

Osteoarthritis and Cartilage



Overexpression of hsa-miR-148a promotes cartilage production and inhibits cartilage degradation by osteoarthritic chondrocytes



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ARTICLE INFO

Article history:

Received 5 March 2013

Accepted 9 November 2013

Keywords:

MicroRNA
Osteoarthritis
Cartilage
Chondrocytes

SUMMARY

Objective: Hsa-miR-148a expression is decreased in Osteoarthritis (OA) cartilage, but its functional role in cartilage has never been studied. Therefore, our aim was to investigate the effects of overexpressing hsa-miR-148a on cartilage metabolism of OA chondrocytes.

Design: OA chondrocytes were transfected with a miRNA precursor for hsa-miR-148a or a miRNA precursor negative control. After 3, 7, 14 and 21 days, real-time PCR was performed to examine gene expression levels of aggrecan (ACAN), type I, II, and X collagen (COL1A1, COL2A1, COL10A1), matrix metalloproteinase 13 (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) and the serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (SERPINH1). After 3 weeks, DNA content and proteoglycan and collagen content and release were determined. Type II collagen was analyzed at the protein level by Western blot.

Results: Overexpression of hsa-miR-148a had no effect on ACAN, COL1A1 and SERPINH1 gene expression, but increased COL2A1 and decreased COL10A1, MMP13 and ADAMTS5 gene expression. Luciferase reporter assay confirmed direct interaction of miR-148a and COL10A1, MMP13 and ADAMTS5. The matrix deposited by the miR-148a overexpressing cells contained more proteoglycans and collagen, in particular type II collagen. Proteoglycan and collagen release into the culture medium was inhibited, but total collagen production was increased.

Conclusion: Overexpression of hsa-miR-148a inhibits hypertrophic differentiation and increases the production and deposition of type II collagen by OA chondrocytes, which is accompanied by an increased retention of proteoglycans. Hsa-miR-148a might be a potential disease-modifying compound in OA, as it promotes hyaline cartilage production.

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Introduction

Osteoarthritis (OA) is a major cause of physical disability due to symptoms as pain, stiffness and loss of mobility. Multiple factors are believed to cause OA, such as trauma, abnormal mechanical loading, failure of nutrient supply and genetic predisposition¹. Available treatments are limited to pain management and in end-stage OA patients, joint replacement surgery is often indicated. OA is characterized by local inflammation, synovitis and proteolytic degradation of cartilage, which correlates with alterations in chondrocyte expression levels of genes involved in synthesis and degradation of cartilage^{1–4}.

Recently, it was proposed that epigenetic mechanisms play a role in modulating cell phenotype in OA^{5–10}, resulting in permanent changes in DNA transcription. One of the epigenetic mechanisms involved is based on microRNA (miRNA) expression^{6,8}. MiRNAs are short (19–24 nucleotide long) non-coding RNA molecules that can silence gene expression by binding to complementary sequences on target messenger RNA (mRNA) transcripts, resulting in translational repression or target degradation. Approximately one-third of all mammalian genes are regulated by miRNAs¹¹.

Changes in miRNA expression patterns are found in many pathological conditions, including several malignancies and neurological, cardiovascular and developmental diseases^{12–16}. The role of miRNAs in joint homeostasis has become evident from studies showing major abnormalities in cartilage development and structure in Dicer-null mice¹⁷. The miRNA expression pattern is also changed in OA and several miRNAs, including hsa-miR-27, 140, 145, 146a and 675 were found to be associated with altered levels of

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cartilage matrix production and degradation^{18–29}. Although the involvements of the abovementioned miRNAs in OA were analyzed, the effects of modulating these miRNAs on cartilage regeneration, in particular at the protein level, is unknown.

Typically, studies on the possible involvement of miRNAs in pathological processes start with miRNA screens to identify potential candidates. Previously, screens of 365 and 115 miRNAs, respectively, were profiled in normal and OA cartilage^{18,19}. In the scope of performing a more extensive miRNA screen, we found that hsa-miR-148a was expressed at 10.6 times lower levels in OA cartilage compared to normal cartilage (unpublished observations), in line with previously published results¹⁹. However, the functional role of hsa-miR-148a in cartilage metabolism or specifically OA has never been studied. Hsa-miR-148a has some predicted targets that are relevant for OA and general chondrocyte biology. Amongst the predicted targets are the mRNAs for type II collagen (COL2A1), type X collagen (COL10A1), matrix metalloproteinase 13 (MMP13), a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) and the collagen chaperone serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 (SERPINH1)³⁰. Therefore, the aim of this study was to investigate the effects of over-expressing hsa-miR-148a on cartilage metabolism of OA chondrocytes during regeneration.

Materials and methods

MiRNA expression screen

Total RNA was isolated from articular cartilage with the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Healthy human cartilage from femoral condyles of knee joints was obtained post-mortem of three male donors and four female donors, the mean age was 65 years (range: 47 years–83 years). OA cartilage was obtained from three male and four female donors (age 53–80, average 69 years) undergoing total knee arthroplasty. cDNA was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Life technologies, Poland) with Megaplex RT Primers, Human Pool A and B v3.0 according to the manufacturer's protocol. The TaqMan low-density Arrays A and B (TaqMan Array Human MicroRNA A v3.0 and B v3.0 Card Sets, Applied Biosystems) were used in a ABI Prism 7900HT sequence detection system (Applied Biosystems). The raw Ct values were calculated using RQ manager software and analyzed using DataAssist software (ABI, Applied Biosystems).

Cell isolation

Chondrocytes were isolated from articular cartilage from patients with OA undergoing total knee arthroplasty. The anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in the University Medical Center Utrecht³¹. The articular cartilage was minced and digested in 0.15% (w/v) collagenase (CLS-2, Worthington, Lakewood, NJ) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco) for 16 h at 37°C.

The cells were filtered through a 100 µm cell strainer (BD Biosciences, San Diego, CA) and washed before culturing or miRNA/mRNA isolation.

Cell culture and transfection

Isolated OA chondrocytes were expanded in T175 tissue culture flasks in DMEM supplemented with 10% FBS, 100 U/ml penicillin,

100 µg/ml streptomycin, and 10 ng/ml bFGF (R&D, Minneapolis, MN), at 37°C in 5% CO₂. At confluency, the cells were trypsinized using 0.25% trypsin/EDTA (Gibco) and replated.

At passage 2, OA chondrocytes were reverse-transfected with a pre-mir miRNA precursor for hsa-miR-148a-5p or a pre-mir miRNA precursor negative control (Ambion) using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA). The reverse-transfection was performed during seeding (density 1.6×10^6 cells/cm²) on Millicell filters (0.4 µm PTFE (Millipore, Bedford, MA)) that were precoated with type II collagen (type II collagen from chicken sternal cartilage (Sigma, St. Louis, MO))^{32,33}. The final concentration of pre-mir miRNA precursor was 10 nM. The cells were retransfected after 1 and 2 weeks of culture on filters.

The filters were cultured in DMEM (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml ascorbate-2-phosphate (Sigma) and culture media were renewed every 3 days.

MiRNA real-time PCR

Total RNA was extracted from chondrocytes with the mirVana miRNA isolation kit (Ambion) according to the manufacturer's protocol. The expression of hsa-miR-148a was verified using a TaqMan miRNA assay for hsa-miR-148a (Applied Biosystems). MiRNA expression was normalized to RNU44 small nuclear RNA.

Real-time PCR

Total RNA was isolated from the cells immersed in Trizol (Invitrogen) as described by the manufacturer. Total RNA (750 ng) was reverse transcribed using an iScript cDNA Synthesis Kit (Biorad, Hercules, CA).

Real-time PCR reactions were performed using the SYBR Green reaction kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) in a LightCycler 480 (Roche Diagnostics). The LightCycler reactions were prepared in 20 µl total volume with 7 µl PCR-H₂O, 0.5 µl forward primer (0.2 µM), 0.5 µl reverse primer (0.2 µM), 10 µl LightCycler Mastermix (LightCycler 480 SYBR Green I Master; Roche Diagnostics), to which 2 µl of five times diluted cDNA was added as PCR template. Primers (Invitrogen) used for real-time PCR are listed in Table 1. Specific primers were designed from sequences available in the data banks, based on homology in conserved domains between human, mouse, rat, dog and cow³⁴. The amplified PCR fragment extended over at least one exon border (except for 18S). Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) and 18S were used as housekeeping genes and the gene expression levels were normalized for the normalization factor calculated with the equation $\sqrt{(YWHAZ \times 18S)}$. With the LightCycler software (version 4), the crossing points were assessed and plotted vs the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by LightCycler software and the data were used only if the calculated PCR efficiency was between 1.85 and 2.0.

Papain digestion

After 3 weeks of culture, filters were digested at 60°C for 18 h in a papain enzyme solution consisting of 5 mM L-cysteine, 50 mM Na₂EDTA, 0.1 M NaAc, pH 5.53 with 2% (v/v) papain (Sigma).

Proteoglycan analysis

To analyze the proteoglycan content of the regenerated cartilage tissue and the amount released into the culture medium, a

Table I
Oligonucleotide sequences used for real-time PCR

Target gene	Oligonucleotide sequence	Annealing temperature (°C)	Product size (bp)
18S	Fw 5' GTAACCCGTTGAACCCCAATT 3'	57	151
	Rev 5' CCATCCAATCGGTAGTAGCG 3'		
YWHAZ	Fw 5' GATGAAGCCATTGCTGAACCTG 3'	56	229
	Rev 5' CTATTGTGGGACAGCATGGA 3'		
ACAN	Fw 5' CAACTACCCGGCCATCC 3'	57	160
	Rev 5' GATGGCTCTGTAATGGAACAC 3'		
COL1A1	Fw 5' TCCAACGAGATCGAGATCC 3'	57	191
	Rev 5' AAGCCGAATTCCTGGTCT 3'		
COL2A1	Fw 5' AGGGCCAGGATGTCGGCA 3'	56	195
	Rev 5' GGGTCCCAGGTTCTCCATCT 3'		
COL10A1	Fw 5' CACTACCAACACCAAGACA 3'	56	225
	Rev 5' CTGGTTTCCCTACAGCTGAT 3'		
MMP13	Fw 5' GGAGCATGGCGACTTCTAC 3'	56	208
	Rev 5' GAGTGCTCCAGGGTCTT 3'		
ADAMTS5	Fw 5' GCCAGCGGATGTGTGCAAGC 3'	57	130
	Rev 5' ACACTTCCCCGGACGCAGA 3'		
SERPINH1	Fw 5' TGATGATGCACCGGACAG 3'	57	212
	Rev 5' GGAGATGGCAACGCCTC 3'		

Forward (Fw) and reverse (Rev) primers for YWHAZ; ACAN; COL1A1, $\alpha 1(I)$ procollagen; COL2A1, $\alpha 1(II)$ procollagen; COL10A1, $\alpha 1(X)$ procollagen; MMP13; ADAMTS5; SERPINH1.

dimethylmethylene blue (DMMB) spectrophotometric analysis was performed to determine the content of sulfated glycosaminoglycans (GAGs)³⁵. DMMB solution and papain digest or medium sample were mixed and the absorbance was read at 540 nm and 595 nm. As reference, chondroitin sulfate C (Sigma) was used. The total amount of proteoglycans produced was defined as the amount of proteoglycans in the papain tissue digest and the amount of proteoglycans released into the culture medium during the entire culture period.

DNA content

Total DNA was quantified in papain digests using Quant-iT Picogreen (Invitrogen) according to the manufacturer's instructions. Picogreen reagent was added to papain digest. This was incubated at ambient temperature for 5 min, protected from light. The fluorescence was measured at ~480 nm excitation and ~520 nm emission and DNA content determined using lambda DNA as standard.

Hydroxyproline assay

To analyze the collagen content, hydroxyproline content was determined in papain digests or medium samples using a modified colorimetric assay³⁶. In short, freeze-dried papain digests or medium samples were hydrolyzed and the free hydroxyprolines were oxidized with Chloramine-T for the production of pyrroles. The addition of Ehrlich's reagent resulted in the formation of chromophores that were measured at 550 nm and collagen content was determined using gelatin (Sigma) as standard. The total amount of collagens produced was defined as the amount of collagens found in the papain digest and the amount of collagens released into the culture medium.

Type II collagen Western blot

After 3 weeks of culture, filters were digested overnight at 4°C with pepsin (Worthington, 100 μ g/ml in 0.2 M NaCl, 0.5 M acetic acid). The digests and 0.05, 0.025, and 0.005 μ g of type II collagen as

reference (chicken sternum, Sigma) were denatured by heating at 95°C for 5 min in NuPage LDS sample buffer (Invitrogen) with NuPage reducing agent (Invitrogen). The digests and collagen standards were resolved by SDS-PAGE (8% resolving gel with 4% stacking gel) and transferred to nitrocellulose membranes (Biorad). The membranes were blocked in 2% (w/v) BSA 0.1% Tween in phosphate buffered saline (PBS) for 1 h and were then incubated with the primary antibody for 2 h. Mouse monoclonal anti-type II collagen (MAB1330, Chemicon, Millipore) was used at 1:1000 dilution. After three washes with 0.1% Tween in PBS, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (DakoCytomation, Glostrup, Denmark) at a 1:5000 dilution for 1 h. Following three washes, immunoreactivity was visualized using Lumi-Light^{plus} (Roche Diagnostics).

Luciferase assay

The 3' untranslated regions (3' UTRs) of type II and type X collagen (COL2A1 and COL10A1), MMP13, ADAMTS5 and SERPINH1 were amplified by PCR with DNA oligonucleotides flanked by XhoI and NotI restriction sites (DNA oligonucleotide sequences are listed in Table II). The fragments were cloned downstream of the Renilla luciferase gene into the pSICHECK2 vector (Promega, Madison, WI) in sense and antisense orientation.

Hela cells were plated into 96-wells plates and cotransfected with the described luciferase reporter constructs and 50 nM pre-miR miRNA precursor for hsa-miR-148a or a pre-miR miRNA precursor negative control (Ambion) using Lipofectamine (Invitrogen). Luminescence was measured 48 h after transfection using Pierce Renilla-Firefly Luciferase Dual Assay Kit (Thermo scientific, Rockford, IL).

Collagenase activity assay

To analyze collagenase activity, the Enzchek Gelatinase/Collagenase Assay Kit (Invitrogen) was used according to the manufacturer's instructions. DQ Collagen Fluorescein conjugate was added to 100 times in reaction buffer (kit component) diluted conditioned medium. This was incubated for 4 h at ambient temperature, protected from light. The fluorescence was measured at ~480 nm

Table II
Oligonucleotide sequences used for amplifying 3' UTRs

3' UTR of target gene	Oligonucleotide sequence
COL2A1 sense	Fw CCGCTCGAGAACTGAACCCAGAAAC
	Rev CGTACGCCGGCGGTACTTTCCAATAATCTTTTC
COL2A1 antisense	Fw GCATGCGGCCGCAAACTGAACCCAGAAACAAC
	Rev CGTACTCGAGGTACTTTCCAATAATCTTTTC
COL10A1 sense	Fw CCGCTCGAGGTACACACAGAGCTAATCTAAATC
	Rev CGTACGCCGGCGCACTTTATGTCTACTTTTTTATTAAC
COL10A1 antisense	Fw GCATGCGGCCGCTACACACAGAGCTAATCTAAATC
	Rev CGTACTCGAGCACTTTATGTCTACTTTTTTATTAAC
MMP13 sense	Fw CCGCTCGAGGTGCTTTTTAAAAAATGTTATT
	Rev CGTACGCCGGCGCTGTGAAATATATTTTTTATTATAAAC
MMP13 antisense	Fw GCATGCGGCCGCGTGTCTTTTTAAAAAATGTTATT
	Rev CGTACTCGAGCTGTGAAATATATTTTTTATTATAAAC
ADAMTS5 sense	Fw CCGCTCGAGCTGTGTTATGATCTTATGAC
	Rev CGTACGCCGGCGCACTTTAACCTAGTTACAATTTATAT
ADAMTS5 antisense	Fw GCATGCGGCCGCGCTGTGTTATGATCTTATGAC
	Rev CGTACTCGAGACTTTAACCTAGTTACAATTTATAT
SERPINH1 sense	Fw CCGCTCGAGGGCTCAGGGTGCACACAGGATG
	Rev CGTACGCCGGCGCCAGCTCCACAAATGTGATTGG
SERPINH1 antisense	Fw GCATGCGGCCGCGCTCAGGGTGCACACAGGATG
	Rev CGTACTCGAGCCAGCTCCAACAAATGTGATTGG

Forward (Fw) and reverse (Rev) primers for COL2A1, $\alpha 1(II)$ procollagen; COL10A1, $\alpha 1(X)$ procollagen; MMP13; ADAMTS5; SERPINH1.

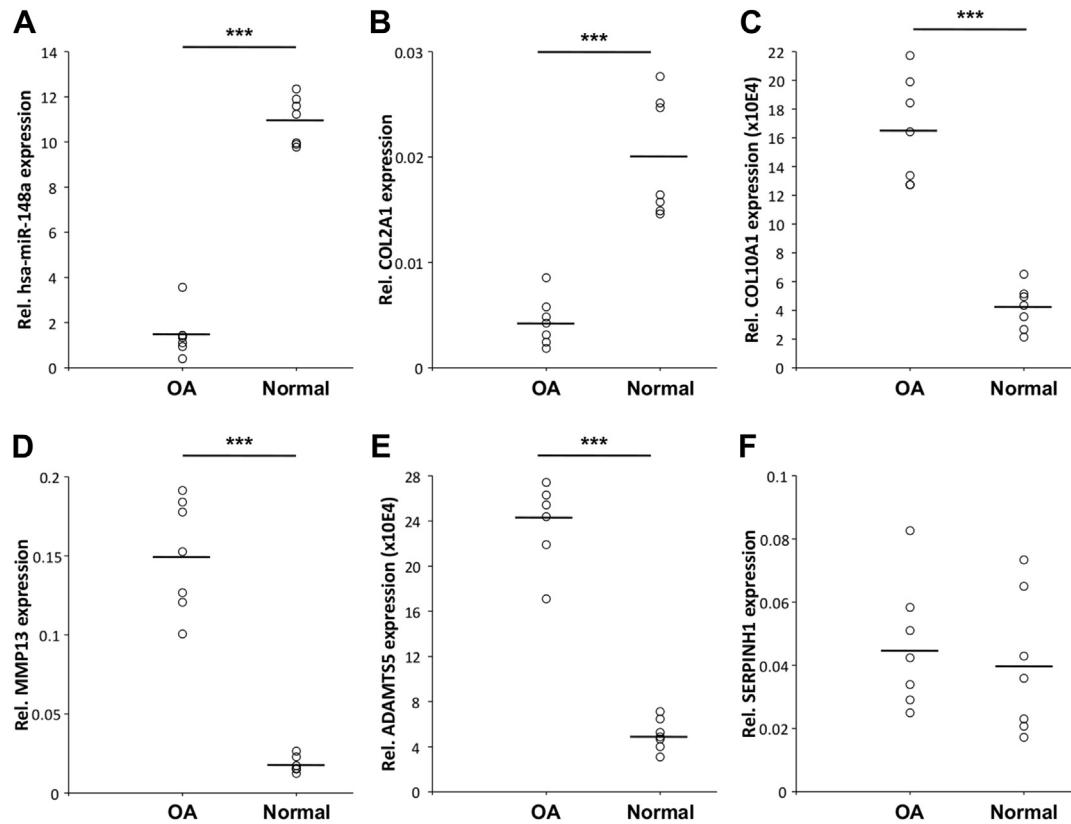


Fig. 1. Real-time PCR was performed for hsa-miR-148a, type II collagen (COL2A1), type X collagen (COL10A1), MMP13, ADAMTS5 and SERPINH1 on reverse transcribed RNA isolated from osteoarthritic cartilage (seven donors) and normal cartilage (seven donors). The data are presented in a dot plot where every dot resembles the value of one donor. The mean value of the samples is indicated by a line. Data are shown as mean \pm SD. ***: $P < 0.001$.

excitation and ~ 520 nm emission and collagenase activity was determined using collagenase type IV from *Clostridium histolyticum* (kit component) as standard.

Statistical analysis

Gene expression data are expressed as mean \pm SD of miR-148a transfected vs mock transfected chondrocytes of target gene expression normalized for the equation $\sqrt{(Y/WHZ \times 18S)}$. Differences in expression ratios were tested with a two-tailed *t*-test for single group mean and compared to 1 (miR-148a transfected/mock transfected = 1, no effect). Data from biochemical assays are expressed in dot plots where every dot resembles the value of one donor and the mean value of the samples is indicated by a line. The data were analyzed using a two-tailed paired *t*-test. The level of significance was set at $P < 0.05$. Normal distribution of the data was confirmed using the Shapiro–Wilk test.

Results

Hsa-miR-148a regulates COL10A1, MMP13 and ADAMTS5

In the scope of performing an extensive miRNA screen between normal and OA cartilage, we had found that hsa-miR-148a was expressed about ten times less in OA cartilage compared to normal cartilage. By real-time PCR it was confirmed that hsa-miR-148a levels were nine fold lower in OA cartilage compared to normal cartilage [Fig. 1(A)].

TargetScan 6.2 and/or microRNA.org identified COL2A1, COL10A1, MMP13, ADAMTS5 and SERPINH1 amongst the predicted

targets of hsa-miR-148a. The gene expression levels of these genes were measured in the same RNA in which hsa-miR-148a levels were determined. The expression level of COL2A1 was decreased in the OA donors [Fig. 1(B)], the levels of COL10A1, MMP13 and ADAMTS5 were increased in OA donors [Fig. 1(C)–(E)], and for SERPINH1 no difference was observed [Fig. 1(F)].

To investigate the effects of upregulation of hsa-miR-148a, a miRNA precursor (pre-mir) for hsa-miR-148a or a pre-mir negative control was transfected in OA chondrocytes. Real-Time PCR analysis confirmed that transfection of the pre-mir increased the expression of hsa-miR-148a compared to transfection with control non-coding pre-miRNA [Fig. 2]. The upregulation of hsa-miR-148a expression was highest 3 days after the initial transfection, with a 5-fold increase compared to the precursor negative control. Just before the re-transfections at days 7 and 14 and at the end of the culture period of 3 weeks, the hsa-miR-148a expression levels were up-regulated about 3.5-fold.

Overexpression of hsa-miR-148a resulted in increased COL2A1, decreased COL10A1, MMP13 and ADAMTS5, and unchanged SERPINH1 gene expression levels [Fig. 2].

To analyze the miRNA–mRNA interactions of the predicted target genes, a luciferase reporter assay was performed. No difference in luciferase activity was observed between HeLa cells cotransfected with the construct containing the 3' UTR of COL2A1 and SERPINH1 and the miRNA precursor for hsa-miR-148a or a miRNA precursor negative control [Fig. 3]. A decrease in luciferase activity was measured in HeLa cells cotransfected with the miRNA precursor for hsa-miR-148a and the luciferase reporter vector containing the 3' UTRs of COL10A1, MMP13 and ADAMTS5 in sense orientation [Fig. 3]. No difference was shown when the 3' UTRs of

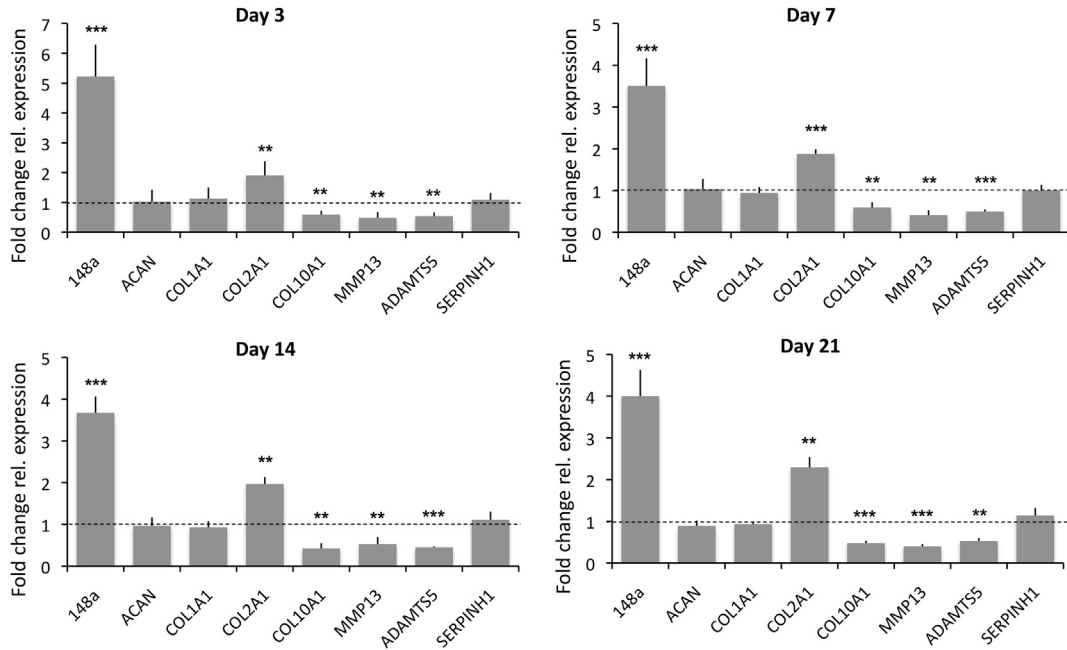


Fig. 2. Real-time PCR was performed on reverse transcribed RNA isolated from OA chondrocytes transfected with a pre-miR miRNA precursor for hsa-miR-148a or a pre-miR miRNA precursor negative control after 3, 7, 14 and 21 days in a regeneration culture. Expression levels of hsa-miR-148a (148a), ACAN, type I collagen (COL1A1), type II collagen (COL2A1), type X collagen (COL10A1), MMP13, ADAMTS5 and SERPINH1 were measured. The results are presented as expression levels of hsa-miR-148a transfected OA chondrocytes relative to negative control transfected OA chondrocytes. Data are shown as mean ± SD. **: $P < 0.01$; ***: $P < 0.001$.

COL10A1, MMP13 and ADAMTS5 were cloned into the luciferase reporter vector in antisense orientation [Fig. 3].

Overexpression of hsa-miR-148a increases proteoglycan content and decreases proteoglycan release

Overexpression of hsa-miR-148a had no effect on ACAN gene expression levels, but did decrease the gene expression levels of the aggrecanase ADAMTS5 [Fig. 2]. The amount of proteoglycans was increased in the tissue generated by hsa-miR-148a overexpressing OA chondrocytes compared to controls [Fig. 4(A)], while proteoglycan release into the culture medium was decreased [Fig. 4(B)].

No difference was found between chondrocytes overexpressing hsa-miR-148a and controls in terms of the total

amount of proteoglycans produced, as determined by adding up the amount found in the deposited matrix at the end of culture period and the total amount released into the medium during culture [Fig. 4(C)].

Overexpression of hsa-miR-148a increases collagen II expression at the mRNA and protein level and decreases the expression of type X collagen and MMP13

However, overexpression of hsa-miR-148a resulted in increased COL2A1 gene expression levels and the gene expression levels of COL10A1 and the collagenase MMP13 were decreased [Fig. 2]. No effect was shown on gene expression levels of COL1A1 and the collagen chaperone SERPINH1 [Fig. 2].

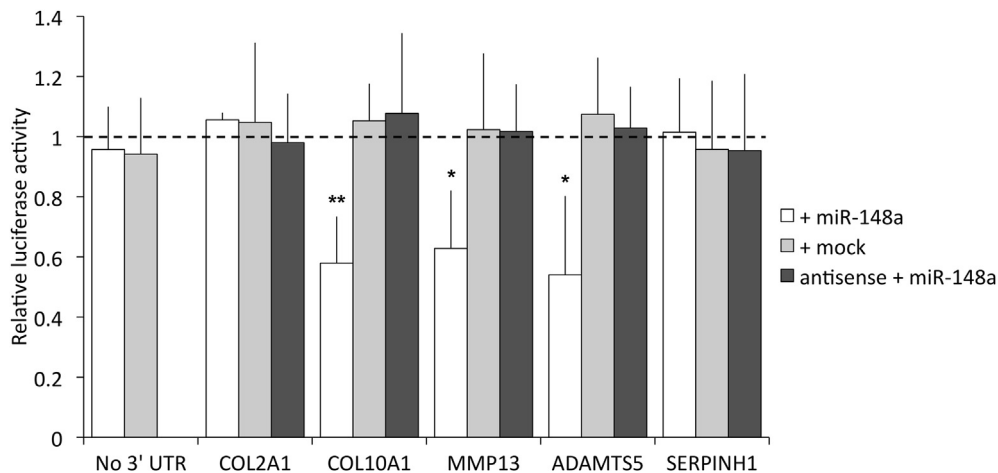


Fig. 3. HeLa cells were cotransfected with 50 nM pre-miR miRNA precursor for hsa-miR-148a or pre-miR miRNA precursor negative control (mock) and pSICHECK2 containing the 3' UTRs of type II collagen (COL2A1), type X collagen (COL10A1), MMP13, ADAMTS5 and SERPINH1 in sense and antisense orientation. Firefly and Renilla luciferase were measured 48 h after transfection. Data are shown as mean ± SD. *: $P < 0.05$; **: $P < 0.01$.

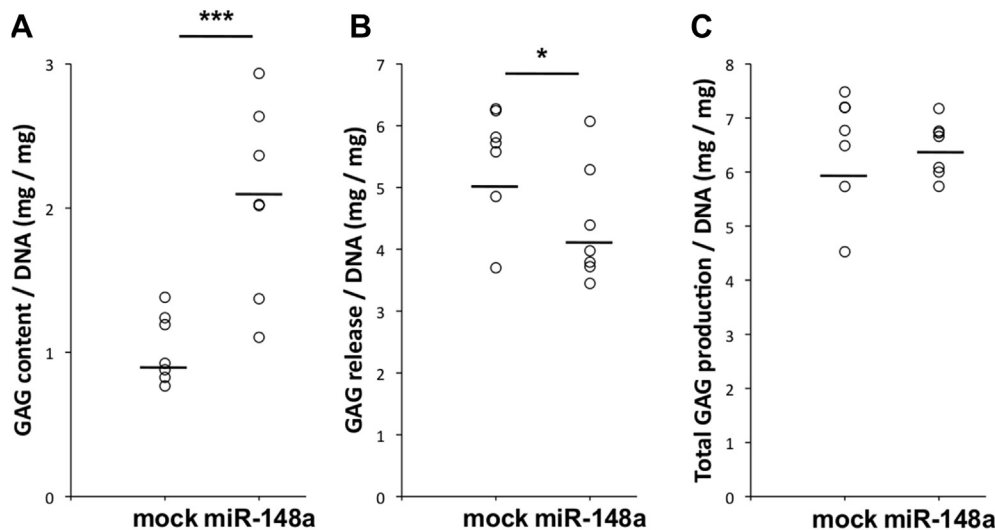


Fig. 4. Proteoglycan content (A), release (B), and total production (C) (determined as GAGs) (normalized for the DNA content) were determined after 21 days in cultures of OA chondrocytes transfected with a pre-mir miRNA precursor negative control (mock) or a pre-mir miRNA precursor for hsa-miR-148a (miR-148a). The data are presented in a dot plot where every dot resembles the value of one donor. The mean value of the samples is indicated by a line. *: $P < 0.05$; ***: $P < 0.001$.

The amount of collagen was increased in the matrix deposited by hsa-miR-148a overexpressing OA chondrocytes compared to controls [Fig. 5(A)], while the amount of collagen released into the culture medium was decreased [Fig. 5(B)].

In contrast to the lack of effect on total proteoglycan production, the total amount of collagen produced was increased by hsa-miR-148a overexpressing OA chondrocytes compared to controls [Fig. 5(C)].

Collagenase activity was decreased in the culture medium of the hsa-miR-148a overexpressing OA chondrocytes [Fig. 5(D)].

To verify whether type II collagen was also specifically up-regulated at the protein level, an immunoblot for type II collagen was performed on the collagen extracted from the cultures, confirming the higher deposition of type II collagen by the hsa-miR-148a overexpressing OA chondrocytes compared to the mock-transfected OA chondrocytes [Fig. 5(E)].

Discussion

In this study hsa-miR-148a, expressed at lower levels in OA cartilage compared to healthy cartilage, is suggested to play an important role in cartilage regeneration. Overexpressing hsa-miR-148a in OA chondrocytes increases extracellular matrix deposition by these cells; not only proteoglycan, but also the collagen and specifically type II collagen content was increased. In addition, matrix degradation was reduced, as reflected by a decreased release of proteoglycans during culture, which was accompanied by downregulation of MMP13, COL10A1 and ADAMTS5, predicted and confirmed targets of miR-148a.

Surprisingly, COL2A1, another postulated target gene was found to be increased upon overexpression of hsa-miR-148a in OA chondrocytes. This suggests that COL2A1 is not a direct target of hsa-miR-148a, as miRNAs are by default negative regulators of gene expression. A luciferase reporter assay also showed no interaction between miR-148a and COL2A1. However, hsa-miR-148a might target a repressor of COL2A1 as was also suggested for miR-675²⁸.

The gene expression patterns for collagen and ACAN were also reflected at the protein level. The gene expression level of ACAN, the main proteoglycan in the cartilaginous extracellular matrix, was unchanged. Although an increased proteoglycan content was found after culturing, total proteoglycan production did not increase. The

decreased proteoglycan release in combination with the decreased ADAMTS5 expression suggests that the increase in proteoglycan content was caused by a diminished breakdown, resulting in increased retention, rather than an increase in synthetic activity.

The increased total collagen and specifically type II collagen production found upon overexpression of miR-148a was in line with the increased gene expression levels of COL2A1. Next to a generalized increase in synthetic activity, a higher portion of collagen was found in the deposited matrix compared to collagen released into the culture medium. This coincided with decreased gene expression of MMP13, the main enzyme involved in cartilage collagen degradation. This is supported by the decreased collagenase activity found in the culture medium of the hsa-miR-148a overexpressed chondrocytes. So, collagen production was not only increased but its degradation was also inhibited. Most likely this increased deposition of collagen was responsible for the increase in proteoglycan content found, as an intact collagen network is required for the retention of proteoglycans inside the cartilage matrix³⁷.

In addition to the stimulatory effects on cartilage matrix formation, the downregulation of COL10A1 gene expression levels by miR-148a overexpression suggests further reversal of the OA chondrocytic phenotype, as hypertrophic differentiation characterized by increased COL10A1 expression levels is a hallmark of OA.

In addition to COL2A1 another predicted miR-148a target that was not affected in line with the sequence-based prediction was SERPINH1, a collagen-specific chaperone. This protein is required for proper intracellular protein folding of the collagens and decreased SERPINH1 levels might lead to improper collagen folding resulting in intracellular accumulation and degradation of collagens. However, miR-148a after all did not seem to target SERPINH1 and increased production and deposition of collagens by miR-148a overexpressed OA chondrocytes suggests that there are no complications with the intracellular folding of collagens. This was confirmed in a luciferase reporter assay, showing that miR-148a interacts with MMP13, COL10A1 and ADAMTS5, but not with SERPINH1 or COL2A1.

In addition to modulating matrix protein expression by binding target mRNA or repressor mRNA of matrix gene expression, another mechanism by which hsa-miR-148a may target cartilage metabolism, is by affecting methylation pathways, as miR-148a directly targets DNA methyltransferase 1 (DNMT1)^{38–41}. Lower expression of miR-148a could increase DNMT1 expression and attenuate DNA

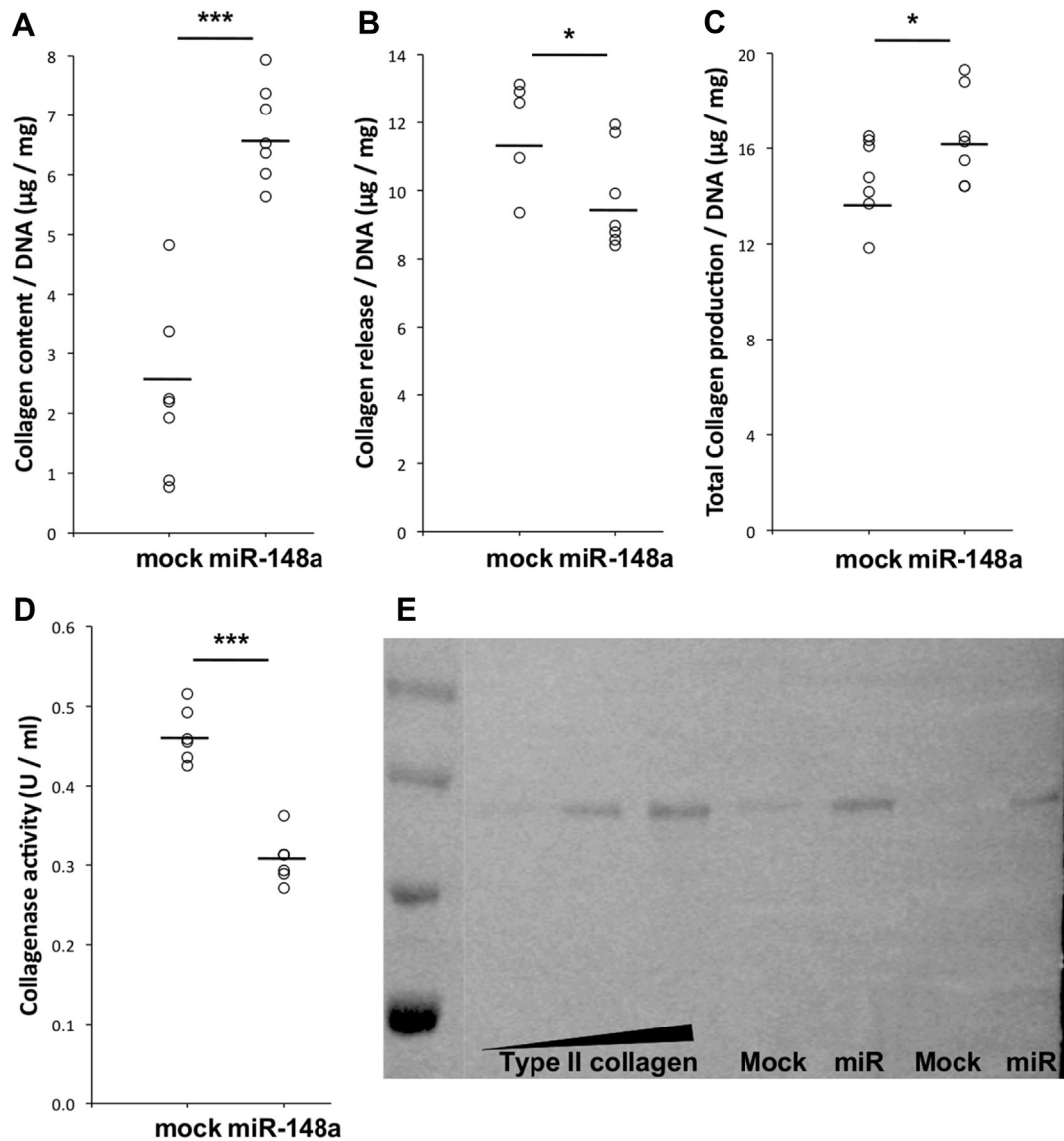


Fig. 5. Collagen content (A), release (B), and total production (C) (determined as hydroxyproline (normalized for the DNA content)), collagenase activity (D) and type II collagen (E, determined by immunoblot) were determined after 21 days in cultures of OA chondrocytes transfected with a pre-mir miRNA precursor negative control (mock) or a pre-mir miRNA precursor for hsa-miR-148a (miR-148a). The data are presented in a dot plot where every dot resembles the value of one donor. The mean value of the samples is indicated by a line. (E) The first lane contains a protein ladder, the following three standards of 0.005, 0.025, and 0.05 μg type II collagen. *: $P < 0.01$; ***: $P < 0.001$.

hypomethylation. It was suggested that changes in DNA methylation also play a role in the gene expression patterns observed in OA¹⁰, and thus hsa-miR-148a might also modulate cartilage matrix production through a more general DNA methylation-based way.

Besides hsa-miR-148a, several other miRNAs are already found to be differentially expressed in OA^{18–21}. Hsa-miR-140 is one of the most studied miRNAs with respect to cartilage and its expression is significantly lower in OA cartilage compared to healthy cartilage^{22–24}. Also hsa-miR-27 and hsa-miR-146a levels are significantly lower in OA cartilage compared to healthy cartilage and they are found to regulate the expression of MMP13^{25–27}. The gene expression of type II collagen (COL2A1) is indirectly regulated by hsa-miR-675 and miR-145 directly targets SOX9^{28,29}.

Although the abovementioned miRNAs were functionally analyzed, the effects of modulating the expression of these miRNAs on actual cartilage regeneration, in particular at the protein level, are unknown. The current study is the first investigating the effect of modulating the expression of a specific miRNA on the production

and degradation of the main components of cartilage, the proteoglycans and collagens.

In the current study, cartilage production was shown to be enhanced by overexpression of hsa-miR-148a in OA chondrocytes, which, in addition to providing information on mechanisms in the pathogenesis and maintenance of OA, may render this miRNA a target for a potential therapy for OA or to induce cartilage repair. Several clinical trials are already ongoing for miRNA-based treatment of hepatitis C⁴², liver cancer^{43,44}, and heart failure⁴⁵. However, a drawback for miRNA-based treatment for cartilaginous tissues is the relative inaccessibility of chondrocytes. Since cartilage is avascular and the chondrocytes are embedded in a dense and charged extracellular matrix, it may be difficult to transfect chondrocytes in the native tissue. However, many advances in transfection of cartilage *in vivo* are currently being achieved^{46–50}, increasing the possibility of application of miRNA in targeting cartilaginous tissues.

In conclusion, overexpression of hsa-miR-148a stimulated the production of collagens, specifically type II collagen, and enhanced

the retention and deposition of collagen and proteoglycans, respectively, in cartilage matrix deposited by OA chondrocytes. Hsa-miR-148a may be a potential target for the treatment of OA, as it promotes cartilage production and prevents cartilage degradation and hypertrophy.

Author contributions

Conception and design: LV, LC, DS.
 Collection and assembly of data: LV, AK.
 Analysis and interpretation of the data: LV, LC.
 Provision of study materials: DS.
 Drafting of the article and reviewing: LV, AK, WD, DS, LC.
 Final approval of submitted version: LV, AK, WD, DS, LC.

Role of funding source

The funding source had no role in study design, collection, analysis or interpretation of data, in writing the manuscript or in submitting the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

This research forms part of the project #SSM06004 Translational Regenerative Medicine of the research program SmartMix, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. L.B. Creemers is funded by the Dutch Arthritis Association. D.B.F. Saris receives trial support from Sanofi/Genzyme and consultancy and teaching fees from Tigenix and Smith & Nephew.

The authors thank Dr G. Krenning for the pSICHECK2 vector.

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