microRNA-495 Inhibits Chondrogenic Differentiation in Human Mesenchymal Stem Cells by Targeting *Sox9*

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The chondrogenic differentiation process of human mesenchymal stem cells (hMSCs) passes through multiple stages, which are carried out by various factors and their interactions. Recently, microRNAs that regulate chondrogenic differentiation have been reported. However, microRNA that regulates SRY-related high mobility group-box gene 9 (*Sox9*), a chondrogenic key factor, has not been identified in hMSC. In this study, we identified that microRNA-495 (miR-495) is an important regulator of hMSC chondrogenic differentiation. In our microarray, miR-495 was downregulated during transforming growth factor (TGF)- β 3-induced chondrogenic differentiation of hMSCs in vitro. We found that there is an miR-495 binding site in the 3' untranslated region (3'UTR) of *Sox9*. We confirmed opposite expression between miR-495 and *Sox9* by using real-time polymerase chain reaction. Further, overexpression of miR-495 inhibited *Sox9* expression, and repression of miR-495 increased expression of *Sox9* in SW1353 cells and hMSCs. Additionally, luciferase analysis revealed that miR-495 directly binds to the *Sox9* 3'UTR, and we confirmed a seed sequence of miR-495 on the *Sox9* 3'UTR. Subsequently, overexpression of miR-495 repressed the expression of the extracellular matrix (ECM) protein, such as type II collagen (Col2A1), aggrecan, and proteoglycan products, whereas inhibition of miR-495 increased their expression. Collectively, this study indicates that miR-495 directly targets *Sox9*, ultimately leading to the regulation of chondrogenic differentiation in hMSCs.

Introduction

CHONDROGENESIS IS AN ESSENTIAL process in cartilage and bone development. This crucial process is initiated with mesenchymal stem cell (MSC) condensation and differentiation into chondrocytes. Condensation of MSCs is crucial for chondrogenic differentiation, and this process is driven by cell-matrix interactions and various factors [1]. The chondrogenic differentiation stage can be characterized by accumulation of cartilage extracellular matrix (ECM) containing type II collagen, type IX collagen, type XI collagen, and aggrecan [2]. On hypertrophic stage of chondrogenic differentiation, chondrocytes express type X collagen [3]. In long bone formation, hypertrophic chondrocytes eventually undergo apoptosis and are replaced to bone. This sequential process of chondrogenic differentiation is modulated by different signaling pathways and various factors, such as transforming growth factor- β (TGF- β), bone morphogenetic proteins (BMPs), fibroblast growth factor, and Indian hedgehog. Among these regulators, TGF- β and BMPs are well known as the most powerful regulator of chondrogenesis both in vitro and in vivo [4,5]. In MSCs, TGF- β induces binding between SRY-related high mobility group-box gene 9 (*Sox9*) and Smad3. Eventually *Sox9* bound to Smad3 en-

hances its transcriptional activity, triggering MSC chondrogenesis [6]. BMPs are members of TGF- β superfamily, and BMP signaling is related to chondrogenic differentiation and chondrocyte proliferation [7]. Specially, BMP2 can induce chondrogenic differentiation and enhance ECM expression in human articular chondrocytes [8]. However, recent report showed that both TGF- β 3 and BMP2 signaling have the distinct effects in fetal and adult MSCs. TGF- β 3 induces chondrogenic differentiation in adult MSCs, whereas BMP2 induces chondrogenic differentiation in fetal MSCs [9]. These results indicate that cytokines that induce chondrogenic differentiation may have another effect depending on the different cell sources.

Sox9 is required at a sequential step of chondrogenic differentiation [10]. *Sox9* acts as a significant regulator in mesenchymal condensation at an early stage of chondrogenic differentiation [11]. In chondrocytes, *Sox9* can bind to the chondrocyte-specific enhancer of type II collagen, and the binding can activate the expression of type II collagen [12,13]. In addition, *Sox9* can stimulate the expression of type XI collagen, aggrecan, and CD-RAP genes, known to be specific markers of chondrogenic differentiation [14–16]. In mouse chimeras, *Sox9*-knockout cells did not express chondrocyte-specific marker genes, such as type II collagen,

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type IX collagen, type XI collagen, and aggrecan [10]. These results strongly show that Sox9 is a critical regulator in chondrogenic differentiation and cartilage formation.

microRNAs that play crucial roles in diverse biological processes, including cell proliferation, apoptosis, and differentiation, have been reported previously [17,18]. microRNAs are small noncoding RNAs that are ~17–23-nucleotide long, and they negatively regulate the expression of target genes at the post-transcriptional level [19]. In addition, recent studies show that specific microRNAs are necessary for the development and maintenance of skeletal tissues. Among various microRNAs that can regulate physiological processes of skeletal development, microRNA-140 (miR-140) is specifically expressed in cartilage tissue during zebrafish embryonic development and homeostasis [20]. In addition, several reports have confirmed that miR-140 can regulate chondrogenesis by targeting histone deacetylase 4, insulin growth factor binding protein 5 (IGFBP5), Adamts-5, Smad3, and Dnpep expression [21–25]. miR-199a is a well-known BMP2-responsive microRNA and a regulator of chondrogenic differentiation via targeting smad1 [26]. Further, our previous report showed that LEF-1 promotes chondrogenic differentiation of hMSCs and revealed that miR-449a regulates LEF-1 expression [27]. Although microRNAs regulating chondrogenesis have been reported, it was not reported whether microRNAs regulate chondrogenic differentiation of human mesenchymal stem cells (hMSCs) by directly targeting Sox9 expression.

In this study, we screened microRNAs associated with chondrogenesis during *in vitro* chondrogenic differentiation of hMSCs by using a microRNA microarray, and found that miR-495 negatively regulates chondrogenic differentiation of hMSCs. Other reports identified that miR-495 is a cancer suppressor or a regulator of liver and pancreas development [28–31]. In our study, we determined that miR-495 plays inhibitory roles during chondrogenic differentiation of hMSCs. Specifically, miR-495 can directly bind to the Sox9 3' untranslated region (3'UTR) and inhibit Sox9 expression, with a corresponding decrease of chondrogenic markers.

Materials and Methods

Bone-marrow-derived hMSC and chondrosarcoma cell line culture

Bone marrow aspirates were obtained from the posterior iliac crest of 12 healthy adult donors between 19 and 63 years of age, after approval from the Institutional Review Board. hMSCs were specifically selected using their natural tendency to adhere to a plastic culture plate surface. Bone marrow aspirates were cultured for 7 days in Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Welgene) with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic solution (Gibco). hMSCs were subcultured up to passages 3–5 to obtain the appropriate number of cells required for the experiment. The SW1353 cell line, a human chondrosarcoma cell line, was cultured in DMEM–high glucose (DMEM-HG; Welgene) containing 10% FBS and 1% antibiotic-antimycotic solution.

Flow cytometry analysis

hMSCs (1×10^6) at passage 4 were stained with FITC-anti-CD34 (Miltenyi Biotec, Inc.), PerCP-anti-CD45 (Miltenyi

Biotec, Inc.), APC-Vio770-anti-CD90 (Miltenyi Biotec, Inc.), and PE-Cy7-anti-CD105 (BioLegend, Inc.) for 30 min at 4°C. Thereafter, the stained cells were washed by phosphate-buffered saline (PBS) at 300 g for 10 min, and then resuspended in 500 μ L of ice-cold PBS (with 1% FBS and 0.05% sodium azide). The samples were subjected to FACS analysis (BD FACSVers flow cytometer; BD Biosciences). With unstained sample (1×10^6) as negative control, they were analyzed using BD FACSuite software (BD Biosciences).

Osteogenic and adipogenic differentiation

To confirm differentiation potentials of cultured hMSCs, the cells were seeded at 8×10^4 cells/well in 12-well culture plates. Osteogenic differentiation was induced for 14 days using osteogenic differentiation medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 100 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma), and 50 μ g/mL ascorbic acid (Gibco)]. For alizarin red S staining, cells were fixed in 1:1 acetone:methanol, and incubated in 3% alizarin red S solution (Sigma) for 30 min. Adipogenic differentiation was induced for 14 days using adipogenic differentiation medium [DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 1 μ M dexamethasone, 0.5 mM isobutylthethylxanthin (Sigma), 5 μ g/mL insulin (Gibco), and 200 μ M indomethasin (Sigma)]. For oil red O staining, cells were fixed in 10% neutral buffered formalin, and incubated in 0.18% oil red O solution (Sigma) for 30 min.

In vitro chondrogenic differentiation of hMSCs using a micromass culture method

A micromass culture method was used for *in vitro* chondrogenic differentiation of hMSCs. The trypsinized hMSCs were resuspended in DMEM-LG with 10% FBS at a density of 1×10^7 cells/mL, and 10 μ L of the resuspended cells was dotted on the center of individual wells of 24-well plates (1×10^5 cells/well). The cells were allowed to adhere at 37°C for 2 h, and then chondrogenic medium, consisting of DMEM-HG supplemented with 1% antibiotic-antimycotic solution, 1% Insulin Transferrin Selenium-A (ITS; Invitrogen), 50 μ g/mL ascorbic acid (Invitrogen), and 10 ng/mL TGF- β 3 (R&D Systems), was gently added. The chondrogenic media containing 10 ng/mL of TGF- β 3 was changed every 2 days during *in vitro* differentiation periods.

Quantitative real-time polymerase chain reaction

Total RNAs from hMSCs and SW1353 cells were isolated using RNA iso Plus (Takara) reagent following the manufacturer's instructions. For cDNA reverse transcription, RNAs were reverse transcribed using an Omniscript Reverse Transcription Kit (Qiagen). The cDNA was used in real-time polymerase chain reaction (PCR) with an SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR was performed using an ABI7500 real-time machine by Applied Biosystems. All primers were purchased from Bioneer. The primers used were GAPDH (P267613) and Sox9 (P232240). There were no validated primers for Col2a1 and aggrecan; thus, the primers were designed as follows: Col2a1, 5'-GT CCTCTCCCAAGTCCACACAG-3' (sense) and 5'-GGGCA CGAAGGCTCATCATTC-3' (antisense); and aggrecan, 5'-CCACTGTTACCGCCACTT-3' (sense) and 5'-GTAGTCT

TGGGCATTGTTGT-3' (antisense). The PCR procedure was initiated for 30 s at 95°C, followed by 40 thermal cycles of 5 s at 95°C and 20 s at 60°C. SYBR fluorescence was detected during the annealing/extension phase, and all real-time PCR products had a final size of ~100 bp. Values from each sample were normalized to GAPDH as an internal control.

For precursor-microRNA reverse transcription, an Mir-X™ microRNA First-Strand Synthesis kit (Clontech) was used following the manufacturer's instructions, and cDNA synthesized from microRNAs was quantified using SYBR qRT-PCR (Clontech). The list of primer sets used for specific mature-microRNA amplification was obtained from Genolution (Genolution) and Bioneer. The primers were designed as follows: U6 small nuclear RNA (snRNA), 5'-CTCGCTT CCGCAGCACA-3' (sense) and 5'-AACGCTTCACGAATT TGCGT-3' (antisense); has-miR-495, 5'-AAACAAACATG GTGCACTTCTT-3' (sense); has-miR-431, 5'-TGTCTTGC AGGCCGTCATGCA-3' (sense). Real-time PCR was performed using an ABI7500 real-time machine. The PCR program was initiated for 10 s at 95°C, followed by 40 thermal cycles of 5 s at 95°C and 20 s at 60°C. U6 snRNA was used to normalize for relative expression intensity for all pre-microRNAs.

microRNA array analysis

The total RNA of undifferentiated hMSCs (cultured during days 0 and 10) and chondrogenic hMSCs from the same donors was isolated by RNA iso Plus (Takara) by following the manufacturer's protocol. The overall quality of the total RNA was validated using spectrophotometry. A Genechip® miRNA 1.0 array (Affymetrix) was used to confirm the expression patterns of different microRNAs. Pearson's correlation analysis was performed to elucidate microRNAs specifically expressed in chondrogenesis-induced MSCs. In brief, the differentially expressed genes were selected using the routine formula of subtracting control groups from the differentiated group of interest; a strict cutoff threshold was implemented to screen genes with at least a 1.5-fold difference in expression intensity. The data have been analyzed for two donors, and the intersection of the results from the two donors is presented.

microRNA transfection

For functional analysis of screened microRNAs and their effects on Sox9 expression, the selected microRNAs were purchased from Genolution. The purchased microRNA mimic was prepared in double-strand form, which would be processed once transfected by endogenous mechanisms and would ultimately yield mature microRNA. Because anti-microRNAs consist of a single-stranded form to target the complementary sequences of microRNAs, scrambled oligonucleotides for anti-microRNA (excluding scrambled oligonucleotides for the microRNA mimic) were separately used as a negative control. Anti-miR-SC and anti-miR-495 were purchased from Bioneer and designed as follows: anti-miR-495, 5'-AAGAAGUGCAC CAUGUUUGUUU-3' (sense); anti-miR-SC, 5'-UCACAACC UCCUAGAAAGAGUAGA-3' (sense). The constructed microRNAs were transfected with Lipofectamine LTX & Plus reagent (Invitrogen) on SW1353 cells and hMSCs by following the provided instructions.

Western blot analysis

Cell lysates were prepared using passive lysis buffer (Promega) containing 10 µg/mL of protease and phosphatase inhibitor and quantified using Pierce™ BCA Protein Assay Kit (Thermo Scientific). The samples (10–30 µg protein) were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membrane (Amersham Pharmacia). Briefly, the membrane was incubated with antibodies targeting Sox9 (Millipore) or GAPDH (Santa Cruz) at a concentration of 1:3,000 for 4 h at room temperature. Following repeated washing of the membrane with 1× TBST, the membranes were incubated with appropriate horseradish-peroxidase-conjugated secondary antibody (Amersham Pharmacia) for 1 h. GAPDH was used as an internal control. Band intensity was measured with the Image J program using image files obtained from Epson image scanners.

Luciferase reporter assay

pEZX-SOX9 3'UTR and pEZX-MT01 vectors were purchased from Genecopoeia to confirm whether miR-495 binds to the Sox9 3'UTR. pEZX-SOX9 3'UTR contains the firefly luciferase gene with the Sox9 3'UTR and the renilla luciferase gene, whereas pEZX-MT01 contains the firefly luciferase and renilla luciferase genes without the Sox9 3'UTR. pEZX-SOX9 3'UTR or pEZX-MT01 was cotransfected with miR-495 or negative microRNA into HeLa cells by using Lipofectamine LTX & Plus reagent (Invitrogen). Firefly and renilla luciferase activities were measured at 24 h after microRNA transfection by using a dual-luciferase reporter assay system (Promega), and the firefly luciferase activities were normalized to renilla expression in triplicate.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized, rehydrated, and washed twice with PBS. To reduce nonspecific background staining due to endogenous peroxidase, the sections were incubated in hydrogen peroxide block for 10 min, and washed two times with PBS. The sections were incubated with rabbit anti-COL2A1 (Santa Cruz) or mouse anti-aggrexin (Santa Cruz) overnight at 4°C, and then washed with PBS. Phycoerythrin (PE)–conjugated goat anti-rabbit secondary antibodies (Santa Cruz) and green fluorescent protein (GFP)–conjugated goat anti-mouse secondary antibodies were used to visualize the primary antibodies. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma). The images were captured using an inverted fluorescence microscope (IX-71; Olympus).

Safranin O staining

The mass pellets were washed twice with PBS, and then fixed for 24 h in 10% formalin solution. After fixation, the pellets were dehydrated in ethanol, and the dehydrated pellets were paraffin-embedded and sectioned. For safranin O staining, the sectioned micromass pellets were deparaffinized and rehydrated. The rehydrated pellets were washed with PBS, and then stained with 1% safranin O solution (Sigma) dissolved in 1% acetic acid for 30 min. Hematoxylin solution (Sigma) was used to stain the background of

the safranin O-stained pellets. The stained pellet sections were mounted and evaluated microscopically.

Glycosaminoglycan content assay

The amounts of sulfated glycosaminoglycans (GAGs) were determined in culture medium obtained from micro-

RNA-transfected chondrogenic micromass cultures by using the Blyscan kit (Biocolor) according to the manufacturer's instructions. In brief, 500 μ L of the culture medium was mixed with 1 mL of Blyscan dye reagent by shaking for 30 min to complete GAG-dye binding. After centrifugation, the dye bound to GAGs was dissolved in a dissociation reagent. The recovered dye concentration was determined at

FIG. 1. Characterization and differentiation potentials of cultured hMSCs. **(A)** Cultured hMSCs were immunostained with antibodies for CD90, CD105, CD34, and CD45. The stained cells were analyzed using flow cytometry to detect the surface markers specific for MSCs. The percentage of cell population with positive or negative staining was represented in each figure. **(B)** hMSCs were seeded at 8×10^4 cells/well in 12-well culture plates. The cells were cultured in osteogenic or adipogenic differentiation medium for 14 days, and then stained with alizarin red S (*left panel*) or oil red O (*right panel*) to determine their osteogenic or adipogenic differentiation potentials. hMSCs, human mesenchymal stem cells.

B

Alizarin red S

**P4 hMSCs cultured in
osteogenic medium**

Oil red O

**P4 hMSCs cultured in
adipogenic medium**

656 nm, and a chondroitin 4-sulfate standard solution (Bio-color) was used to generate standard curves. All samples and standards were tested in triplicate.

Statistical analysis

The statistical analysis for the results obtained from human bone marrow-derived MSCs, SW1353, and HeLa cells was carried out using the Student's *t* test, and the data are expressed as the mean $\pm$ standard deviation. Values of $P < 0.05$ or 0.01 were considered statistically significant.

Results

Characterization of cultured cells and multilineage differentiation potential

To confirm whether hMSCs cultured up to passages 3–5 have characteristics of general MSCs, the hMSCs were analyzed using flow cytometry. It is well known that MSCs express CD90 and CD105, whereas do not express CD34 and CD45, hematopoietic stem cell marker [32–34]. More than 98% of the cultured cells were positive for CD90 and CD105, but negative for

CD34 and CD45 (Fig. 1A). To identify multilineage differentiation potential of hMSCs, osteogenic and adipogenic differentiation was induced in the cells cultured up to passages 3–5. After induction of osteogenic and adipogenic differentiation for 14 days, the cells were positively stained for alizarin red S and oil red O staining, respectively (Fig. 1B). These results showed that the cells used in current study have the characteristics considered as requirements of MSCs.

Conflicting expression between Sox9 and selected microRNAs during chondrogenic differentiation of hMSCs

To confirm the Sox9 expression pattern, we examined the mRNA expression of Sox9 genes during in vitro chondrogenic differentiation of micromass-cultured hMSCs. The mRNA expression of Sox9 was increased 8-fold (± 0.9) at day 5 and 24-fold (± 0.7) at day 10 of chondrogenic differentiation compared with that of undifferentiated hMSCs (Fig. 2A). We next performed microRNA array analysis to screen the up- and downregulated microRNAs during in vitro chondrogenic differentiation of hMSCs. Pearson correlation analysis was used

FIG. 2. mRNA expression pattern of Sox9 and a hierarchical clustering map of microRNAs during in vitro chondrogenic differentiation of hMSCs. (A) To observe the expression pattern of Sox9 mRNA during in vitro chondrogenic differentiation, quantitative real-time PCR was performed at days 0, 5, and 10 of chondrogenic differentiation. The mRNA expression levels of Sox9 were normalized to GAPDH expression and were performed in triplicate. The data presented have been confirmed in three donors tested, and representative data are shown (** $P < 0.01$ compared with day 0). (B) microRNA array analysis was performed to investigate expression changes of microRNAs during in vitro chondrogenic differentiation of hMSCs. In CH10 sample, dark gray and black indicates upregulated microRNAs, and light gray indicates downregulated microRNAs in the above hierarchical clustering data (one-way ANOVA $P < 0.05$). CT0 and CT10 mean the samples were analyzed in undifferentiated hMSCs at days 0 and 10, respectively, whereas CH10 means the sample was analyzed in TGF- β 3-induced chondrogenic hMSCs at day 10. (C) microRNA real-time PCR was performed to investigate the changes in expression of miR-495 and miR-431 during in vitro chondrogenic differentiation. The expression levels of miR-495 and miR-431 were normalized to U6 expression and were performed in triplicate. The data presented have been confirmed in two donors tested, and representative data are shown (** $P < 0.01$ compared with day 0). miR-495, microRNA-495; PCR, polymerase chain reaction; Sox9, SRY-related high mobility group-box gene 9; TGF, transforming growth factor.

FIG. 3. Effects of 2 selected microRNAs on Sox9 mRNA and protein expression of human chondrosarcoma cell line, SW1353. **(A)** Real-time PCR was performed to investigate the effect of miR-495 and miR-431 overexpression on endogenous Sox9 mRNA expression of SW1353. The expression level of Sox9 mRNA was normalized to GAPDH expression and was performed in triplicate. The data presented have been confirmed in two independent experiments in the SW1353 cell line (* $P < 0.05$ compared with miR-SC). miR-SC indicates scrambled control microRNA. **(B)** Western blot analysis was performed to investigate the protein expression of Sox9 in miR-SC-, miR-495-, or miR-431-transfected cells. The above data have been confirmed in three independent experiments, and representative data are shown. GAPDH was used as the internal control.

to classify the differentially expressed microRNAs between undifferentiated groups (CT0 and CT10) and chondrogenic differentiated groups (CH10). A hierarchical clustering map showed the differentially expressed microRNAs, and these results showed at least 1.5-fold differences between experimental groups with strict cutoff criteria (Fig. 2B). We have

identified 17 microRNAs that showed higher or lower expression in chondrogenic differentiated hMSCs (CH10) than in undifferentiated hMSCs (CT0 and CT10) during in vitro chondrogenic differentiation. Among differentially expressed microRNAs, we found that miR-495 and miR-431 have binding sites at the 3'UTR of the human *Sox9* gene (data not

FIG. 4. Sox9 is the direct target of miR-495. **(A)** The above figure shows the map of the pEZ-MT01 vector, which consists of SV40, firefly luciferase, *Sox9* 3'UTR, CMV, and renilla luciferase. The pEZ-MT01 vector containing the *Sox9* 3'UTR has a whole plasmid size of 9,460 bp, whereas the *Sox9* 3'UTR length is 2,030 bp. The miR-495 binding site (GUUUGUU) is located in the 1,900 to 1,906 bp region in the *Sox9* 3'UTR. **(B)** HeLa cells were cotransfected with the *Sox9* 3'UTR-containing pEZ-MT01 vector and each microRNA (50 nM miR-SC, 25 nM miR-495, or 50 nM miR-495). **(C)** HeLa cells were cotransfected with the *Sox9* 3'UTR-containing pEZ-MT01 vector and microRNA (50 nM miR-SC, 50 nM miR-495, or 50 nM mutant-miR-495). **(B, C)** After 24 h, dual-luciferase assays were performed, and relative firefly activities were normalized to renilla luminescence (* $P < 0.05$ compared with miR-SC). The above data have been confirmed in three independent experiments. 3'UTR, 3' untranslated region.

shown), according to the TargetScan program and microRNA target prediction database (www.targetscan.org), and their expression pattern was decreased during chondrogenic differentiation. In addition, downregulated expression of miR-495 and miR-431 was confirmed by real-time PCR (Fig. 2C). Based on these results, we hypothesized that miR-495 and miR-431 bind to the 3'UTR of *Sox9* to downregulate expression of their target gene, resulting in suppression of chondrogenic differentiation in hMSCs.

miR-495 decreases Sox9 expression in a chondrosarcoma cell line

To identify whether miR-495 and miR-431 regulate *Sox9* expression, miR-SC, miR-495, or miR-431 was, respectively, transfected into a chondrosarcoma cell line, SW1353. miR-495 and miR-431 decreased the mRNA expression of *Sox9* compared with miR-SC in SW1353 (Fig. 3A). However, only miR-495 decreased the protein expression of *Sox9* (Fig. 3B). These results suggest that miR-495 may play an important role in chondrogenic differentiation, as seen by the reproducibility of results from multiple experiments. Therefore, the latter part of this study focused on miR-495.

miR-495 directly targets Sox9 through binding to its complementary sequences present in the 3'UTR of Sox9

It is well known that microRNA suppresses the mRNA expression of target genes by binding to its complementary sequences present in the 3'UTR of the target genes. To identify whether miR-495 binds to their seed sequence present in *Sox9* 3'UTR, we performed the luciferase reporter assay using the pEXZ-*Sox9* vector containing the *Sox9* 3'UTR. We confirmed that the 3'UTR of the *Sox9* gene has a seed site of miR-495 (Fig. 4A). microRNA mimic and pEXZ-*Sox9* were cotransfected into HeLa cells, and then luciferase activities were measured at 24 h after transfection. Luciferase activities showed a dose-dependent decrease in pEXZ-*Sox9* vector-transfected HeLa cells by 25 and 50 nM miR-495 transfection compared with miR-SC-transfected HeLa cells (Fig. 4B).

Following such observations, we then aimed to confirm whether the predicted sequences between miR-495 and *Sox9* are an important seed site for their binding. miR-SC, miR-495 with normal sequences (AAACAAACAUGGUGC ACUUCUU), and miR-495 with seven mutated sequences (ACCACCCAAUGGUGCACUUCUU) were transfected

FIG. 5. Effects of miR-495 and anti-miR-495 on Sox9 expression of hMSC and SW1353 cell lines. (A) Western blot analysis was performed to investigate the protein expression of Sox9 in miR-SC-, miR-495-, anti-miR-SC-, or anti-miR-495-transfected hMSCs. Sox9 was not expressed without TGF-β₃. After 24 h of treatment with TGF-β₃, miR-495 suppressed the increase of Sox9 expression compared with miR-SC, but anti-miR-495 increased Sox9 expression compared with anti-miR-SC. The above data have been confirmed in three independent donors, and representative data are shown. GAPDH was used as the internal control. (B) miR-SC, miR-495, anti-miR-SC, or anti-miR-495 was individually transfected in SW1353 cells expected to maintain constant expression of Sox9 and miR-495. The western blot shows decreased expression of Sox9 protein after miR-495 transfection, whereas Sox9 protein expression of anti-miR-495-transfected cells was increased compared with anti-miR-SC-transfected SW1353 cells. The above data have been confirmed in three independent experiments, and representative data are shown. GAPDH was used as the internal control. The band intensity of Sox9 expression was measured with the Image J program, and normalized to the GAPDH band density (**P* < 0.05 compared with miR-SC or miR-495).

with the pEZ-Sox9 vector in HeLa cells, respectively. As a result, miR-495 induced an ~40% reduction of luciferase activities, whereas miR-495 with mutated sequences (mut-miR-495) had no effects on the luciferase activities of pEZ-Sox9-vector-transfected cells, indicating that miR-495 can bind to the predicted seed sequence with its complementary sequences (Fig. 4C).

miR-495 suppresses TGF- β -induced Sox9 expression in hMSCs

To investigate the effect of miR-495 on TGF- β -induced Sox9 expression, miR-495 and anti-miR-495 were transfected into hMSCs. We induced Sox9 expression by adding 10 ng/mL of TGF- β 3 in micromass-formed cells because Sox9 was not expressed in undifferentiated hMSCs. After 24 h of TGF- β 3 treatment, Sox9 expression was significantly decreased in miR-495-transfected hMSCs compared with miR-SC-transfected hMSCs, whereas anti-miR-495 inhibited increase of Sox9 expression against to anti-miR-SC-transfected hMSCs (Fig. 5A). The equivalent study was performed in SW1353 to confirm the effects of miR-495. The miR-495 and anti-miR-495 were transfected into SW1353 cells to investigate the effect of endogenous miR-495 suppression on Sox9 expression. Oligonucleotides with complementary sequences to miR-495 (anti-miR-495) were used to block the expression of endogenous miR-495. The results showed that the protein expression of endogenous Sox9 was decreased in miR-495-transfected SW1353 cells compared

with miR-SC-transfected cells, whereas its expression of endogenous Sox9 was significantly increased in anti-miR-495-transfected SW1353 cells compared with anti-miR-SC (Fig. 5B). These results suggest that miR-495 has negative effects on Sox9 expression, whereas inhibition of miR-495 increases Sox9 expression.

miR-495 regulates expression of chondrogenic differentiation markers in hMSCs

Ultimately, to identify the effects of miR-495 on chondrogenic differentiation of hMSCs, we examined the expression of Col2A1 and aggrecan mRNA, late chondrogenic markers, in miR-SC-, miR-495-, anti-miR-SC-, and anti-miR-495-transfected hMSCs during in vitro chondrogenic differentiation using real-time PCR. At day 5 of chondrogenic differentiation, miR-495 suppressed mRNA expression of Sox9, Col2A1, and aggrecan compared with the miR-SC-transfected hMSCs, whereas anti-miR-495 increased the mRNA expression of Sox9, Col2A1, and aggrecan compared with the anti-miR-SC-transfected hMSCs (Fig. 6A). To determine whether protein levels of Col2A1 and aggrecan can be regulated by miR-495 during chondrogenic differentiation of hMSCs, an immunohistochemical analysis was performed in paraffin-embedded sections of micromass cultures at day 10 of chondrogenic differentiation. Immunohistochemistry showed that miR-495 suppressed protein expression of Col2A1 and aggrecan during chondrogenic differentiation of hMSCs (Fig. 6B). In contrast to miR-495, anti-miR-495

FIG. 6. Immunocytochemistry for Col2A1 and aggrecan in the chondrogenic mass cultures of miR-495- or anti-miR-495-transfected hMSCs. To identify the effect of miR-495 on chondrogenic differentiation, 50 nM of miR-SC-, miR-495-, anti-miR-SC-, or anti-miR-495-transfected hMSCs was induced to undergo chondrogenic differentiation for 7 days. At day 7, chondrogenic differentiation was evaluated with real-time PCR of (A) Sox9 and chondrogenic marker genes (Col2A1 and aggrecan). The expression levels of Sox9, Col2A1, and aggrecan were normalized to GAPDH expression and were performed in triplicate. The data have been confirmed in three donors tested, and representative data are shown (* P < 0.05 compared with miR-SC or miR-495). (B) At day 10, the effects of miR-495 and anti-miR-495 on chondrogenic differentiation were analyzed by immunohistochemistry using anti-Col2A1 (PE: fluorescence) or anti-aggrecan (FITC: fluorescence) antibody. The nucleus was stained with DAPI (fluorescence). The above figures have been confirmed in three independent donors, and representative data are shown. Col2A1, type II collagen; PE, phycoerythrin.

FIG. 7. Histological analysis of the PGs and GAGs in the chondrogenic mass cultures of miR-495- or anti-miR-495-overexpressed hMSCs. (A) miR-SC, miR-495, anti-miR-SC, and anti-miR-495-transfected hMSCs (1×10^5 cells per mass) were incubated in chondrogenic medium containing 10 ng/mL TGF- β 3 for 7 days. After incubation, safranin O staining was performed to detect GAGs or PGs. The background was stained with hematoxylin. The above figures have been confirmed in three independent donors, and representative data are shown. (B) GAG contents were measured with a Blyscan sulfate GAG assay in miR-SC-, miR-495-, anti-miR-SC-, and anti-miR-495-transfected hMSCs. The sulfate GAGs were quantified in supernatant, and standardized by a chondroitin 4-sulfate standard solution (* $P < 0.05$ compared with miR-SC or anti-miR-SC). GAGs, glycosaminoglycans; PGs, proteoglycans.

increased the protein expression of Col2A1 and aggrecan at day 10 of chondrogenic differentiation.

Taken together, these results revealed that miR-495 is a negative regulator of hMSC differentiation to chondrogenic lineage through suppression of Sox9, resulting in a decrease of chondrogenic markers, such as Col2A1 and aggrecan.

miR-495 inhibits chondrogenic differentiation in hMSCs

We next examined the chondrogenic differentiation potential of miR-SC-, miR-495-, and anti-miR-495-transfected hMSCs by cultivating the cells in chondrogenic medium for 10 days. Subsequently, synthesis of proteoglycans (PGs) and GAGs in chondrogenic differentiated micromass pellets was visualized using safranin O staining. During chondrogenic differentiation of hMSCs, safranin O staining showed a significant decrease in PG synthesis in miR-495-transfected hMSCs compared with miR-SC-transfected hMSCs. In contrast to the result in miR-495-transfected hMSCs, anti-miR-495 slightly increased the PG synthesis at 10 days of chondrogenic differentiation compared with anti-miR-SC (Fig. 7A). To confirm the results of safranin O staining, we performed the Blyscan sulfated GAG assay in miR-SC-, miR-495-, anti-miR-SC-, and anti-miR-495-transfected hMSCs at day 10 of chondrogenic differentiation. Quantification of GAG showed that miR-495 inhibited chondrogenic differentiation of hMSCs compared with that of miR-SC-transfected hMSCs, whereas anti-miR-495 slightly increased the accumulation of GAG during chondrogenic differentiation compared with anti-miR-SC (Fig. 7B). These results indicate that miR-495 regulates chondrogenic differentiation of hMSCs by suppressing the expression of Sox9.

Discussion

microRNAs have been considered with a lot of interest in various mechanisms, and they have been shown to play

crucial roles in cellular proliferation and differentiation [19]. Such evidence suggests that microRNAs can be good candidates in controlling various cellular processes; however, their roles and function in MSC chondrogenic differentiation are largely unknown. In this study, we aimed to investigate microRNAs that regulate the chondrogenic differentiation process of hMSCs. It is important to understand the processes that regulate chondrogenic differentiation of hMSCs because they are an ideal candidate for cartilage regeneration and they possess chondrogenic differentiation potential. Accordingly, it can be meaningful to investigate the correlation between major factors and microRNAs related to chondrogenic differentiation of hMSCs.

It is clear that Sox9 is a strong regulator in chondrogenesis, and the formation of cartilage does not occur without it [11]. To find a microRNA that has a regulatory effect on Sox9 expression during chondrogenic differentiation of hMSCs, we performed microRNA microarray analysis by using an undifferentiated hMSC group or a TGF- β 3-induced chondrogenic differentiated hMSC group. We selected the microRNAs that showed an expression pattern opposite to that of Sox9 (Fig. 2). Among the downregulated microRNAs, miR-495 has a binding sequence to the Sox9 3'UTR and it inhibited Sox9 expression in SW1353 cells (Fig. 3). miR-101 and miR-145 are well known as regulators targeting Sox9 in rat chondrocytes and murine MSCs [35]. However, expression of miR-101 and miR-145 was not changed significantly during chondrogenic differentiation of hMSCs in our microarray data. Therefore, we studied miR-495, which showed a significantly decreased expression pattern during chondrogenic differentiation of hMSCs. Further, we confirmed that miR-495 directly binds to the Sox9 3'UTR and identified the exact seed sequence of miR-495 to the Sox9 3'UTR by using a luciferase reporter assay (Fig. 4). We confirmed that miR-495 has negative effects on Sox9 expression, and inhibition of miR-495 enhanced Sox9 expression in hMSCs (Fig. 5). TGF- β 3 induces transcriptional activity of Sox9 through the Smad signaling pathway, and then

promotes chondrogenic differentiation of hMSCs [36]. In our results, TGF- β 3 significantly induced Sox9 expression, but miR-495 impaired TGF- β 3-induced Sox9 expression. Further, Sox9 binds to the chondrocyte-specific enhancer in the HMG domain-binding sites of Col2a1 and activates *Col2a1* gene expression [13]. Further, promoter/enhancer activity of the *aggrecan* gene is enhanced by Sox9 in TC6 cells [15]. In our data, miR-495-transfected hMSCs showed a decrease of Sox9 expression, and miR-495-inhibited hMSCs showed an increase of Sox9 expression. Following these results, miR-495-overexpressed or -inhibited hMSCs increased or inhibited expression of Col2a1 and aggrecan, respectively (Fig. 6). Further, miR-495 regulated the chondrogenic phenotype of hMSCs. We confirmed that miR-495 inhibited PG production via measurement of GAG contents and safranin O staining (Fig. 7). Taken together, the findings in this study show that blockade of miR-495 may be a way to enhance chondrogenic potential of hMSCs via targeting Sox9.

Recent research showed that miR-495 regulates cyclin-dependent kinase 6, is involved in inhibition of glioma cell proliferation, and decreases proliferative S phase cells [37]. In metastatic prostate cancer cells, miR-495 inhibits cell cycle progression and decreases proliferative S phase cells together with increases in G1 cells [38]. In our data, miR-495 had no effect on the proliferation rate in hMSCs (data not shown). These results indicate that miR-495 can regulate chondrogenic differentiation of hMSCs without affecting cell proliferation. In conclusion, our results showed that miR-495 functions as a negative regulator of hMSC chondrogenic differentiation by repressing Sox9 expression.

Currently, a number of in vivo studies have been performed to confirm the effect of microRNAs validated in vitro. A variety of delivery methods, such as locked nucleic acid (LNA), nanoparticle, viral vector, and polyethylenimine, have been used to effectively overexpress or inhibit the validated microRNAs in in vivo [39–42]. Recently, miravirsin, known as LNA-modified anti-miR drug that inhibits biogenesis of miR-122, has been developed in cartilage disease area [43]. Therefore, developing anti-miR-495 that can inhibit miR-495 endogenously expressed in hMSCs would be useful in applying to cartilage defect models. Further, it is a prerequisite to utilize an appropriate animal model with cartilage defect as well as to select efficient methods to deliver microRNA or anti-microRNA targeting it.

Taken together, this study suggests that miR-495 may have potentially important therapeutic implications for cartilage regeneration.

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Author Disclosure Statement

No competing financial interests exist.

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