patients (aged 23, 62, and 53 years), or after two sequential passages (P2). The extent of dedifferentiation was confirmed by assessing the expression of the most important molecule for cartilage matrix function, COL2A1, whose expression was greatly reduced with passage for the three patients (data not shown), in line with our previous observations (9, 18). We simultaneously analyzed the expression of 754 miRNAs using TaqMan-based RT-qPCR arrays, of which almost 400 were detected in chondrocytes. From these, 29 miRNAs were up-regulated >2-fold during dedifferentiation, whereas 18 miRNAs were down-regulated (Fig. 1A). To validate our results, we performed selected individual RT-qPCR assays with the same samples used for the array experiments in addition to a further four samples. The results were confirmed for all miRNAs investigated, i.e. they showed the same directional change with passage as observed in the array experiments (Fig. 1B). Because hypoxia is a physiological condition of articular cartilage (19) and promotes expression of the differentiated HAC phenotype (9, 20), we also investigated miRNA expression in passaged HACs exposed to hypoxia. For miR-140-5p and -3p and miR-1247, there was a significant change (increase) in miRNA expression levels in HACs exposed to hypoxic treatment (Fig. 1B). The importance of miR-140-5p for the development and homeostasis of cartilage has been demonstrated (7), but the function of miR-1247 is completely unknown.

miR-1247 Is Expressed in Mouse Cartilage and Correlates with miR-675 Expression in a Range of Murine Tissues—The expression of miR-1247 was measured in total RNA isolated from 15 different murine tissues extracted from six different mice. As expected, the expression of Col2a1 and Sox9 was relatively high in the cartilage and trachea samples (Fig. 2, A and B). In addition, we measured known cartilage-associated miRNAs miR-140-5p (6) and miR-675 (10). Levels of miR-140-5p were highest in cartilaginous tissues (i.e. cartilage and trachea), but interestingly, this miRNA was also highly expressed in every tissue investigated (~60,000 copies/ng RNA) (Fig. 2C). miR-675 is differentially expressed in cartilage, with very low or undetectable levels in many of the other tissues studied (Fig. 2D). Similarly to miR-675, miR-1247 expression was very low in several tissues, whereas cartilage and trachea were two of the tissues presenting the highest levels (~2500 copies/ng RNA) (Fig. 2E). Thus, miR-1247 and miR-675 showed a very similar expression pattern across the various tissues analyzed (Fig. 2F), with the exception being the adrenal gland, whose expression was relatively high only for miR-1247.

miR-1247 Modulates Expression of Cartilage Matrix Gene COL2A1—We next investigated mechanism of action of miR-1247 in HACs by screening for its direct targets. Ten putative miR-1247 target mRNAs (as predicted by bioinformatics analyses) were investigated by RT-qPCR in HACs transfected with...
miR-1247 mimic or inhibitor. Of the 10 analyzed genes (Fig. 3), only a small but significant change in mRNA levels was detected for ADAM15 in response to miR-1247 overexpression. Conversely, ADAM15 mRNA levels were significantly upregulated upon miR-1247 inhibition (Fig. 4A). However, this effect was not observed at the protein level (Fig. 4B). In addition, luciferase binding assays failed to show any evidence of direct binding of miR-1247 to the 3′-UTR of ADAM15 (Fig. 4C), suggesting this gene may not be a biologically relevant miR-1247 target in chondrocytes. The function of ADAM15 in cartilage is unclear, and ADAM15 knockdown experiments showed that it was not regulating COL2A1 expression in HACs (Fig. 4, D–F).

We next investigated phenotypic effects of miR-1247 in HACs by measuring cartilage matrix gene COL2A1 expression after transfection of miR-1247 mimic or inhibitor (Fig. 5, A and B, respectively). The levels of COL2A1 mRNA were significantly reduced upon miR-1247 overexpression and increased in inhibitor experiments. Interestingly, miR-140 expression was modulated in a similar manner to COL2A1 upon miR-1247 overexpression and inhibition (Fig. 5, C and D). Because neither COL2A1 nor miR-140 (primary transcript) are predicted to be direct targets of miR-1247 but are both transcriptionally activated by SOX9 (22, 23), we hypothesized that their regulation by miR-1247 may be via targeting of SOX9 by this miRNA.

**miR-1247 Directly Targets SOX9 via Its Coding Sequence**—We first assessed the levels of SOX9 in chondrocytes overexpressing miR-1247, finding a strong decrease in SOX9 protein (but not mRNA) levels (Fig. 6, A and B, respectively), suggesting regulation at the level of translation. Furthermore, SOX9 protein levels were increased upon miR-1247 inhibition (Fig. 6C). No miR-1247 binding sites are predicted in the 3′-UTR of SOX9 mRNA. However, to discard the possibility of miR-1247 targeting SOX9 through a non-canonical binding site in the 3′-UTR, we cloned the latter downstream of the ORF of firefly luciferase in the pmiRGlo vector that was co-transfected in HACs with a control or a miR-1247 mimic. As expected, no changes in the luciferase activity were observed in the presence of the miR-1247 mimic, suggesting that miR-1247 does not, indeed, target the SOX9 3′-UTR. However, because miRNAs can, in many cases, target the coding sequences (CDS) of mRNAs (24), we screened for possible miR-1247 binding sites in the SOX9 CDS using MicroInspector (25) and RNAhybrid (26), and both programs detected overlapping putative binding sites in positions 525 and 540 of the CDS, respectively (Fig. 6E).

Interestingly, this region is highly conserved across six species analyzed (Fig. 6F). We thus cloned the region of the CDS (160 nucleotides) containing the predicted binding sites downstream of the luciferase ORF. Transfection of miR-1247 mimics significantly reduced luciferase activity of the construct containing the putative miR-1247 binding sites, whereas no effect was observed in reporters containing the inverted sequence (Fig. 6G). To monitor both the extent of the miR-1247 mimic repression and sensitivity of the system, we generated a reporter containing three consecutive perfect matches for miR-1247 downstream of the luciferase ORF. Repression of this construct by miR-1247 was consistent (35% repression). Furthermore, a 15% reduction in luciferase expression was observed for the construct containing the SOX9 CDS fragment. These results confirm that SOX9 is targeted by miR-1247, not through its 3′-UTR but through a highly conserved region in its ORF.

**miR-1247 Expression Is Inhibited by SOX9**—Because SOX9 is an essential transcription factor with multiple targets in cartilage, we considered the possibility that it could itself be regulating the expression of miR-1247. Initial experiments attempting to overexpress SOX9 using a DNA plasmid were unsuccessful due to cell toxicity issues (data not shown). However, we efficiently depleted SOX9 levels in HACs by transient transfection of gene-specific siRNA (Fig. 7, A and B), leading to a strong decrease in the expression of its direct transcriptional target COL2A1 (Fig. 7C), thus confirming a functional depletion of SOX9. Most interestingly, we observed a significant increase in the levels of mature miR-1247 following SOX9 depletion (Fig. 7D), indicating that this transcription factor was acting as a miR-1247 repressor in HACs. To give some insights into the mechanism of action and investigate whether this involved transcriptional regulation, we measured the level of expression of the primary transcript of miR1247 (pri-miRNA) in SOX9-depleted cells. If SOX9 represses miR-1247 transcription, a concomitant increase in the pri-miR-1247 level should be observed upon SOX9 depletion. To test this hypothesis, we used specific qPCR primers recognizing the stem loop present in pri-miR-1247 (and pre-miR-1247), but not the mature miR-1247. Surprisingly, pri-miR-1247 levels were, in fact, decreased by SOX9 depletion (Fig. 7E). The same result was obtained when the reverse transcription reaction was performed with oligo dT primers, which recognize only the primary (polyadenylated) transcript, confirming that SOX9 depletion was in fact specifically decreasing the levels of the primary transcript (data not shown). Because we were unable to specifically detect the ~70-nucleotide intermediate form (pre-miR1247) by RT-qPCR, we performed Northern blotting using an LNA oligonucleotide probe against miR-1247 capable of detecting both pre-miR-1247 and the mature miR-1247. Although the low expression of miR-1247 limited its detection by Northern blot, we clearly detected the ~70-nucleotide band corresponding to the miRNA intermediate (pre-miR-1247), which was unaffected by SOX9 depletion (Fig. 7F). That the detected band specifically corresponded to pre-miR-1247 was confirmed by reblotting the membrane with a probe against miR-1247-3p, whose sequence is also present in the precursor (data not shown). Thus, because SOX9 depletion results in decreased levels of primary tran...
FIGURE 3. Analysis of the effect of miR-1247 overexpression on mRNA levels of 10 putative miR-1247 targets (ADAM15, ADAMTS5, BMP1, DVL1, ELAC2, ETS2, FAM20C, RARA, RSPO1, and SULF1). Only ADAM15 levels were significantly reduced upon miR-1247 overexpression. Control (denoted C) or miR-1247 mimics were transfected in P1–P2 HACs that were subsequently cultured in 20% or 1% O2 tension for 44 h. Values are presented as relative to that obtained in cells transfected with control and cultured in 1% O2. Data represent average ± S.E. from five to seven different experiments, each performed with different donor cells. *, p < 0.05; **, p < 0.01; ns, not significant.
FIGURE 4. Investigation of ADAM15 in HACs. A, RT-qPCR analysis of ADAM15 mRNA levels following miR-1247 inhibition. Control (denoted C) or miR-1247 inhibitors were transfected in P1–P2 HACs that were subsequently cultured in 20% or 1% O2 tension for 44 h. Values are presented as relative to that obtained in cells transfected with control cultured in 1% O2. Data represent average ± S.E. from five experiments performed with different donor cells. **, *p* < 0.01. B, representative Western blot of ADAM15 following miR-1247 overexpression in HACs. C, HACs from two different donors were transfected with luciferase reporters containing full-length ADAM15 3′-UTR with (3′UTR(mut)) or without (3′UTR(w/t)) mutations of the predicted miR-1247 binding-site downstream of the firefly luciferase ORF. Control (C) or miR-1247 mimics were co-transfected in the cells. Values are normalized to the levels of co-transfected Renilla luciferase and are expressed relative to those obtained for each construct co-transfected with control miRNA precursor (± S.E.). D, representative Western blot showing reduced ADAM15 levels following transfection of two different siRNAs against ADAM15. E and F, RT-qPCR analysis of ADAM15 and COL2A1 mRNA levels, respectively, after transfection with ADAM15 siRNA. ADAM15 depletion had no effect on COL2A1 expression. Values are presented as relative to that obtained in cells transfected with the control (5 nM) cultured in 1% O2. Data represent average ± S.E. from three different experiments, each performed with different donor cells. **, *p* < 0.01; ***, *p* < 0.001; ns, not significant.
script, but concomitantly increased levels of mature miR-1247 our data suggest that SOX9 may negatively regulate miR-1247 processing, representing a negative feedback loop between the miRNA and its target (Fig. 7G).

**DISCUSSION**

This study gives the first insight into the function and mechanism of action of miR-1247. MiR-1247 modulates the expression of cartilage master transcriptional regulator SOX9 in human chondrocytes. The skeleton develops from a cartilage anlage that acts as a template for subsequent bone formation and growth (27). Transcription factor SOX9 is essential for this process and subsequently for maintaining the permanent articular cartilage, which lines the long bone ends of joints enabling weight-bearing and near friction-free locomotion (28–30). Consequently, mutations in SOX9 result in severe dwarfism in humans (31), and murine studies have shown that this transcription factor directly targets the genes encoding the main structural components of cartilage that are essential for its function (22, 29). Therefore, understanding the regulation of SOX9 expression is of fundamental importance from a biological, pathological, and therapeutic perspective.

Only minor changes were observed at the SOX9 mRNA level upon miR-1247 modulation, suggesting that the greater effect observed at the protein level may be occurring as a direct result of translational repression mediated by miR-1247. Because putative miR-1247 binding sites were not detected in the SOX9 3'-UTR, we speculated that the interaction could occur through a highly conserved region predicted to be targeted by miR-1247 in the CDS of SOX9. Luciferase binding assays subsequently confirmed this hypothesis. Although early experiments in the field indicated that miRNAs preferentially target mRNAs in their 3'-UTR, most recent studies have shown that interaction through the CDS is not only possible but occurs frequently (24, 32). It has been proposed that binding sites in coding regions are, on average, less effective than those located in 3'-UTRs (33), but it has also been suggested that miRNA targets in coding regions enhance regulation mediated by 3'-UTR binding sites (34). Although no additional miR-1247 binding sites have been detected in the 3'-UTR of SOX9, we have recently shown that at least one functional binding site for miR-145 is present (14). The prospect that both miRNAs (miR-145 and miR-1247) and others, as yet unidentified, may act in conjunction to regulate the expression of this key transcription factor represents a very interesting possibility that will be addressed in future studies.

Our initial approach to identify functionally important miRNAs in cartilage was based on the rationale that miRNAs showing altered expression during the process of chondrocyte de-differentiation may have an important role in the maintenance of the chondrocyte phenotype. We have previously successfully adopted a similar approach to identify important genes in cartilage (9). The validity of our approach is further supported by the presence, in our list of candidates, of several miRNAs whose role in cartilage has already been demonstrated. Thus, miR-140 has been shown to be key for cartilage development and homeostasis (7); miR-221-3p regulates chondrogenic differentiation by targeting Mdm2 (35); miR-145 targets master transcription factor SOX9 in HACs (14); and the
expression of several other miRNAs identified (miR-137, miR-34a-5p, miR-337-5p, miR-342-3p, miR-210, miR-195-5p, miR-483-5p, miR-146a-5p, or miR-140-5p) is altered in osteoarthritis (11, 36, 37). Interestingly, some of the selected miRNAs are clustered in the genome (such as miR-654-5p and miR-543 or miR-143-3p and miR-145-5p) or are processed from the same
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FIGURE 7. SOX9 modulates miR-1247 levels. A, Western blot showing SOX9 depletion following transfection of a siRNA against SOX9 in HACs. B and C, RT-qPCR analysis showing reduced SOX9 and COL2A1 mRNA levels, respectively, following SOX9 depletion. D, mature miR-1247 levels were increased in response to SOX9 depletion. E, pri-miR-1247 expression levels following SOX9 depletion. F, Northern blot analysis of miRNA intermediate pre-miR-1247 following SOX9 depletion. The blot represents three different experiments performed with cells from three different donors. RNU24 is shown as a loading control. G, proposed mutual negative feedback loop between miR-1247 and SOX9 in HACs. Note that in all experiments, P1–P2 HAC cells were transfected with control (C) or SOX9 siRNA and subsequently cultured in 20% or 1% O₂ for 44 h. RT-qPCR data are presented as relative to that obtained in cells transfected with control and represent average ± S.E. from six different experiments, each performed with different donor cells.*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

pre-miRNA (such as miR-140-5p and miR-140-3p or miR-34a-5p and miR-34a-3p), indicating that common regulatory networks may be affecting their expression during cartilage differentiation. The role of other miRNAs from this list is currently being investigated in our laboratory.

ADAM15 is a trans-membrane glycoprotein shown to be involved in cell adhesion (38) and protection from apoptosis (39). ADAM15 has a predicted miR-1247-binding site in its 3’-UTR, and Yan and colleagues (40) suggest that the effect in migration, which they observed upon modulation of this miRNA could be mediated by its targeting ADAM15; however, they did not test this hypothesis. Although we detected subtle modulation of ADAM15 at the mRNA level upon overexpression and inhibition of miR-1247, this effect was not observed at the protein level and luciferase assays did not reveal any direct binding of miR-1247 to the 3’-UTR of ADAM15. Therefore, we conclude that ADAM15 may not be a biologically relevant miR-1247 target in HACs.

According to early observations of miRNA expression profiles, miRNAs tend to negatively correlate with target gene expression (41). However, we find a positive correlation between the expression of miR-1247 and its target SOX9 during HAC dedifferentiation and partial redifferentiation in hypoxia. In recent years, studies have revealed that miRNAs are involved in various kinds of regulatory networks that provide biological systems with the ability to maintain their function. One frequent example is the occurrence of both negative and positive feedback loops between a
miRNA and its targets, in many cases where a miRNA targets a transcriptional regulator (42). Our data reveals that SOX9 inhibits the expression of its own repressor, miR-1247 in human chondrocytes, thus representing a negative feedback loop form of regulation. The expression of SOX9 is tightly regulated during joint development (43) and is thought to be altered in joint disease (44). SOX9 regulates the expression of several key genes required for normal cartilage function and maintenance (28, 27) and is also necessary for regulation of genes associated with cartilage hypertrophy (45, 46). In addition to targeting cartilage matrix genes, SOX9 has more recently been shown to regulate expression of two miRNAs important for cartilage homeostasis, namely miR-140 (7, 23) and miR-675 (10). Thus, it is perhaps not surprising that regulation of its own expression can occur and may need to be finely regulated. Because miR-1247 and SOX9 show a similar expression pattern upon modulation of the HAC phenotype, we propose that miR-1247 acts to finely tune SOX9 expression, reducing undesirable fluctuations in levels of this key transcription factor.

Although we cannot rule out effects on miRNA stability, our data suggest that the mechanism by which SOX9 inhibits miR-1247 may be at the level of miRNA processing because levels of the primary transcript (pri-miR-1247) are reduced by SOX9 depletion, whereas those of the final, processed form are concomitantly increased suggesting an increased rate of processing. Regulation of miRNA processing has been reported at different levels. For example, ADAR enzymes are able to edit pri- and pre-miRNAs resulting in altered overall stability or a double-stranded RNA structure that could affect the processing into the mature miRNA (47). Also, levels of Drosha, DGC8R, Dicer, or of other co-factors of these enzymes can be modulated in response to different signals (48). Another level of regulation includes RNA binding proteins or factors capable of binding to the terminal loop of pri- and pre-miRNAs, resulting in altered processing efficiency (49). Most recent studies have also reported that mature miRNAs can directly regulate the processing of other miRNAs through binding to their precursor (49). miR-1247 is transcribed from the DLK1-DIO3 genomic region on human chromosome 14, which contains several paternally and maternally imprinted genes, miRNAs, small nucleolar RNAs, long intergenic non-coding RNAs, and pseudogenes. miR-1247 is the only miRNA located on the reverse strand, overlapping with exons of eight different transcripts annotated for the long intergenic non-coding RNA DIO3OS, whose function is unknown. The function of this long intergenic non-coding RNA could be related to the generation of the mature miR-1247, although whether miR-1247 is processed from any of these transcripts or whether it originates as an independent pri-miRNA remains to be studied. SOX9 is capable of activating the transcription of both protein-coding and non-coding RNAs and so, in theory, could indirectly repress miR-1247 processing by any of the aforementioned mechanisms. This thus opens the door to a whole new area of investigation of this cartilage master regulator gene.

SOX9 is present and functional in tissues other than cartilage, most notably the testes (50), the developing nervous system (51), the heart (52), and the eye (53). Furthermore, SOX9 expression can be regulated by other miRNAs in different tissues; for example, brain-enriched miR-124 targets SOX9 during adult neurogenesis (21), and miR-145 also targets SOX9 in cartilage (14). In addition to cartilage, we report relatively high expression of miR-1247 and of SOX9 in the eye and brain of mice. Potentially, miR-1247 could regulate SOX9, alone or in co-operation with other miRNAs in such tissues, with important functional implications. Such studies will be an interesting area for future investigation.

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