MicroRNA-199a-3p, microRNA-193b, and microRNA-320c Are Correlated to Aging and Regulate Human Cartilage Metabolism

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ABSTRACT: MicroRNAs (miRNAs) are small RNAs of ~22 base pairs that regulate gene expression. We harvested cartilage tissue from patients with polydactylism, anterior cruciate ligament injury, and osteoarthritis undergoing total knee arthroplasty and used microarrays to identify miRNAs whose expression is upregulated or downregulated with age. The results were assessed by real-time PCR and MTT assay in a mimic group, in which synthetic double-stranded RNA from the isolated miRNA was transfected to upregulate expression, and in an inhibitor group, in which the miRNA was bound specifically to downregulate expression. The expression of two miRNAs (miR-199a-3p and miR-193b) was upregulated with age and that of one miRNA (miR-320c) was downregulated with age. A real-time PCR assay showed that type 2 collagen, aggrecan, and SOX9 expression were downregulated in the miR-199a-3p mimic group but was upregulated in the inhibitor group. Similar results were observed for miR-193b. By contrast, ADAMTS5 expression was downregulated in the miR-320c mimic group and upregulated in the inhibitor group. Cell proliferative activity was upregulated significantly in the miR-193b inhibitor group compared with the control group. We believe that miR-199a-3p and miR-193b are involved in the senescence of chondrocytes, and miR-320c is involved in the juvenile properties of chondrocytes. J Orthop Res 30:1915–1922, 2012

Keywords: miR-199a-3p; miR-193b; miR-320c; cartilage metabolism; osteoarthritis

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of ~22 base pairs that regulate gene expression by repressing translation. In 1993, the first miRNA, lin-4 was discovered in nematodes, and subsequent studies have shown that miRNAs regulate ~30% of all human genes.[1] miRNA is transcribed initially from miRNA genes into a long primary transcript (pri-mRNA), which is then cleaved by Drosha to produce precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm using exportin-5, and there it is cleaved by Dicer to yield miRNA. The miRNA then combines with an argonaute protein to form RISC, which sequence-specifically recognizes and represses the translation of target RNAs.[2] Abnormalities in cartilage development have been observed in Dicer gene dysfunction models, and miRNA is known to play a key role in cartilage differentiation.[3] miRNA plays a key role in differentiation and growth in organs and tissues,[2] and miRNAs expressed specifically in cartilage[4–8] have been reported. Till date, miR21,[4] miR22,[9] miR27a,[6] miR140,[10] and miR146[8] have been reported as miRNAs that are associated with cartilage metabolism. Many studies have reported the use of a microarray analysis for isolating miRNAs that are expressed in a tissue-specific manner. For example, Miyaki et al.[10] performed microarray analysis of MSC and articular chondrocytes, and they observed a high expression of miR140 in chondrocytes. They also observed the expression of miR-140 increased during chondrogenesis. In addition, they reported that miR140 expression was lower in the OA group than in the normal group. Iliopoulos et al.[9] performed microarray analysis using normal chondrocytes and chondrocytes isolated from OA. They reported an increased expression of miR22 and a decreased expression of miR140 in the OA group. They also reported that the expressions of IL-1β and MMP13 were increased, whereas the expressions of aggrecan was decreased in the group that showed increased miR22 expression. In contrast, using microarray analysis, we focused on three types of miRNAs, that is, miR199a-3p, miR193b, and miR320c, and their expressions were observed to vary with age.

The purpose of this study was to identify an miRNA that could be used as a biomarker to evaluate cartilage in OA or regenerative medicine. To identify an miRNA that was involved with cartilage metabolism, we isolated miRNAs with expression levels that increased or decreased with age, by performing microarray analysis using chondrocytes isolated from patients with three different conditions, that is, polydactylism(PD), anterior cruciate ligament (ACL) injury, and OA undergoing total knee arthroplasty (TKA). We evaluated whether the extracted miRNAs were involved with cartilage metabolism by comparing two groups, that is, the overexpression group where miRNA levels increased and the inhibitor group where miRNA levels decreased. Cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, while gene expression was evaluated using real-time polymerase chain reaction (PCR). For real-time PCR, we focused on three anabolic factors, that is, aggrecan, type 2 collagen, and SOX9, which affect cartilage synthesis. We also focused on three catabolic factors, that is, MMP3, MMP13, and ADAMTS5, which affect cartilage matrix

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destruction. In addition, we focused on type 1 collagen, which is expressed in high levels in fibrocartilage. We also analyzed miRNA-controlled genes, OA-related genes, and cartilage-related genes by pathway analysis to verify whether miRNAs had direct or indirect effects on cartilage.

MATERIALS AND METHODS

Cartilaginous Tissue

The protocols were performed under the approval and guidance of the Clinical Research Review Committee of the Tokai University School of Medicine. Cartilaginous tissue was obtained from 17 patients (age, 60–79 years; average age, 73.4 years) who had been diagnosed with OA and who had undergone TKA at the Tokai University Hospital from April 2010 to June 2011; three patients (age, 15–31 years; average age, 22.3 years) who had an ACL injury and who had undergone surgery at the same hospital during the same period; and six patients (age, 11–16 months; average age: 13 months) who had been diagnosed with polydactylism and who had undergone plastic surgery at the National Center for Child Health and Development during January and February 2010. We used all layers of the normal articular cartilage from PD, ACL, and TKA. Cartilage tissues were harvested from the joints of the resected fingers in patients with polydactylism, arthroscopically excised non-loading parts of injured ACLs, and femur and tibia from patients with knee OA who underwent total knee arthroplasty.

Isolation and Culture of Chondrocytes

The harvested cartilaginous tissue of each patient was sliced finely with scissors and treated with enzymes in a Petri dish for 4 h using Dulbecco's modified Eagle medium F12 (DMEM/F12; GIBCO, Invitrogen Corporation, Carlsbad, CA) containing 0.05% collagenase type 1 (Worthington, Inc., Lakewood, NJ). The enzyme-treated cartilaginous tissue was passed through a cell strainer (BD Falcon^{TM}; BD Biosciences, Bedford, MA) with a pore size of 100 μm, and the cells were retrieved by centrifugation. The chondrocytes were seeded into a 1×10^4 cells/cm^2 plate and incubated in culture medium of DMEM/F12 supplemented with 20% fetal bovine serum (FBS; Gibco, NY) and 1% antibiotic–antimycotic solution (Gibco). After 4 days, the culture was maintained by adding a further 50 μg/ml ascorbic acid (Wako Junyaku Kougyou Corp, Kanagawa, Japan). All incubations were performed at 37°C in 5% CO_2 and 95% air. The isolated chondrocytes were seeded at passage 0 and subcultured until passage 2.

Microarray

Microarray analysis was performed using a total of 15 specimens, that is, articular cartilage from the resected fingers of patients with polydactylism ($N = 6$), excised non-loading parts of injured ACLs ($N = 3$), and knee articular cartilage from patients with knee OA ($N = 6$). RNA was isolated from the chondrocytes after they were seeded into 1×10^4 cells/cm^2 24-well plates and incubated in 5% CO_2 and 95% air. An SV Total RNA Isolation System (Promega Corp., Madison, WI) was used to perform total RNA extraction, according to the manufacturer's instructions. The quality of RNA in each sample was verified using the A260/280 absorbance ratio. Using miRNA kits (Qiagen, Tokyo, Japan), 10 μl of RNA was isolated according to the manufacturer's instructions. Intensity values of each scanned feature were quantified using Agilent feature extraction software version 10.7.3.1, which performs background subtractions. Normalization was performed using Agilent GeneSpring GX version 11.0.2. (per chip: quantile normalization; per gene: normalization to median of all samples). There are total of 939 probes on Agilent Human miRNA Microarray (Design ID: 021827) without control probes. The probes which were differentially expressed between the three conditions (PD, ACL, and TKA) were extracted.

Target Scan: Target Gene Prediction

We used the GeneSpring GX11 *TargetScan* function (Agilent) to predict the miRNA target genes. The context percentile was set at 90.0, and conserved and nonconserved databases were chosen.

Pathway Analysis

Pathway analysis of the association between the miRNA-regulated genes and the OA- and cartilage-related genes, which were detected using MetaCore, was performed using GenMAPP (http://www.genmapp.org/) pathway analysis software.

cDNA Synthesis

The incubated chondrocytes were rinsed twice in phosphate-buffered saline (PBS), placed in 700 μl of QIAzol Lysis Reagent (Qiagen, Inc., Valencia, CA), and the RNA was isolated using a miRNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocols. RNA at a concentration of 1 μg/μl that was isolated using the miScript Reverse Transcription Kit (Qiagen) was mixed with 4 μl of miScript RT Buffer, 1 μl of miScript Reverse Transcriptase Mix, and RNase-free Water. The solution was incubated for 60 min at 37°C and then 5 min at 95°C, and the cDNA was prepared.

Real-Time PCR

In the PCR used to confirm the miRNA, 25 μl of QuantiTect SYBR Green PCR Master Mix, 5 μl of miScript Universal Primer, 5 μl of miScript Primer Assay (Qiagen), a suitable quantity of RNase-free Water, and template cDNA were mixed for 15 min at 95°C, 15 s at 94°C, 30 s at 55°C, 30 s at 70°C (40 cycles), 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C (dissociation step) using a miScript Primer Assay kit (Qiagen) and a miScript SYBR Green PCR kit (Qiagen). In the calculations, the values were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the expression level was calculated using the $2^{-\Delta\Delta CT}$ values.

In the PCR used to confirm the gene expression, 12.5 μl of SYBR Green Master Mix, 0.5 μl of 20 nmol of both Primer Front and Primer Reverse, 1 μl of cDNA and 10.5 μl of distilled water were mixed for 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, 1 min at 60°C (60 cycles), 15 s at 95°C, 30 s at 60°C, and 15 s at 95°C (dissociation step). In the calculations, the values were standardized to GAPDH, and the expression level was calculated using the $2^{-\Delta\Delta CT}$ values. Real-time PCR was used to confirm the gene expression of aggrecan ($N = 6$), ADAMTS5 ($N = 8$), type 1 collagen ($N = 9$), type 2 collagen ($N = 6$), SOX9 ($N = 7$), MMP3 ($N = 7$), and MMP13 ($N = 7$). The primer sequences used in the real-time PCR are shown in Table 1.

MTT Assay

We used samples of knee articular cartilage tissue that were harvested from a total of 12 patients with knee OA who had

Table 1. List of Primers Used in Real-Time PCR

Primer ID	Accession No.	Sequence	Expect Size (bp)
Aggrecan-F	NM_001135	TCG AGG ACA GCG AGG CC	94
Aggrecan-R		TCG AGG GTG TAG GCG TGT AGA GA	
ADAMTS5-F	NM_007038	GAG CCA AGG GCA CTG GCT ACT A	120
ADAMTS5-R		CGT CAC AGC CAG TTC TCA CAC A	
Type I collagen-F	NM_000088	AAG GGT GAG ACA GGC GAA CAA	170
Type I collagen-R		TTG CCA GGA GAA CCA GCA AGA	
Type II collagen-F	NM_033150	GGA CTT TTC TTC CCT CTC T	113
Type II collagen-R		GAC CCG AAG GGT CTT ACA GGA	
SOX9-F	NM_009428	AAC GCC GAG CTC AGC AAG A	138
SOX9-R		CCG CGG CTG GTA CTT GTA ATC	
MMP3-F	NM_002422	ATT CCA TGG AGC CAG GCT TTC	138
MMP3-R		CAT TTG GGT CAA ACT CCA ACT GTG	
MMP13-F	NM_002427	TCA CGA TGG CAT TGC TGA CA	77
MMP13-R		AGG GCC CAT CAA ATG GGT AGA	
GAPDH-F	NM_002046	GCA CCG TCA AGG CTG AGA AC	142
GAPDH-R		ATG GTG GTG AAG ACG CCA GT	

undergone TKA. We prepared a solution by dissolving DMEM High Glucose 1× (Invitrogen) at a concentration of 4 mg/ml. Subsequently, we seeded the cells into 24-well plates at a density of 4.0×10^4 cells/cm^2. Before adding the MTT solution, the DMEM medium was removed completely and the cells were washed with PBS once. To each 24-well plate, 500 μl of MTT solution was added, and the plate was incubated for 2 h at 37°C in the dark. After incubation, the MTT solution was removed, 300 μl of dimethyl sulfoxide was added to each well, 100 μl of the supernatant liquid was transferred to each well of a 96-well plate, and the absorbance was measured at 590 nm.

miRNA Overexpression

1×10^5 chondrocytes were seeded in 24-well plates along with synthetic double-stranded RNA (Syn-rno-miR-199a-3pmi miScript miRNA Mimic, Syn-hsa-miR-193bmi miScript miRNA Mimic, and Syn-hsa-miR-320c miScript miRNA Mimic), a 2 nM solution and 5 μl/well of HiPerFect Transfection Reagent (Qiagen) was added, and the cells were incubated for 6 h at 37°C in 5% CO_2 and 95% air. In the control group, we added only 5 μl/well of the HiPerFect Transfection Reagent (Qiagen) after cell seeding (Table 2).

Repression of miRNA Expression

The chondrocytes were seeded at a density of 1×10^5 cells/cm^2 into 24-well plates, and anti-rno-miR-199a-3pmi miScript miRNA inhibitor, anti-hsa-miR-193bmi miScript miRNA inhibitor, or anti-hsa-miR-320c miScript miRNA inhibitor (Qiagen) and 5 μl/well of HiPerFect Transfection Reagent (Qiagen) was added. A 2 nM solution was made, and the cells were incubated for 6 h at 37°C in 5% CO_2 and 95% air. In the control group, we added only 5 μl/well of the HiPerFect Transfection Reagent (Qiagen) after cell seeding. The chondrocytes used in the miRNA overexpression group and inhibitor group were harvested from the knee joints of 10 patients with knee OA who had undergone TKA (Table 2).

Statistical Analysis

Steel's test, a nonparametric multiple-comparison test, was used to evaluate the real-time PCR and MTT assay data. One-way ANOVA was used to evaluate microarray analysis.

RESULTS

Microarray Analysis

Microarray analysis was performed to isolate the miRNA whose expression differs between PD, ACL, and TKA, and with age. A test microarray to compare the three groups (PD, ACL, and TKA) showed a significant difference ($p < 0.05$) by analysis of variance. Isolation of the miRNAs whose expression varied consistently showed that expression was upregulated incrementally with age in two types (miR-199a-3p and miR-193b) and was downregulated incrementally with age in one type (miR-320c; Fig. 1).

Table 2. List of the RNA Sequences Used for Transfection

Name	Sequence
rno-miR-199a-3p Mimic	ACAGUAGUCUGCACAUUGGUUA
rno-miR-199a-3p Inhibitor	TGTCATCAGACGTGTAACCAAT
hsa-miR-193b Mimic	CGGGGUUUUGAGGGCGAGAUGA
hsa-miR-193b Inhibitor	TTGACCGGGAGTTTCAGGGCGA
hsa-miR-320c Mimic	AAAAGCUGGGUUGAGAGGGU
hsa-miR-320c Inhibitor	TTTTCGACCCAACTCTCCCA

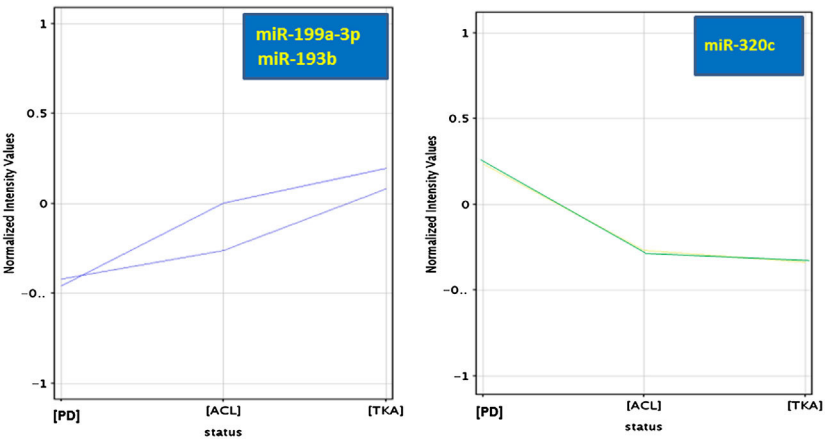

Figure 1. Result of microarray. Microarray analysis was performed to isolate the miRNA whose expression differs between PD, ACL, and TKA, and with age. MiR-199a-3p and miR-193b were incrementally upregulated, and miR-320c was incrementally downregulated with age.

Target Scan

A target scan was performed to investigate the genes regulated by the identified miRNAs: seven genes were regulated by miR-199a-3p, 10 were regulated by miR-193b, and 19 were regulated by miR-320c (Table 3).

Pathway Analysis

Analysis of differences in the pathways between the isolated miRNAs and the OA-related genes and cartilage-related genes revealed that the miRNA-regulated genes were those involved in the metabolism of cartilaginous tissue, such as type 1 collagen, MMP13, stromelysin 1(MMP3), and aggrecanase 2 (ADAMTS5; Fig. 2A,B).

Real-Time PCR

Gene expression analysis was performed using miRNAs that were isolated by microarray (miR-199a-3p, miR-193b, and miR-320c). MiR-199a-3p and miR-193b, for which expression is upregulated with age, regulated anabolic factors such as aggrecan, type 2 collagen, and SOX9. MiR-320c, in which expression is downregulated with age, regulated the catabolic factor ADAMTS5 (Table 4).

MTT Assay

The MTT assay showed no significant difference in the cell proliferation capacity between miR-199a-3p and miR-320c. By contrast, the cell proliferation capacity was upregulated significantly in miR-193b in the inhibitor group on day 7 (Fig. 3).

MiRNA Expressions

Each mimic group increased in the miRNA expression. On the other hand, each inhibited group decreased (Table 5).

DISCUSSION

MiRNAs are noncoding RNAs of ~22 base pairs that are involved in cell proliferation, differentiation, development, and tumorigenesis. miRNAs bind to the 3′-end of the untranslated region of mRNA (the target) where they induce posttranscriptional repression and mRNA degeneration, and modulate gene expression.[11–18] Since Lee et al.[19] reported the first miRNAs, several hundred have been discovered in plants and animals, and miRNAs specific to certain animal species and tissues are also known. Several types of miRNA have been discovered in fetal skeletal tissue, and it is believed that they play a key role in cartilage development.[20]

In terms of the miR-199a, which was the focus of this study, in experimental models using articular chondrocytes from mice, Lin et al.[21] reported that Col2A1, COMP, and SOX9 expression was downregulated in the miR-199a expression-enhanced group and upregulated in the miR199a expression-repressed group compared with the control group. Because the expression of miR-199a after BMP2 induction declines initially and then increases gradually for several days, it is probably essential in the final stages of cartilage development, including cartilage hypertrophy and cartilage maturity.[21] MiR-193b may also target SOX5. In miRNA expression analysis on differentiated and dedifferentiated chondrocytes, miR-193b expression is about 7.6-fold higher in dedifferentiated chondrocytes than in differentiated chondrocytes (Table 5).[22] However, there is no report on the functional analysis of miR-193b. Our study is the first report that miR-193b affects human cartilage metabolism by regulating type 2 collagen, aggrecan, and SOX9 expression. Although an association between miR-320c expression and other abnormalities such as impaired glucose tolerance[23] has been reported, no relationship with cartilage has been reported.

The miRNAs identified by microarray varied with age, and this pattern may affect articular cartilage metabolism. The results of the real-time PCR analysis of the three types of miRNA (Table 4) suggest that miR-199a-3p and miR-193b expression is upregulated with age and may be involved in chondrocyte senescence by downregulating anabolic factors such as type 2 collagen, aggrecan, and SOX9. Conversely, the results also suggest that miR-320c expression is downregulated with age and may be involved in the juvenile properties of chondrocytes by downregulating the

Table 3. Results of Target Scan Analysis

Probe name	miRNA	Gene Symbol
(A)		
A_23_P19987	hsa-miR-199a-3p	IGF2BP3
A_23_P134125	hsa-miR-199a-3p	MAP3K5
A_33_P3377130	hsa-miR-199a-3p	MAP3K5
A_33_P3389188	hsa-miR-199a-3p	TFAM
A_33_P3276475	hsa-miR-199a-3p	CHMP1B
A_23_P30995	hsa-miR-199a-3p	CYBSR4
A_23_P307392	hsa-miR-199a-3p	DPF3
A_32_P160883	hsa-miR-199a-3p	NEDD4
A_33_P3619171	hsa-miR-193b	PMAIP1
A_23_P47614	hsa-miR-193b	PHLDA2
A_33_P3357949	hsa-miR-193b	ETV1
A_32_P78491	hsa-miR-193b	ETV1
A_33_P3401156	hsa-miR-193b	ETV1
A_33_P3357954	hsa-miR-193b	ETV1
A_32_P192376	hsa-miR-193b	ENPP1
A_23_P156880	hsa-miR-193b	ENPP1
A_23_P48339	hsa-miR-193b	IFT88
A_33_P3408320	hsa-miR-193b	LASS1
A_23_P502312	hsa-miR-193b	CD97
A_32_P12610	hsa-miR-193b	E2F6
A_23_P88580	hsa-miR-193b	ARID3B
A_23_P30655	hsa-miR-193b	NFKBIE
(B)		
A_23_P329198	hsa-miR-320C	OBFC2A
A_32_P181638	hsa-miR-320C	BVES
A_23_P68031	hsa-miR-320C	STAT4
A_33_P3312182	hsa-miR-320C	C10orf47
A_23_P82990	hsa-miR-320C	OGN
A_33_P3519683	hsa-miR-320C	ZBTB8OS
A_24_P77904	hsa-miR-320C	HOXA10
A_33_P3231297	hsa-miR-320C	CREG1
A_24_P99838	hsa-miR-320C	ZNF223
A_23_P127140	hsa-miR-320C	RAB11FIP2
A_23_P219084	hsa-miR-320C	ZNF3
A_33_P3289113	hsa-miR-320C	COX11
A_23_P309865	hsa-miR-320C	ZNF449
A_23_P81650	hsa-miR-320C	C5orf15
A_33_P3304372	hsa-miR-320C	TMEM144
A_23_P97700	hsa-miR-320C	TXNIP
A_23_12896	hsa-miR-320C	FANCF
A_23_P343398	hsa-miR-320C	CCR7
A_33_P3392537	hsa-miR-320C	TK2

(A) Of the two types of miRNA whose expression is upregulated with age, miR-199a-3p regulated 7 types of genes and miR-193b regulated 10 types of genes. (B) miR-320c, whose expression is downregulated with age, regulates 19 types of genes.

expression of the catabolic factor ADAMTS5. MiR-199a-3p and miR-193b may be involved in cartilage degeneration, and we found nothing that contradicts previous reports.[21,22] ADAMTS5 is an efficient aggrecanase and induces cartilage degeneration. Although miR-320c has not been reported in relation to cartilage, the expression of ADAMTS5 was downregulated in the miR-320c mimic group and was upregulated in the inhibitor group. This suggests that miR-320c is involved in the metabolism of chondrocytes by regulating ADAMTS5.

We used the TargetScan program to investigate the genes regulated by the three identified types of miRNA. ETV1 (ER81), which is regulated by miR-193b, has also been reported as a transcription factor unique to mesenchymal stem cells (MSCs).[24] In the study by Kubo et al.,[24] ETV1 expression was upregulated by 5–10 times in MSC compared with fibroblasts, chondrocytes, synovial cells, and adipose cells. Kubo et al. also reported that cell differentiation and juvenile properties are affected when ETV1 is knocked down using short interfering RNA; for example, the self-renewal capacity of MSCs was repressed. Another study reported that the expression of STAT4, which is regulated by miR-320c, was upregulated in cells in which chondrocyte differentiation has been induced compared with a control group,[25] suggesting that STAT4 affects cartilage differentiation.

In the analysis of the pathways between the OA-related genes and cartilage-related genes detected by MetaCore and the miRNAs we identified in this study, ER81 (ETV1), which is controlled by miR-193b, produced positive feedback on the OA-related genes MMP1, stromelysin-1(MMP3), and MMP13 (Fig. 2A). Although the pathway of the E2F6 gene, which is regulated by miR-193b, is unclear, the pathway analysis suggested that this gene is involved in the feedback on aggrecanase-2(ADAMTS5) and transforming growth factor β1 (TGFβ1; Fig. 2A). NEDD4, which is regulated by miR-199a-3p, produced negative feedback on connexin 43, another OA-related gene (Fig. 2A). On the basis of the comparison of pathway analysis between genes regulated by miRNAs and cartilage-related genes, we believe that HOXA10, which is regulated by miR-320c, may regulate MMP13 through RUNX2 (Fig. 2B).

Although there is a great deal of literature on miRNAs, there are few reports on the relationship between miRNAs and the cell proliferation capacity of chondrocytes. The MTT assay performed in this study (Fig. 3) suggests that the effects on cell replication capacity did not differ between miR-199a-3p and miR-320c. Although miR-193b was upregulated significantly in the inhibitor group compared with the control group on day 7, mimic group was no significant difference. Thus these three miRNAs(miR-199a-3p, miR-193b, miR-320c) may not affect the cell replication capacity of chondrocytes.

Our findings suggest that miR-199a-3p and miR-193b may be involved in chondrocyte aging by regulating aggrecan, type 2 collagen, and SOX9, and that miRNA-320c may be involved in the juvenile properties of chondrocytes by regulating ADAMTS5. Our findings suggest that miR-199a-3p, miR-193b, and miR-320c may be useful for a marker of cartilage as donor tissue of cartilage graft and tissue culture for cell therapy (Fig. 4).

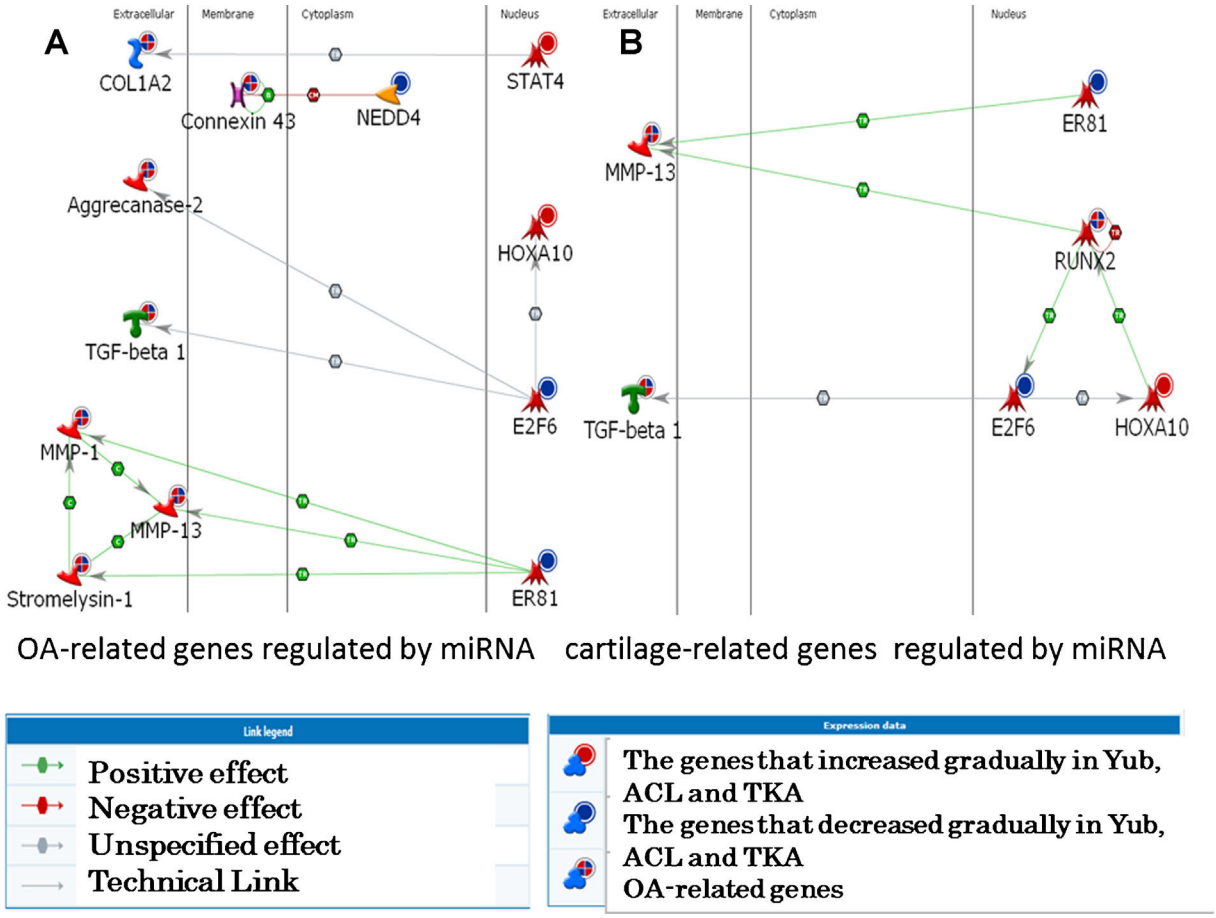

Figure 2. Pathway analysis. (A) Pathway analysis between OA-related genes and the genes regulated by miRNA. ER81 (ETV1) controlled by miR-193b produced positive feedback on MMP-1, stromelysin-1(MMP3), and MMP-13. Similarly, although the pathway of E2F6 gene, which is controlled by miR-193b, is unclear, it is involved in aggrecanase-2(ADAMTS5) and TGFβ1 feedback. NEDD4, which is controlled by miR-199a-3p, produced negative feedback on connexin 43, an OA-related gene. (B) Pathway analysis between cartilage-related genes and the genes regulated by miRNA. In the pathway analysis of genes regulated by miRNA and cartilage-related genes, HOXA10, which is regulated by miR-320c, regulates MMP13 through RUNX2.

Table 4. Relative Expression of mRNA

	miR199a-3p			miR193b			miR320c		
Gene Name	Fold Change	*p* Value	SE	Fold Change	*p* Value	SE	Fold Change	*p* Value	SE
Mimic COL1	0.5	<0.05	0.16	2.34	0.88	0.94	0.80	0.07	0.24
Inhibit COL1	0.79	<0.01	0.33	2.11	0.72	1.38	3.59	<0.01	1.56
Mimic COL2	0.5	<0.05	0.18	0.34	<0.05	0.16	0.80	0.13	0.22
Inhibit COL2	8.5	<0.01	4.21	4.07	<0.05	5.65	1.18	0.42	0.30
Mimic MMP3	1.08	0.85	0.09	1.11	0.06	0.35	0.79	<0.05	0.27
Inhibit MMP3	0.73	0.10	0.20	0.75	<0.05	0.10	1.63	0.57	0.79
Mimic MMP13	0.58	<0.05	0.15	1.03	0.34	0.33	0.47	<0.01	0.19
Inhibit MMP13	0.75	1.00	0.36	0.95	<0.01	0.29	1.35	1.00	0.28
Mimic aggrecan	0.86	<0.01	0.05	0.68	<0.01	0.09	1.59	0.07	0.31
Inhibit aggrecan	6.12	<0.05	5.66	2.66	<0.01	0.90	0.70	0.07	0.38
Mimic ADAMTS5	3.53	0.27	2.21	1.05	0.58	0.40	0.39	<0.05	0.17
Inhibit ADAMTS5	2.61	1.00	1.59	0.80	0.08	0.37	2.81	<0.05	0.70
Mimic SOX9	0.7	<0.05	0.15	0.73	<0.05	0.13	0.95	1.00	0.30
Inhibit SOX9	1.24	<0.05	0.11	2.61	<0.01	0.82	1.05	0.78	0.38

Expression of mRNA in the type 2 collagen, aggrecan, and SOX9 mimic group was downregulated by miR-199a-3p but was upregulated in the inhibitor group. Similarly, expression in the type 2 collagen, aggrecan, and SOX9 mimic group was downregulated by miR-193b but was upregulated in the inhibitor group. Expression of miR-320c was downregulated in the ADAMTS5 mimic group but upregulated in the inhibitor group.

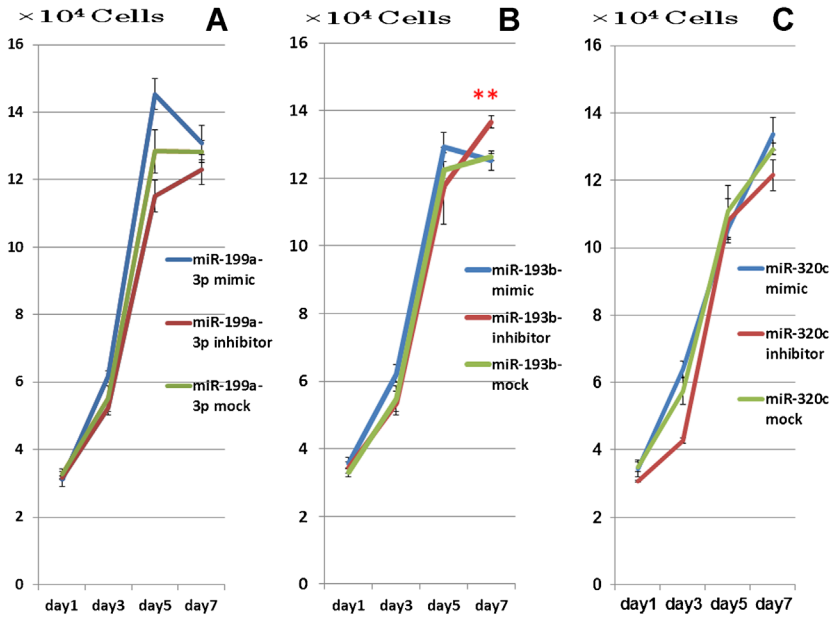

Figure 3. MTT assay. The cell proliferation potential did not differ between the mimic and the inhibitor group for miR-199a-3p (A) and miR-320c (C), but cell proliferation was upregulated significantly in the inhibitor group on day 7 (B). $^{**}p < 0.01$.

Table 5. MiRNA Expressions

	Fold Change		
	miR199a-3p Expession	miR193b Expression	miR320c Expression
Mimic	37.1533	141.9286	114.9257
Inhibitor	0.728932	0.372904	0.5014

Expression levels of the three miRNAs (miR-199a-3p, miR-193b, and miR-320c) isolated by the microarray analysis were increased by miScript miRNA Mimic, which had a similar function to the intrinsic mature miRNA. Expression levels were decreased by the miScript miRNA inhibitor, which specifically inhibited the function of the miRNA.

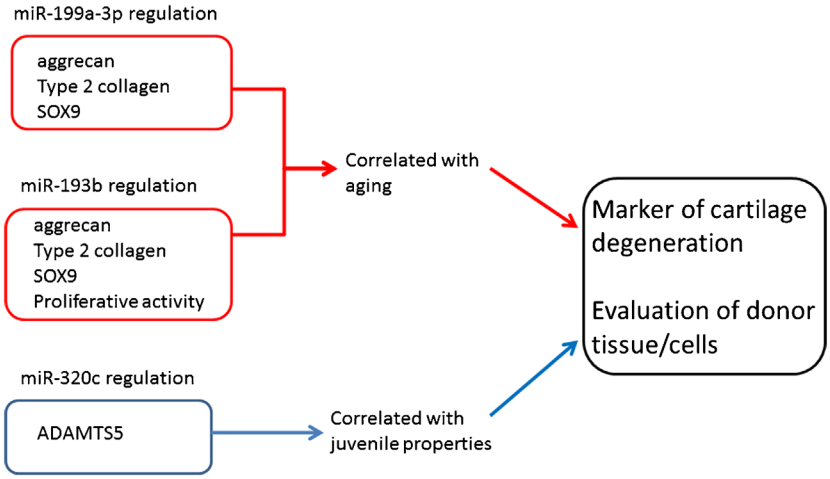

Figure 4. Three miRNAs (miR-199a-3p, miR-193b, and miR-320c) correlate with chondrocytes. MiR-199a-3p and miR-193b may be involved in chondrocyte aging by regulating aggrecan, Type 2 collagen, and SOX9, and miRNA-320c may be involved in the juvenile properties of chondrocytes by regulating ADAMTS5. MiR-199a-3p, miR-193b, and miR-320c may be useful for a marker of cartilage degeneration and evaluation as donor tissue of cartilage graft.

In summary, three types of miRNA whose expression correlates with age were identified in chondrocytes. These findings suggest that miR-199a-3p and miR-193b are involved in chondrocyte aging by regulating aggrecan and type 2 collagen, and that SOX9 and miRNA-320c are involved in the juvenile properties of chondrocytes by regulating ADAMTS5.

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