Identification of the microRNA which regulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cell

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Identification of the microRNA which regulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cell

Directed by Professor Jin Woo Lee

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석사 학위를 시작한지 어느덧 2년 반이라는 시간이 흘러 졸업을 하려니 그 동안 연구실에서 지냈던 시간들과 추억들이 너무나 감사하게 느껴집니다. 그 동안 저와 함께 하고 도움을 주신 분들께 감사의 마음을 전합니다.

부족한 저를 지도해주시고 연구자로써의 자세를 가르쳐 주신 이진우 교수님께 깊은 감사를 드리며 세심한 조언과 격려를 해주신 박상욱 교수님, 이재면 교수님께 감사를 드립니다.

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Abstract

Identification of the microRNA which regulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cell

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Human bone marrow mesenchymal stem cells (hMSCs) differentiate into multiple connective tissue cell types including adipocyte, chondrocyte, myoblast, osteoblast, depending on specific condition. Chondrogenic differentiation of human MSC passes through multiple stages and it carried out by various factors and their interactions. As a possible regulator of MSC chondrogenic differentiation, microRNA has identified recently. microRNA is consisted of 20-24 nucleotide and inhibits its target gene expression through target mRNA cleavage or translational repression. Current reports indicate that microRNAs play critical roles in diverse biological process including cell proliferation, apoptosis and differentiation. In this study, we showed that miR-495 regulates chondrogenic differentiation. High throughput screening though

microRNA microarray analysis was performed to find microRNA expression change during chondrogenic differentiation. By statistical analysis We have found that miR-495 was especially down-regulated in hMSCs induced chondrogenic differentiation. Using microRNA target prediction database, we have selected its target, Sox9, SRY-related high mobility group-Box gene 9. Sox9 has been extensively reported its critical role in overall progression of chondrogenic differentiation. We have confirmed opposite expression of miR-495 and Sox9 using realime PCR. Furthermore, over-expression of miR-495 has inhibited Sox9 expression in cell line and hMSCs. Additionally, Luciferase analysis data revealed that miR-495 directly binding to Sox9 3'UTR. Subsequently, over-expression of miR-495 has repressed expression of chondrogenic marker genes, such as type II collagen (Col2A1), aggrecan and proteoglycan products. Thus, our finding suggests that miR-495 regulates chondrogenic differentiation of hMSCs by directly binding Sox9.

Key words : MSC, chondrogenic differentiation, Sox9, microRNA, miR-495

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I. INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent stromal cells and can differentiate into multiple connective tissue cell types, including fat, tendon, cartilage and bone[1-5]. Chondrogenic differentiation of human MSC passes through multiple stages. The initial step of chondrogenic differentiation is mesenchymal stromal cell proliferation and pre-cartilage condensation. Mesenchymal stromal cell condensation is crucial for chondrogenic

differentiation and this process is driven by cell–cell and cell–matrix interactions as well as secreted factors[6]. Chondrogenic differentiation stage is characterized by accumulation of cartilage extracellular matrix including type II collagen, type IX collagen, type XI collagen and aggrecan[7]. Chondrocytes arrive hypertrophic stage and express type X collagen that is unique to the hypertrophic stage of chondrogenic differentiation[8]. In long bone formation, hypertrophic chondrocytes eventually undergo apoptosis and are substituted with bone. The temporal expression pattern of extracellular matrix and transcription factor during chondrogenic differentiation represented schematically(Fig 1)[7].

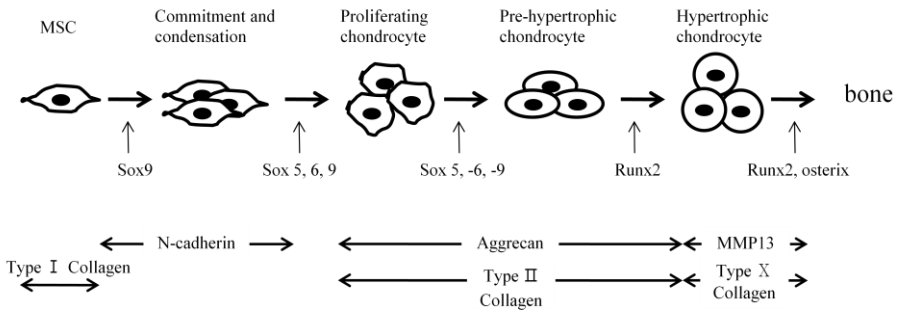

Figure 1. The expression patterns of chondrogenic key factors and extracellular matrix during chondrogenic differentiation.

Chondrogenic differentiation is very complicate and modulated several transcription factors and cytokines. SRY-related high mobility group-Box gene 9 (Sox9), a high-mobility-group box (HMG)-containing transcription

factor, is required at sequential chondrogenic differentiation steps. HMG domain contributes to DNA binding in the minor groove, bending DNA, interaction with other transcription factors[9]. Sox9 is significant for mesenchymal condensation in early stage of chondrogenic differentiation, but Sox9 expression is inhibited in hypertrophic chondrocytes[10-13]. In chondrocytes, Sox9 bindis to type II collagen chondrocyte-specific enhancer and activates activates type II collagen expression, a specific marker of chondrogenic differentiation[14,15]. In addition, Sox9 stimulates type XI collagen, Aggrecan, CD-RAP gene[16-18]. Human skeletal dysmorphology syndrome, campomelic dysplasia, is caused by heterozygous mutations in and around the Sox9 gene. This disease is marked by anomalies of the ribs and vertebral column and bowing of the long bones. Sox9 heterozygous mutant mouse die in a couple of hours after birth from respiratory distress[19]. Heterozygous mutants exhibited the similar skeletal abnormalities to campomelic dysplasia and showed delayed chondrogenic mesenchymal condensation and enlargement of the hypertrophic zone in association with premature mineralization[19]. In mouse chimaeras, $Sox9^{-/-}$cells are excluded from all cartilages and did not express the chondrocyte-specific markers such as type II collagen, type IX collagen, type XI collagen and aggrecan[20]. These evidences strongly suggest that Sox9 is essential for chondrogenic differentiation and cartilage formation. However, overall mechanisms of chondrogenic differentiation still remain to be defined.

MicroRNAs(miRNAs) are short noncoding RNAs of ~22 nucleotides that negatively regulate gene expression at the post-transcriptional level[21]. In the nucleus, primary microRNA transcript (pri-miRNA) is produced by RNA polymerase II or III and cleaved by the microprocessor complex Drosha-DGCR8[22]. The resulting Pre-miRNA is transported to cytoplasm by Exportin and then pre-miRNA is cleaved into imperfect dsRNA duplex (miRNA:miRNA*) containing mature miRNA strand and its complementary strand[23-25]. Mature microRNA become complex with RNA-induced silencing complex (RISC), the cytoplasmic effector and microRNA guiding to target mRNAs[23,26]. By forming functional microRNA-RISC complex, microRNA recognizes complementary sequence of target mRNA 3'UTR and represses mRNA expression by mRNA cleavage or translational repression or mRNA deadenylation [27-29].

Many current reports indicate that microRNAs play critical roles in diverse biological process including cell proliferation, apoptosis and differentiation[30,31]. And recent evidence suggests that microRNAs are necessary for normal skeletal development and skeletal tissue maintenance. miR-140 is revealed cartilage specific expression during embryonic development and regulates cartilage development and homeostasis[32,33]. Several reports have been idenfied that miR-140 regulates histone deaceylase 4 (HDAC4), insulin growth factor binding protein 5 (IGFBP5), Adamts-5, Smad3 and Dnpep expression [33-37]. Further, miR-140$^{-/-}$ mice have mild

skeletal phenotype with short stature and craniofacial changes[35].

In this study, we screened for chondrogenic-specific microRNAs using microRNA microarray and found miR-495 regulating chondrogenic differentiation of human MSCs. Other reports identified that miR-495 is cancer suppressor and regulators of liver and pancreas development[38-41]. In our study, we determined that miR-495 plays inhibitory roles during chondrogenic differentiaton of human MSCs. Specially, miR-495 directly binds to Sox9 3'UTR and inhibits Sox9 expression.

II. Materials and methods

1. Primary mesenchymal stromal cell and chondrosarcoma cell line culture

Bone marrow aspirates were obtained from the posterior iliac crest of 10 healthy adult donors ranging from 20-69 years of age under the approval of the Institutional Review Board (IRB). Mesenchymal stromal cells were specifically selected using their natural tendency to adhere to a plastic culture plate surface. After 7 days of culture in Dulbecco's modified Eagle's medium-Low Glucose (DMEM-LG, Welgene, Daegu, Korea) with 10% fetal bovine serum (FBS) (Gibco, , *Invitrogen*, Carlsbad, Calif, *USA*) and 1X antibiotic-antimycotic solution (Gibco), non-adherent hematopoietic cells were removed. The cells were grown to 70% confluence over an average culture period of 10 days and were then promptly harvested by incubation with 0.25% trypsin/1mM EDTA (Gibco) followed by a 3-minute centrifugation at 1,300 RPM. This initial batch of cultured cells was designated as Passage 1, and the cells were subcultured in a new $10cm^2$ dish up to passage 6-7. Two human chondrosarcoma cell lines, SW1353, were cultured in Dulbecco's modified Eagle's medium-High Glucose (DMEM-HG, Welgene) with 10% fetal bovine serum (FBS) and 1X antibiotic-antimycotic solution (Gibco).

2. Chondrogenic differentiation of hMSCs by micromass culture method

The cultured MSCs between passages 3-5 were harvested using the method described previously. For micromass culture, cells were re-suspended on DMEM-LG with 10% FBS at the specified density of 10000 cells/μL of media, and 10μL of the suspended cells were dotted on the center of each well on the 24-well plates. Single drop of 1X Dulbecco's Phosphate-Buffered Saline (DPBS, Welgene) was placed in between each well to suppress over-drying of the cells. Cells were then placed on 37℃ incubator maintained with 5% CO_2 for 2 hours to stimulate adherence of the cells to the plate. For control group, 1mL of control medium consisted of DMEM-HG (Welgene), 1X antibiotic-antimycotic solution, 1X Insulin Transferrin Selenium-A (Invitrogen) and 50μg/mL ascorbic acid (Invitrogen) was used while the chondrogenic medium contained 10ng/mL of TGF-β3 (R&D systems, Minneapolis, MN, USA) as well. The culture medium was changed every 2-3 days. All experiments were carried out in triplicate using MSCs from three donors.

3. Quantitative real-time polymerase chain reaction (PCR)

Total RNA from MSCs were isolated using RNAiso Plus (Takara, Shiga, Japan) reagent following the manufacturer's instruction. Briefly, 1mL of RNA iso Plus solution was added to the collected cells and was repeatedly pipette to

completely dissolve cells. The cells were left on room-temperature for 10 minutes, and 200 μL of chloroform was added and were vortexed until the solution became milky. The solution was then kept at room-temperature for 5 minutes, and was centrifuged at 13,000 RPM for 15 minutes at 4℃. Top liquid layer was then transferred to new tube and 500 μL of 100% isopropanol was added. After brief vortex, the solution was kept at room-temperature for 10 minutes and was promptly centrifuged at 13,000 RPM for 10 minutes at 4℃. The supernatant was removed without disturbing the RNA pellet, which was then washed with cold 70% ethanol via centrifugation at 10,000 RPM for 5 minutes at 4℃. Finally, the RNA pellet was re-suspended on 30μL of diethylpyrocarbonated-water (DPEC-water). The overall quality and concentration of each RNA sample was confirmed using spectrophotometry. For cDNA reverse transcription, Omniscript Reverse-Transcription Kit (Qiagen, Venlo, Netherlands) was used. Quantitative real time PCR have been carried out using Applied Biosystems (ABI, Carlsbad, CA, USA), following the instruction as given. ABI7500 real time by Applied Biosystems (ABI) was used for all quantitative real time PCR procedures. The primers used for quantitative real time PCR as well as universal quantitative real time PCR conditions are as shown on Table 1 and Table 2. All primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). SYBR fluorescence was detected during the annealing/extension phase, and all real-time PCR products had a final size of approximately 100 bp. GAPDH was used to normalize for

relative expression intensity of all genes.

For precursor-microRNA reverse transcription, MicroRNA First-Strand Synthesis and Quantitation kit (Clontech, Mountain View, CA, USA) was used following the manufacturer's instructions. The list of primer sets used for specific mature-microRNA amplification was obtained from genolution and listed on Table 3. U6 snRNA was used to normalize for relative expression intensity for all pre-microRNAs.

Table 1. Primer sequences for quantitative real time PCR

Gene	Strand	Primer Sequence
Col2A1	S	5' GTCCTCTCCCAAGTCCACACAG 3'
	AS	5' GGGCACGAAGGCTCATCATTC 3'
Aggrecan	S	5' CCACTGTTACCGCCACTT 3'
	AS	5' GTAGTCTTGGGCATTGTTGT 3'
SOX5	S	5′-AGCCCCACATAAAGCGTCCAAT-3′
	AS	5′-GGTCCTCCTCCTCCTCATCGTA-3′
SOX6	S	5′-AGCAGAGCCTGTGAAGTCC-3′
	AS	5′-CTTTGCTTCCTTCTG CGTCC-3′
GAPDH		P267613 (Bioneer)
SOX9		P232240 (Bioneer)

S: Sense Strand AS: Antisense Strand

GAPDH and SOX9 each used validated primer from Bioneer and their sequences were not open to public.

Table 2. Conditions for quantitative real-time PCR

Process	Temperature	Time
Initial Denaturation	95℃	30 sec
Denaturation	95℃	5 sec
Annealing/Extension	60℃	20 sec
Dissociation Stage		

Total of 40 cycles (Denatruation – Annealing/Extension) has been carried out

Table 3. Sequence of primers used for microRNA PCR analysis

microRNA		Primer Sequence	Size (bp)
Hsa-miR-495-3p	S*	5'-AAACAAACATGGTGCACTTCTT-3'	60-90
U6 snRNA	S	5'-CTCGCTTCGGCAGCACA-3'	94
	AS	5'-AACGCTTCACGAATTTGCGT-3'	

S*: sense strand used for microRNA PCR reactions.

Universal anti-sense primer was provided by Invitrogen.

S and AS for U6 snRNA refers to Sense and Antisense, respectively.

4. Microarray analysis

The total RNA of cultured MSCs was isolated by RNAiso Plus (Takara) by following the manufacture's protocol. The overall quality of the total RNA was validated using spectrophotometry. For cDNA expression, AffymetrixGeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) was used. Pearson's correlation analysis has been performed to elucidate genes specifically expressed in chondrogenesis-induced MSCs. Briefly, the Differentially Expressed Genes (DEGs) have been selected using the routine formula of subtracting control groups from the differentiated group of interest:

DEGs = [Day 10 Chondrogenesis – (Day 0 Control + Day 10 Control)]

Strict cut-off threshold have been implemented to screen for genes with at least two fold difference in expression intensity.

5. Western blot analysis

Passive lysis buffer (Promega, Madison, WI, USA) containing 10 μg/ml of protease and phophatase inhibitor was used for preparation of the cell lysates. Clarified lysates from centrifugation of samples at 13,000 rpm at 4℃ for 15 minutes were quantified using modified Bradford assay. Total 30μg aliquots of the cell lysates were separated in 10% SDS-PAGE in reducing condition. The samples were promptly transferred onto PVDF membrane (Amersham, Pharmacia, Piscataway, NJ, USA) for 90 minutes at 50V in transfer buffer containing 1.4% glycine, 20% methanol and 25 mMTris-HCL (pH 8.3). 1% skimmed milk in 1X TBST (50 mM Tris-HCl, 150mM NaCl, 0.1% Tween-20) was used for blocking over night at 4℃. Sox 9 (Millipore, Billerica, MA, USA), COL2A1 (Santa Cruz, CA, USA), Aggrecan (Santa Cruz) primary antibody was used at a concentration of 1:3000, for 3hr at room temperature. Following repeated washing of the membrane with 1X TBST, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham Pharmacia). GAPDH antibody (Santa Cruz) was for internal control.

6. Over-expression and inhibition of microRNA for Sox9

For functional analysis of screened microRNA and its effect on the target gene, Sox9, the identified microRNA has been over-expressed via microRNA-mimic purchased from Genolution (Genolution, Seoul, South Korea). The purchased microRNA mimic was prepared in double-strand form, which would be processed once transfected by endogenous mechanisms which would ultimately yield mature microRNA. For negative control, scramble microRNA has been used. The exact sequence of the microRNA mimic used were obtained from microRNA database (miRbase) and the duplex sequences are as shown on Table 4. The constructed microRNAs have been transfected with Lipofectamin LTX&Plus reagent (Invitrogen) on human chondrosarcoma cell lines, human mesenchymal stromal cells, following the provided instructions.

Table 4. microRNA mimics used for over-expression

microRNA	Strand	Duplex Sequence
hsa-miR-SC	S	5' UUACCAGACGUGUCUUCACUCCC 3'
	AS	5' GGGAGUGAAGACACGUCUGGUAA3'
hsa-miR-495-3p	S	5' AAACAAACAUGGUGCACUUCUU 3'
	AS	5' AAGAAGUGCACCAUGUUUGUUU 3'
mut-miR-495-3p	S	5’ ACCACCCAAUGGUGCACUUCUU 3’
	AS	5’ AAGAAGUGCACCAUUGGGUGGU 3’

S: Sense Strand AS: Antisense Strand

7. Luciferase reporter assay

SOX9 3'UTR containing luciferase vector is purchased from Genecopoeia (Rockville, MD, USA) and was termed pEZX-SOX9 3'UTR. pEZX-SOX9 3'UTR contains firefly luciferase gene with SOX9 3'UTR and renilla luciferase gene. For mutated pEZX-SOX9 3'UTR, miR-495-3p seed sequence was mutated using Quikchange site-directed mutagenesis kit (Stratagene, Piscataway, NJ, USA). pEZX-SOX9 was cotransfected with miR-495-3p or negative microRNA into Hela cell using Lipofectamin LTX&Plus reagent. Firefly and Renilla luciferase activity was measured after 24hr using Dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) and firefly luciferase activity was normalized to renilla luciferase expression.

8. Immunohistochemistry

Paraffin tissue sections were deparaffinized, rehydrated, and washed twice in buffer. To reduce nonspecific background staining due to endogenous peroxidase, the slides were incubated in hydrogen peroxide block for 10 minutes, and washed 2 times in buffer. Cells were incubated with rabbit anti-COL2A1 (Santa Cruz) or mouse anti-aggrecan (Santa Cruz) for overnight at 4℃ temperature and washed with PBS. Phycoerythrin (PE) conjugated goat anti-rabbit secondary antibodies (Santa Cruz) and green fluorescent protein (GFP) confugated goat anti-mouse secondary antibodies were used to visualize the primary antibodies. The nuclei were stained with DAPI (4,6-

diamidino-2-phenyindole, Sigma). The images were captured using an inverted fluorescence microscope (IX-71, Olympus).

9. Statistical Analysis

In all applicable settings, appropriate statistical analysis had been carried out to derive at statically significant result. In all figures, graphs and tables, * indicates p-value < 0.05, while ** indicates p-value < 0.01.

III. RESULTS

1. Microarray analysis revealed distinctive cluster of genes with specifically enhanced or reduced expression in chondrogenesis-induced MSCs.

MSCs were induced chondrogenic differentiaton in chondrogenic medium for 10 days. As a control, MSCs were cultivated in control medium for 10 days. Sox9 was increased during chondrogenic differentiation of MSCs. (Figure 2A.) Afterwards, microRNA microarray analysis has been performed to screen for microRNAs with distinctive expression pattern in chondrogenic differentiation-induced MSCs. Using Pearson correlation analysis and with strict cut off criteria of at least 1.5 fold difference in expression between groups, the differently expressed microRNAs were classified as shown on hierarchical clustering map. Overall, 17 microRNAs are identified which were expressed significantly higher or lower than the Day 0 CT or Day 10 CT control and 4 microRNAs are significant down-regulated during chondrogenic differentiation of MSC(Figure 2B). After that, we selected 2 microRNAs, miR-495 and miR-431, using microRNA target prediction database, TargetScan (www.targetscan.org) (Figure 3A). miR-495 and miR-431 has binding site in Sox9 3'UTR which is up-regulated and play a key role during chondrogenic differentiation.

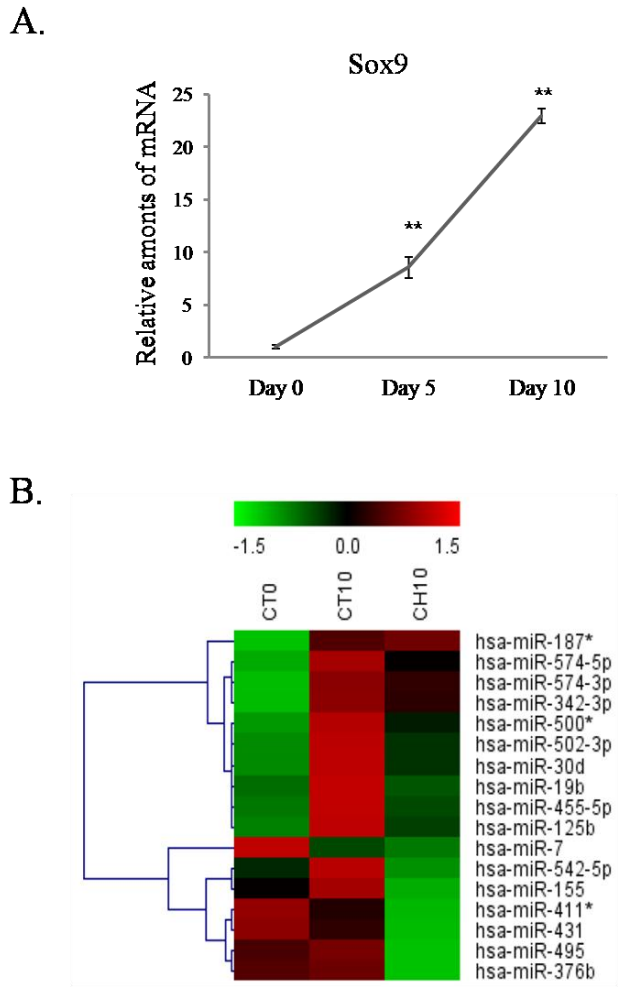

Figure 2. The expression of Sox9 and microRNA during chondrogenic differentiation. Sox9 mRNA expression during chondrogenic differentiation of MSCs was measured using realtime PCR (A). Differential expression of microRNAs during chondrogenic differentiation, compared to control (one-way ANOVA p-value < 0.05) (B). CT0, CT10, CH10 mean Control Day 0, Control Day 10 and Chondrogenesis-induced Day 10, respectively. Red and green color refer to up- and down- regulated microRNAs.

A.

```
Sox9 3'UTR    5'  ...UCGUACCCAAAUUUCCAAGACAC... 3'
                                    ||||||
miR-431       3'     ACGUACUGCCGGACGUUCUGU

Sox9 3'UTR    5'  ...CCAGCAGUUAACCUUCAAGACAU... 3'
                                    ||||||
miR-431       3'     ACGUACUGCCGGACGUUCUGU


Sox9 3'UTR    5'  ...UUGUGUUCGUGUUUUGUUUGUUU... 3'
                                    |||||||
miR-495       3'     UUCUUCACGUGGUACAAACAAA
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B.

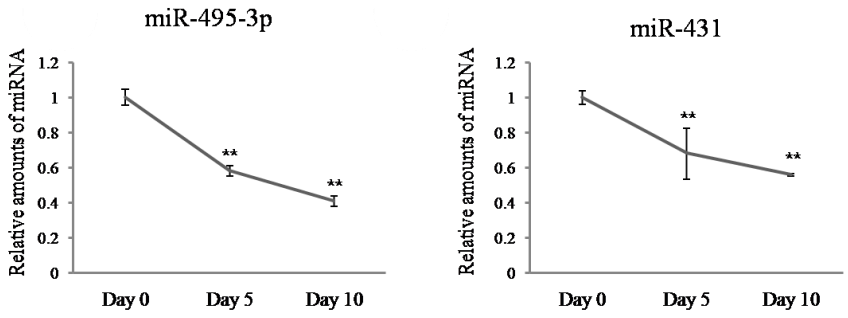

Figure 3. miR-495 and miR-431 expression during chondrgenic differentiation. Predicted binding sites of miR-431 and miR-495 (A). miR-431 has two binding sites and miR-495 has one binding site in Sox9 3'UTR. Expression of miR-495, 431 and Sox9 during chondrogenic differentiation of hMSC have been quantified using real time PCR (B). miR-495 and miR-431 was down-regulated with passage of day (Day0, Day 5, Day 10). ** indicates statistical significance of p-value < 0.01 when compared with control.

We hypothesized that miR-495 and miR-431 bind to Sox9 3'UTR and

regulate Sox9 expression. Furthermore miR-495 and miR-431 may have effect on chondrogenic differentiation of MSC. In addition to microRNA microarray results, real time PCR was performed to confirm miR-495, miR-431 and Sox9 expression (Figure 3B). miR-495 and miR-431 expression was decreased during chondrogenic differentiation.

2. miR-495 regulates Sox9 expression in cell line.

To study whether miR-495 and miR-431 regulates Sox9 expression, SW1353, chondrosarcoma cell line, was transfected with miR-495 and miR-431 respectively. Both miR-495 and 431 was inhibit Sox9 mRNA expression (Fig 4A), only miR-495 showed meaningful decrease of endogenous Sox9 protein expression in SW1353 (Fig 4B). While miR-495 and miR-431 displayed inverse expression profile against Sox9 in micoRNA microarray data and realtime PCR, only miR-495 regulated Sox9 protein expression in SW1353. Therefore further study was pursued using miR-495.

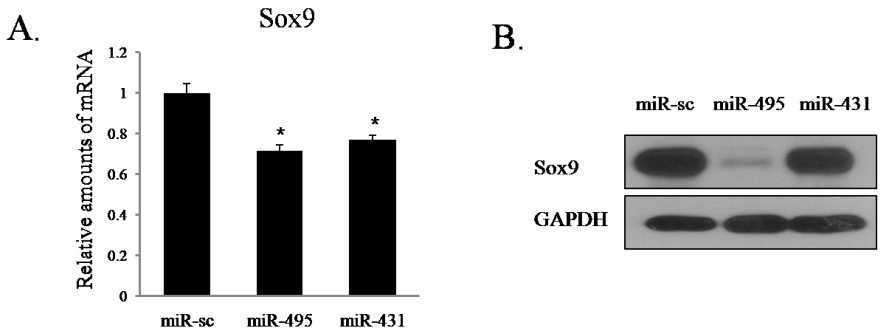

Figure 4. Effects of miR-495 on Sox9 expression in human chondrosarcoma cell line, SW1353. Overexpression of miR-495, miR-431 led to decrease of endogenous Sox9 mRNA expression (A), whereas miR-431 overexpression has any effect on Sox9 protein expression in chondrosarcoma cell line, SW1353 (B). GAPDH is internal control in realtime PCR and western blot analysis. * indicates statistical significance of p-value < 0.05 and ** is < 0.01 when compared with control.

3. miR-495 directly targets Sox9 through binding to Sox9 3'UTR.

microRNA regulates target mRNA expression via binding to 3'UTR. We found that Sox9 3'UTR has a seed site of miR-495(Fig 5A). To identify whether miR-495 binding to Sox9 3'UTR, we applied luciferase vector contataining Sox9 3'UTR (Fig 5B). microRNA mimic and pEZX-Sox9 are transfected into Hela cell. The pEZX-Sox9 luciferase activity was dose dependently decreased by miR-495 comopared with control microRNA in Hela cell(Fig 5C). To identify the seed sequence of miR-495, miR-495 seed sequence mutation formed mimic, mut-miR-495, is transfected in Hela cell with pEZX-Sox9. Luciferase activity showed complete recovery when transfected with mut-miR-495. In addition, the luciferase activity was dose dependently decreased by miR-495 (Fig 5D).

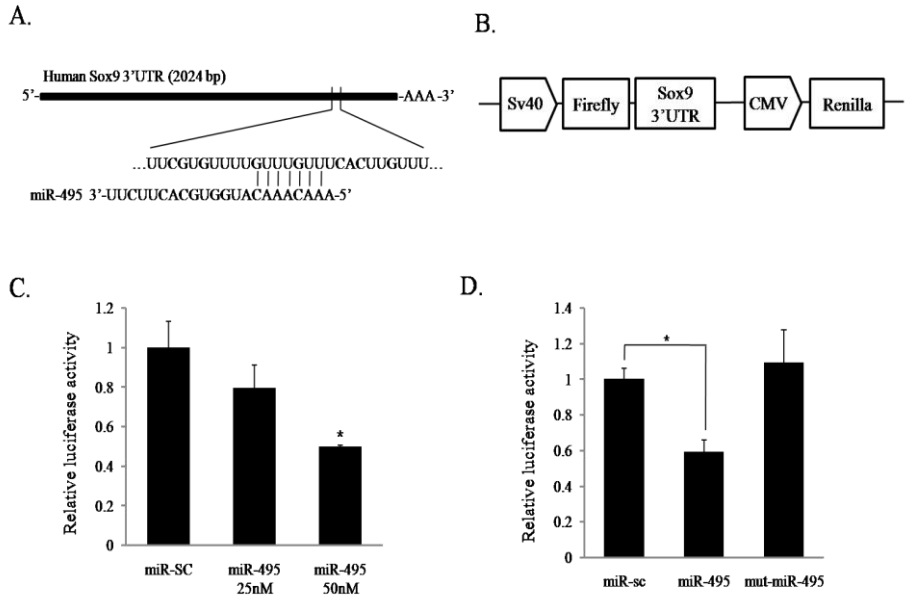

Figure 5. miR-495 specially binds to 3'UTR of Sox9. Luciferase reporter assay using pEZX-Sox9 co-transfected with miR-495 has showed reduction of luciferase activity by miR-495. Schematic diagram of the predicted miR-495 binding site of Sox9 3'UTR (A). pEZX-Sox9 vector contains entire 2kb of Sox9 3'UTR (B). HEK 293T cells were trasfected with pEZX-Sox9 and 50nM miR-SC, 25nM miR-495 and 50nM miR-495 mimic respectively (D). Firefly luciferase activity is normalized to renilla luciferase activity

4. miR-495 inhibits Sox9 expression in hMSCs.

To confirm whether miR-495 regulates Sox9 expression in hMSC, hMSCs were transfected with miR-495 and treated by TGF-β. hMSC induced chondrogenic differentiation using TGF-β because hMSCs showed different Sox9 expression level when undifferentiation stage. Sox9 was up-regulated significantly in negative microRNA transfected hMSCs, whereas miR-495 transfected hMSCs showed less Sox9 expression than negative control (Fig. 6).

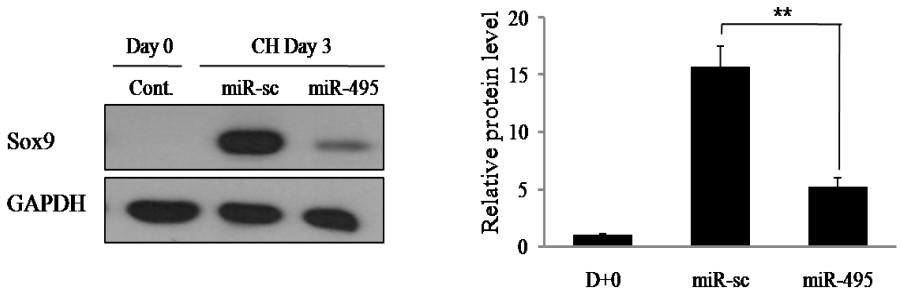

Figure 6. Effects of miR-495 on endogenous Sox9 expression of hMSC. hMSC were transfected with negative control microRNA (miR-sc) and miR-495 individually. After treated TGF- β for 3days, Sox9 expression was measured using western blot. miR-495 has inhibited increase of Sox9 protein expression by TGF- β. ** indicates statistical significance of p-value < 0.01 when compared with control.

5. miR-495 regulates chondrogenic differentiation marker gene expression of hMSCs.

Ultimately, to identify effects of miR-495 on chondrogenic differentiation of hMSCs, we examined the expression of *Col2A1* and *aggrecan* mRNA, late chondrogenic markers, in miR-sc and miR-495 transfected hMSCs during chondrogenic differentiation *in vitro* using real-time PCR. miR-495 decreased the expression of *Col2A1* and *aggrecan* mRNA compared to the miR-sc transfected hMSCs (Figure 7A, B). To determine whether protein levels of Col2A1 and aggrecan can be regulated by miR-495 during chondrogenic differentiation of hMSCs, an immunohistochemical analysis was perfomed in paraffin-embedded section of micromass cultures. Immunohistochemistry showed that miR-495 supressed the protein expression of Col2A1 and aggrecan during chondrogenic differentiation of hMSCs *in vitro* (Figure 7C, D). Taken together, these results revealed that miR-495 is a negative regulator of hMSC differentiation to chondrogenic lineage through suppressing Sox9, resulting in decrease of the chondrogenic markers, such as Col2A1 and aggrecan.

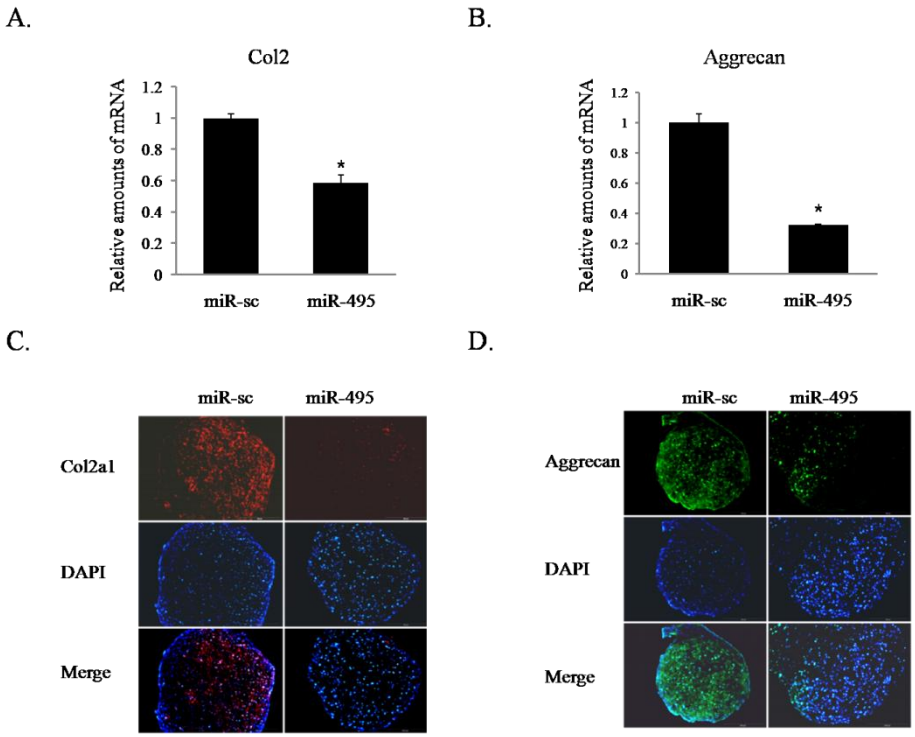

Figure 7. Effects of miR-495 on the expression of chondrogenic marker genes. hMSCs were transfected with miR-SC and miR-495 respectively and induced chondrogenic differentiation. Chondrogenic differentiation was measured by chondrogenic differentiation marker genes (Col2A1 and aggrecan) using realtime PCR after chondrgenic differentiation at day 5 (A, B). The effect of miR-495 on chondrogenic differentiation was analyzed by immunohistochemistry using anti-Col2A1 and anti-aggrecan antibodies at day 10 (C, D).

6. miR-495 has inhibitory roles in chondrogenic differentiation of hMSCs.

We measured sulfated proteoglycans and glycosaminoglycans (GAGs) production to evaluate chondrogenic differentiation of hMSCs. miR-495 transfected hMSCs were lower in GAG production than negative control (Figure 8A). And safranin O staining was performed to visualize proteoglycan. miR-495 transfected hMSCs were revealed reduced staining intensity compared with control (Figure 8B). Taken together, our results suggest that miR-495 has a negative role during chondrogenic differentiation of hMSCs.

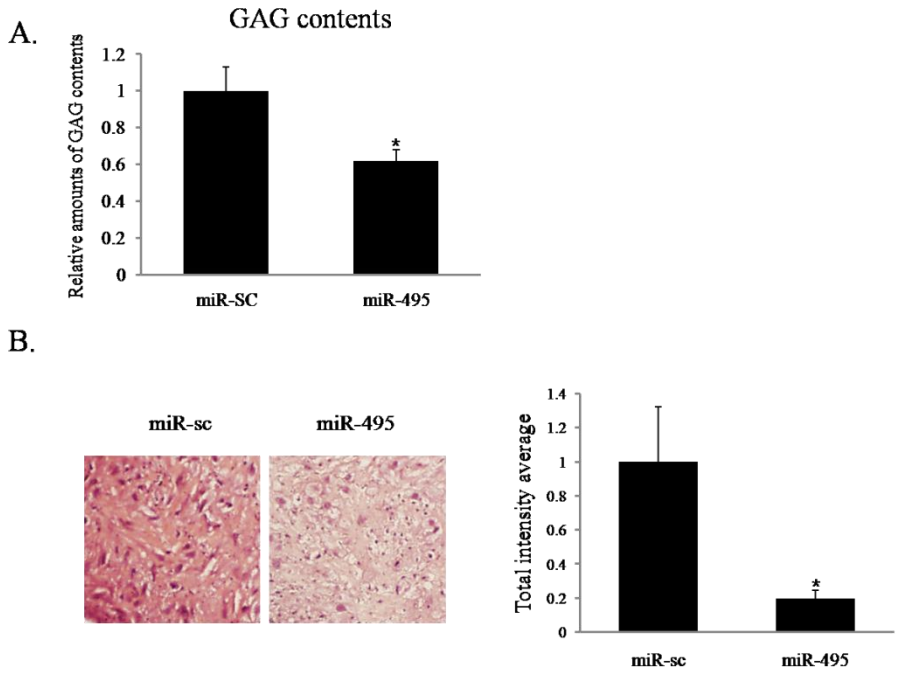

Figure 8. Effects of miR-495 overexpression on chondrogenic differentiation of hMSCs. Over-expression of miR-495 had significantly reduced GAG production in hMSC (A). Reduced proteoglycan by miR-495 is visualized using safranin O staining (B). * indicates statistical significance of p-value < 0.05 compared with control.

IV. DISCUSSION

Bone marrow derived stromal stromal cells have ability to differentiate multiple cell type and can differentiate cartilage in specific condition[5]. Chondrogenic differentiation is regulated by many transcription factors and their interaction. One of them, Sox9 is well known for critical regulator of chodnrogenic differentiation [9-11]. And in the previous studies have reported that microRNA is important regulator of cartilage homeostasis differentiation [32]. Dicer-null chondrocytes have showed decrease of chondrocyte proliferation and promote to hypertrophic chondrocyte [42]. miR-221 inhibits chondrogenic differentiation targeting Mdnm2 and miR-675 has positive role in chondrogenic differentiation[43,44]. miR-140 is showed cartilage specific expression and it has several targets involved cartilage related genes, HDAC4, IGFBP5, Adamts-5, Smad3 and Dnpep [33-37]. miR-140 null mouse manifested age-related osteoarthritis-like change with proteoglycan loss and fibrillation of articular cartilage [35]. These studies suggest that microRNAs are crucial for cartilage development and may have potentially important therapeutical implications.

In order to investigate potential microRNA related chondrogenic differentiation of hMS, we have utilized microRNA microarray analysis. In this result, 4 microRNAs are down-regulated during chondrogenic differentiation of hMSCs. Among them, we found that miR-431 and miR-495

have predicted seed site in Sox9 3'UTR using target prediction database, TargetScan and miRanda. And Sox9 is up-regulated during chondrogenic differentiation whereas miR-495 and 431 is down-regulated with passage of day in microRNA realtime PCR data. These results suggest that miR-495 and 431 may be regulator of chondrogenic differentiation targeting Sox9. In human chondrosarcoma cell line tested, SW1353, both miR-495 and miR-431 inhibit Sox9 mRNA expression whereas only miR-495 significantly represses Sox9 protein expression in western analysis (Figure 4). Further, to study whether miR-495 directly targets to Sox9 3'UTR, we have performed luciferase reporter assay. Luciferase activity has been decreased in dose-dependent manner when pEZX-Sox9 luciferase vector was co-transfected with miR-495 mimic. (Figure 5). Moreover, miR-495 repressed Sox9 expression in chondrogenic induced hMSCs. Sox9 was up-regulated by chondrogenic medium containing TGF-β3 for 3 days, but miR-495-transfected hMSC has showed less or any increase of Sox9 expression compared with control (Figure 6). These results revealed that miR-495 directly binds to Sox9 3'UTR and regulates Sox9 expression during early stage of chondrogenic differentiation.

Finally, we confirmed the effects of miR-495 on chondrogenic differentiation. Over-expression of miR-495 resulted in inhibition of chondrogenic differentiation in hMSCs, as reflected by a decrease of Col2A1 and aggrecan expression. And we have confirmed that miR-495 inhibited

proteoglycan production via measurement of GAG contents and safranin O staining. Following these over-expression study, miR-495 is key regulator of chondrogenic differentiation of hMSC through targeting Sox9.

Recently, one study has observed that miR-145 represses Sox9 expression and regulates chondrogenic differentiation in murine embryonic mesenchymal cell line C3H10T1/2 cells [46]. microRNA may have multiple mRNA targets and one mRNA might be targeted by multiple microRNs [47,48].

Ultimately, our results showed that miR-495 functions as a negative regulator of hMSC chondrogenic differentiation by repression of Sox9 expression.

V. CONCLUSION

In this study, we have identified role of miR-495 during chondrogenic differentiation. We have found that miR-495 is down-regulated during chodnrogenic differentiation of hMSC using microRNA microarray analysis and we have predicted its target as Sox9 by prediction database, such as Targetscan and miRanda. Sox9 and miR-495 have shown inverse expression pattern in realtime PCR data. Furthermore, we have elicited that miR-495 is negative regulator of Sox9 expression in SW1353 cell line and hMSC. Through luciferase analysis, directly binding of miR-495 in Sox9 3'UTR was observed. miR-495 has inhibited luciferase activity when transfected with luciferase vector containing Sox9 3'UTR, and effect of miR-495 on luciferase activity occurred in dose-dependent manner. Additionally, miR-495 treated hMSC have shown significantly reduction of chondrogenic marker genes such as Col2A1, aggrecan and proteoglycan. Collectively, these data identified that miR-495 is critical regulator of chondrogenic differentiation through targeting Sox9.

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ABSTRACT (IN KOREAN)

골수유래 중간엽줄기세포의 연골분화를 조절하는 microRNA 규명

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이 슬 기

사람 골수 유래 중간엽 줄기세포는 배양 조건에 따라 지방세포, 연골세포, 골 세포 등의 다양한 조직으로 분화 할 수 있는 능력을 가지고 있다. 줄기세포의 여러 단계를 거쳐 진행되며 각각의 단계는 다양한 인자와 그들의 상호작용에 의해 이루어지나 그 정확한 경로는 아직 규명되지 않았다. 최근 연골분화를 조절하는 물질로서 microRNA가 발견되었고 심도있게 연구되고 있다. microRNA는 20-24 nucleotide정도의 작은 RNA로 표적 mRNA를 분해시키거나 전사 후 과정을 억제시킴으로써 표적 유전자를 조절한다고 보고되어 있다. 현재 많은 연구에서 microRNA가 세포 증식과 사멸, 분화를 포함한 다양한 생물학적 과정에 중요한 역할을 하는 것이 밝혀지고 있다. 본 연구에서는 miR-495가 사람 줄기세포의 연골분화 과정에서 어떠한 작용을 하는지 밝히고자 하였다. 연골분화가 유도된 유도군과 대조군의 MSC에서 추출한

RNA를 microRNA microarray analysis를 통해 각 군에서의 microRNA 발현을 비교하였다. 통계적 분석을 통하여 연골분화 시킨 유도군에서 miR-495가 대조군과 대비하여 유의하게 감소함을 확인하였다. 이 후 데이터베이스 프로그램을 이용하여 miR-495가 Sox9을 표적으로 하는 것을 예측하였다. Sox9은 이미 연골분화의 전반적 과정에 중요한 역할을 하는 핵심요소로 많은 연구가 되어 있다. Realtime PCR를 이용하여 선별한 miR-495가 Sox9과 반대 발현 패턴을 보이는 것을 확인하였고 cell line과 사람 중간엽 줄기세포에서 miR-495에 의해 Sox9의 발현이 감소하는 것을 확인하였다. 또한 luciferase reporter 실험을 통하여 miR-495가 Sox9의 3'UTR에 결합하여 Sox9을 억제한다는 것을 확인하였다. miR-495를 줄기세포에 처리한 후 연골분화를 시켰을 때, 대조군과 비교하여 연골분화에 관련된 유전자들이 감소하였다. 이러한 결과를 토대로 miR-495가 Sox9의 발현을 억제시킴으로써 줄기세포의 연골분화를 조절한다는 것을 확인하였다.

핵심되는 말 : 중간엽 줄기세포, 연골분화, Sox9, microRNA, miR-495