miR-449a Regulates the Chondrogenesis of Human Mesenchymal Stem Cells Through Direct Targeting of Lymphoid Enhancer-Binding Factor-1

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microRNAs are small molecules, about 17–23 nucleotides in length, that act as translational regulators of their target gene. By binding to a target, microRNAs are known to either inhibit translation or induce degradation of the target. Despite the great interest in microRNAs, however, the exact targets of each individual microRNA in different processes remain largely unknown. In this study, we determined that the lymphoid enhancer-binding factor-1 (*LEF-1*) was expressed during the chondrogenesis of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) and sought to identify a novel microRNA targeting this gene. Through subsequent studies, we have identified, for the first time, one particular microRNA, miR-449a, that recognizes and regulates the expression of *LEF-1* in a dose-dependent and sequence-specific manner. In addition, we observed that the inhibition of *LEF-1* via miR-449a led to the subsequent repression of *Sox 9*, which is a well-established regulator of chondrogenesis. Collectively, this study demonstrated that miR-449a directly targets *LEF-1*, which in turn affects the expression of *Sox 9*, ultimately leading to the proper regulation of the differentiation and chondrogenesis of human MSCs (hBM-MSCs).

Introduction

MESENCHYMAL STEM CELLS (MSCs) are plastic-adherent, fibroblast-like cells that were first identified and described by Friedenstein [1]. Following their initial discovery, various groups have identified the cells' ability to differentiate into multiple connective tissue cell types, including fat, tendon, cartilage, and bone [2–6]. Chondrogenesis occurs via multiple stages that are initiated by cell condensation driven by upregulated N-cadherin, neural cell adhesion molecule, and fibronectin, which promote cell-to-cell and cell-to-extracellular matrix (ECM) interactions. Once the differentiating cells reach the chondroblast phase, the cells change the major components of the ECM from *type I collagen*, which is characteristic of undifferentiated human bone marrow-derived mesenchymal stem cells (hBM-MSCs), to a more cartilaginous make-up, involving *type II collagen*, *type IX collagen*, *type XI collagen*, *cartilage oligomeric matrix protein-1* (COMP-1), and *aggrecan*. Subsequently, the chondrocytes reach a hypertrophic state in which the cells begin to express *type X collagen*, which is unique to the hypertrophic stage of chondrogenesis. Eventually, as part of natural bone growth and development, the hypertrophic chondrocytes undergo apoptosis and are replaced by bone. This long and complicated process of chondrogenesis is directed by a number of signaling path-

ways that involve transforming growth factor β (TGF- β), fibroblast growth factor, Wnt, and Indian hedgehog signaling. Among these potent regulators of chondrogenesis, Wnt-signaling has been of particular interest to many researchers because of its involvement in a vast range of biological processes, including embryonic development, tissue morphogenesis, body patterning, and tumorigenesis [7,8].

Small molecules of 17–23 nucleotides in length, microRNAs have been found to be involved in various processes, including cell proliferation [9,10], apoptosis [11,12], immunoresponses [13], protein expression [14–16], and tissue development [17–23]. By forming a functional RNA-induced silencing complex [24], microRNAs recognize a complementary sequence of the target genes' 3' untranslated region (UTR) region and induce the inhibition of gene expression by 1 of 2 mechanisms: mRNA cleavage [25] or translational repression [26]. Currently, only a handful of microRNAs have been experimentally validated as key regulators in chondrogenesis. One study reported that microRNA18a targets the CCN family protein 2/connective tissue growth factor, which is a central player in endochondral bone formation [27]. miR-199a* profoundly inhibited the early stages of chondrogenesis in pluripotent C3H10T1/2 stem cells grown in pellet culture. When treated with miR-199a*, the authors observed a significant reduction in key chondrogenic markers, namely,

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COMP, Sox 9, and type II collagen, whereas inhibition of miR-199a* increased these genes [28]. In this study, we used high-throughput screening along with experimental validation using semiquantitative and quantitative real-time polymerase chain reaction (PCR) and found that few genes exhibited a distinctive expression pattern during chondrogenesis. Of the genes that did show specific increases during chondrogenesis, we noticed that a critical gene involved in Wnt signaling and known to be a direct target of β -catenin, lymphoid enhancer-binding factor-1 (LEF-1), was distinctively expressed during chondrogenesis. Subsequent studies have confirmed that LEF-1 indeed elicits a significant effect on the overall progression of chondrogenesis, and further study was then carried out to elucidate a novel microRNA that targeted LEF-1. Considering that Wnt signaling in general, along with LEF-1, has been shown to play a critical role in numerous biological pathways and is not solely restricted to chondrogenesis, the identification of a potent microRNA capable of regulating the expression of LEF-1 may allow us to gain a better understanding of human physiology as a whole. Accordingly, this study was conducted to identify a novel microRNA that specifically regulates the expression of LEF-1, which in turn would exhibit a specific function in the overall chondrogenesis process. We identified, for the first time, a microRNA, miR-449a, that specifically targets LEF-1 in a sequence-specific and dose-dependent manner.

Materials and Methods

Primary MSC and chondrosarcoma cell line culture

Bone marrow aspirates were obtained from the posterior iliac crest of 10 healthy adult donors ranging from 20 to 69 years of age under the approval of the Institutional Review Board. MSCs 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; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1× antibiotic–antimycotic solution (Invitrogen), nonadherent 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/1 mM ethylenediaminetetraacetic acid (Invitrogen) followed by a 3-min centrifugation at 1,300 rpm. This initial batch of cultured cells was designated as passage 1, and the cells were subcultured in a new 10-cm² dish up to passage 6–7. Two human chondrosarcoma cell lines, SW1353 and JJ, were cultured in Dulbecco's modified Eagle's medium–High Glucose (DMEM-HG; Invitrogen) with 10% FBS and 1× antibiotic–antimycotic solution (Invitrogen).

Chondrogenesis in a micromass culture

The cultured hBM-MSCs between passages 3 and 5 were harvested using the method described previously. For micromass culturing, the cells were resuspended in DMEM-LG with 10% FBS at a specified density of 8,000 cells/ μ L of media, and 10 μ L of the suspended cells was dotted on the center of each well of 24-well plates. A single drop of 1× phosphate-buffered saline (PBS; Thermo Scientific, Logan, UT) was placed in between each well to suppress overdrying of the cells. The cells were then placed in an incubator at

37°C and maintained at 5% CO₂ for 2 h to stimulate the adherence of the cells to the plate. For the control group, 1 mL of the control medium consisting of DMEM-HG (Invitrogen), 1× antibiotic–antimycotic solution, 1× insulin transferrin selenium-A (Invitrogen), and 50 μ g/mL of ascorbic acid (Invitrogen) was used, while the chondrogenic medium contained 10 ng/mL of TGF- β 3 (R&D systems, Minneapolis, MN) as well. The culture medium was changed every 2–3 days. All experiments were carried out in triplicate using hBM-MSCs from 3 donors.

Validation of gene expression at the mRNA level

The total RNA from hBM-MSCs was isolated using RNAiso Plus reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. Briefly, 1 mL of the RNAiso Plus solution was added repeatedly with a pipette to the collected cells to completely dissolve the cells. The cells were left at room temperature for 10 min, 200 μ L of chloroform was added, and the resulting mixture was vortexed until the solution became milky. The solution was then kept at room temperature for 5 min, after which it was centrifuged at 13,000 rpm for 15 min at 4°C. The top liquid layer was then transferred to a new tube, and 500 μ L of 100% isopropanol was added. After brief vortex-mixing, the solution was kept at room-temperature for 10 min, and was then promptly centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was removed without disturbing the RNA pellet, which was washed with cold 70% ethanol via centrifugation at 10,000 rpm for 5 min at 4°C. Finally, the RNA pellet was resuspended in 30 μ L of diethylpyrocarbonated-water. The overall quality and concentration of each RNA sample was confirmed using spectrophotometry. For cDNA reverse transcription, an Omniscript Reverse-Transcription Kit (Qiagen, Hilden, Germany) was used. The primer sets for the amplification of specific genes, obtained from Gene Bank and/or the manufacturer, are listed in Table 1. GAPDH was used to normalize the relative expression intensity of all genes for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

In addition to RT-PCR, quantitative real-time PCR was carried out using Takara Ex TaqTM (Takara) according to the manufacturer's instruction. Quantitative real-time PCR was performed using an ABI7500 real-time machine by Applied Biosystems (ABI, Carlsbad, CA). The primers used for quantitative real-time PCR are shown in Table 2. All primers

TABLE 1. PRIMER SEQUENCES FOR REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Gene		Primer sequence	Size (bp)
LEF-1	S	5'-GTCTGGTAAGTGCTTCTCTGT-3'	104
	AS	5'-GAGTGGGTTTGGCTATTACATT-3'	
SOX-9	S	5'-GAACGCACATCAAGACGGAG-3'	631
	AS	5'-TCTCGTTGATTTCGTGCTC-3'	
Type II collagen	S	5'-TTCAGCTATGGAGATGACAATC-3'	472
	AS	5'-AGAGTCCTAGAGTGACTGAG-3'	
Type X collagen	S	5'-GCCCCAAGAGGTGCCCTGGA-3'	570
	AS	5'-CCTGAGAAAGAGGAGTGAG-3'	
GAPDH	S	5'-GAAGGTGAAGGTCCGAGTC-3'	220
	AS	5'-GAAGATGGTGATGGGATTTC-3'	

LEF-1, lymphoid enhancer-binding factor-1; S, sense strand; AS, antisense strand.

TABLE 2. PRIMER SEQUENCES FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene	Strand	Primer sequence
<i>β-Actin</i>	S	5' GTCCTCTCCCAAGTCCACACAG 3'
	AS	5' GGGCACGAAGGTCATCATTC 3'
<i>LEF-1</i>	S	5' GCCACGGACGAGATGATCC 3'
	AS	5' TGTCTGGCCACCTCGTGTC 3'
<i>Aggrecan</i>	S	5' CCACTGTTACCGCCACTT 3'
	AS	5' GTAGTCTTGGGCATTGTTGT 3'
<i>SOX 9</i>	S	5' CGACTACGCTGACCATCAGA 3'
	AS	5' AGACTGGTTGTTCCCAAGTGC 3'
<i>Type X collagen</i>	S	5' CCAGGACAGCCAGGCATCAA 3'
	AS	5' ATGCCTTCTGGCCCTCGTTC 3'

were purchased from 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 100bp.

Microarray analysis

The total RNA of cultured hBM-MSCs was isolated by RNAisoPlus (Takara) according to the manufacturer's protocol. The overall quality of the total RNA was validated using spectrophotometry. For cDNA expression, an Affymetrix GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) was used. Pearson's correlation analysis was performed to examine genes specifically expressed in chondrogenesis-induced hBM-MSCs. A strict cutoff threshold was implemented to screen for genes with at least a 2-fold difference in the expression intensity.

Western blot analysis

A lysis buffer (Promega, Madison, WI) with 10 µg/mL of protease inhibitors and phosphatase inhibitors was used to prepare whole-cell lysates. Clarified lysates from samples centrifuged at 13,000 rpm at 4°C for 15 min were quantified using a modified Bradford assay. Aliquots of 30 µg of the cell lysates were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. The samples were promptly transferred to a polyvinylidene fluoride membrane (Amersham Pharmacia, Piscataway, NJ) for 90 min at 50 V in a transfer buffer containing 1.4% glycine, 20% methanol, and 25 mM of Tris-HCl (pH 8.3). For blocking, 1% skimmed milk in 1×TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) was used for 1 h at room temperature. *LEF-1* (Cell Signaling, Danvers, MA) and *Sox 9* (Santa Cruz, Santa Cruz, CA) primary antibodies were used at concentrations of 1:10,000 and 1:5,000, respectively, overnight at 4°C. Following repeated washing with 1×TBST, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia). The *β-Actin* antibody (Santa Cruz) was used for internal control.

Overexpression and inhibition of microRNA and siRNA for LEF-1

For functional analysis of the screened microRNA and its effect on the target gene, *LEF-1*, the identified microRNA

was overexpressed via microRNA-mimic purchased from Genolution (Genolution, Seoul, South Korea). The purchased microRNA mimic was prepared in a double-strand form, which is processed by endogenous mechanisms once transfected, ultimately yielding mature microRNAs. miR-1 was used as a positive control, as it is known to repress its target gene *twinfilin-1*. For a negative control, scrambled microRNA was used. The exact sequences of the microRNA mimics used were obtained from the microRNA database (miRanda), and the duplex sequences are shown in Table 3. The constructed microRNAs were transfected with 4 µL of lipofectamine 2000 (Invitrogen) into the human chondrosarcoma cell lines, SW1353 and JJ, according to the provided instructions. For transfection of microRNA into hBM-MSCs, few modifications had to be made, as hBM-MSCs are generally more difficult to transfect and show a lower ability to differentiate from the stress induced during the transfection procedure. Briefly, 7 µL of lipofectamine 2000 (Invitrogen) was used for transfection into hBM-MSCs, and the cells were cultured in growth media for an additional day post-transfection to induce stabilization.

We used 2'-O-methyl RNA with sequence complementary to the microRNA (ST Pharm Co., Seoul, South Korea) to repress the activity of the selected microRNA. For the inhibition of the microRNA, only the antagomir of miR-449a, which showed significant repression of *LEF-1*, was used. The antagomir was modified by attaching a FAM fluorescent dye on the 5' end for visualization after transfection, and the exact sequence of the miR-449a antagomir was 5' ACCAGCUAACAUAACACUGCCA 3'. For siRNA transfection, a prevalidated siRNA duplex with fluorescein isothiocyanate attached to the 5' end for visualization was used. The exact sequence of the siLEF-1 was 5' GUUAUUCGGGUACAUAU 3', and the exact sequence of the siControl was 5' CCUACGCCACCAAUUUGGC 3'.

Reporter vector construction

The 3' UTR of the *LEF-1* gene was cloned into the *XbaI* site of a pGL3 Luciferase Reporter Vector with a SV40 promoter (Promega) and was called pGL3-LEF-1-UTR. The construct was then cotransfected with the microRNA mimic in SW1353 and JJ cell lines to evaluate the efficacy of the selected

TABLE 3. MICRORNA MIMICS USED FOR OVEREXPRESSION

microRNA	Strand	Duplex sequence
hsa-miR-1	S	5' UGGAAUGUAAAGAAGUAUGUAU 3'
	AS	5' AUACAUACUUCUUUACAUUCCA 3'
hsa-miR-SC	S	5' UUACCAGACGUGUCUUCACUCCC 3'
	AS	5' GGGAGUGAAGACACGUCUGGUA 3'
hsa-miR-138	S	5' AGCUGGUGUUGUGAAUCAGGCCG 3'
	AS	5' CGGCCUGAUUCACAACACCAGCU 3'
hsa-miR-411	S	5' UAGUAGACCGUAUAGCGUACG 3'
	AS	5' CGUACGCUAUACGGUCUACUA 3'
hsa-miR-449a	S	5' UGGCAGUGUAUUGUUAGCUGGU 3'
	AS	5' ACCAGCUAACAUAACACUGCCA 3'
hsa-miR-548c-5p	S	5' AAAAGUAAUUGCGUUUUUGCC 3'
	AS	5' GCGAAAAACCGCAAUACUUU 3'
hsa-miR-767-3p	S	5' UCUGCUCAUACCCCAUGGUUUCU 3'
	AS	5' AGAAACCAUGGGGUAUGAGCAGA 3'

microRNA based on its degree of luminescence. The primer sequences used for *LEF-1* 3' UTR cloning are as follows, with the underlined regions marking the sequence for the *Xba*I recognition site.

Sense: 5'-ATGCTCTAGAGTGGAAAACGAAGCTCATTCC-3'

Antisense: 5'-ATGCTCTAGATTAAAAAATGCTCAGCACATTAAC-3'

Safranin-O staining of monolayer chondrogenic cells

Proteoglycan synthesis of chondrogenic cells was visualized using safranin-O staining. Chondrogenic cells in 24-well plates were briefly washed twice with PBS, and then 200 μ L of 1% safranin-O staining solution dissolved in 1% acetic acid was added and incubated for 3 h to stain. The cells were then repeatedly rinsed with 1% acetic acid. The safranin-O stained cells were destained with 10% cetylpyridinium chloride monohydrate, and the absorbance value was measured at a wavelength of 490 nm.

Statistical analysis

The statistical analysis for the results of the staining test and the gene expression pattern obtained from hBM-MSCs was carried out using the Student's *t*-test, and the data are expressed as the mean $\pm$ standard deviation. Values of $P < 0.05$ were considered to be statistically significant.

Results

Chondrogenesis of hBM-MSCs using micromass cultures

Chondrogenesis of the hBM-MSCs acquired by the previously mentioned methods was induced by micromass culturing. During the 14-day differentiation process, the total RNA was collected on days 7, 10, and 14, and the expression profiles of key chondrogenic marker genes, such as *type II collagen* and *Sox 9*, along with the key hypertrophic marker gene *type X collagen*, were observed using semiquantitative RT-PCR (Fig. 1A). *Type II collagen* and *Sox 9* showed progressively increasing expression, indicating the proper progression of chondrogenesis, while the hypertrophic marker *type X collagen* showed relatively unchanged expression. To better assess the overall expression profile of genes that showed the most distinctive expression patterns during the chondrogenesis of hBM-MSCs, high-throughput screening with microarray techniques was used. hBM-MSCs from 3 separate individuals were collected and cultured in either the control medium without TGF- β 3 or in the chondrogenic medium with TGF- β 3 for 10 days. From these samples, microarray analysis was carried out to screen for the genes that were significantly increased only in the differentiated group throughout all 3 donors. The scatter plot and quantitative real-time data show the distinctive expression of *LEF-1* in the differentiated group (Fig. 1B, C). *Sox 9* and *aggrecan*, 2 distinctive marker genes that should increase upon the progression of chondrogenesis, were expressed at a significantly higher intensity compared to the day 0 and 10 control hBM-MSCs, further confirming the efficacy of chondrogenesis induced by micromass culturing (Fig. 1D, E). *LEF-1* exhibited a dramatic increase specifically in

chondrogenesis-induced hBM-MSCs. All experiments have been carried out in triplicate using hBM-MSCs derived from 3 separate donors.

LEF-1 plays a significant role in the progression of chondrogenesis

Of the 418 genes from the initial high-throughput screening whose expression profiles were considered significant, we examined the established ontology of each identified gene for possible implications for the overall progression of chondrogenesis. Briefly, an Affymetrix GeneChip Human gene 1.0 ST Array was used to identify the genes that had altered expression profiles from the controls during the differentiation process. For statistical analysis, Pearson correlation was performed to acquire statistically meaningful candidates. Of the genes, *LEF-1* was distinguished from the rest of the genes for its known role in various developmental processes and, more specifically, its role in Wnt signaling. For experimental validation of these expression patterns, we performed quantitative real-time PCR to confirm the expression data obtained from the microarray and found that the 2 results were completely aligned (Fig. 1F).

Based on previous studies that reported the significance of Wnt signaling in the chondrogenesis of hBM-MSCs, we decided to focus specifically on *LEF-1*, which is a crucial mediator of Wnt, and more specifically, β -catenin signaling. Microarray data indicated that *LEF-1* was expressed at 10.4-fold higher abundance in day 10 chondrogenesis-induced hBM-MSCs compared to the day 10 control, which was the sixth highest expression difference among the screened genes (Table 4).

After confirming the accuracy of the high-throughput data with quantitative real-time PCR results, we then moved on to further evaluating the specific effect of *LEF-1* in chondrogenesis by performing an inhibition experiment. Human BM-MSCs were transfected with 100 nM of either siControl (purchased from Bioneer) or siLEF-1 and were cultivated in a chondrogenic medium for a period of 10 days. The total RNA was harvested at specific time points: days 0, 1, 3, 5, 7, and 10. RT-PCR was carried out to monitor the efficacy of *LEF-1* on the chondrogenesis of human BM-MSCs (Fig. 2, day 10 data not shown because the gene expressions showed rapid recovery). When *LEF-1* was inhibited, it was clearly evident that genes involved in differentiation, including *type II collagen* and *Sox 9*, were repressed.

Screening of microRNAs specifically targeting LEF-1 during the chondrogenesis of hBM-MSCs

After confirming a significant expression pattern of *LEF-1* during the early phase of chondrogenesis, we then aimed to identify the microRNA that specifically controls the expression of *LEF-1*. Using the 2 most-widely used microRNA-target prediction search engines, TargetScan (www.targetscan.org) and miRanda (www.microRNA.org), we screened for microRNAs that were predicted to target *LEF-1*. From the derived list, we crosschecked the microRNAs that were highly ranked to the target gene of interest in both databases. In addition to observing the expression profile of microRNAs during chondrogenesis, it was crucial to identify whether the microRNAs actually targeted the gene of interest, *LEF-1*, and

FIG. 1. Chondrogenesis of human MSC and identification of *LEF-1* as distinctively expressed gene. Human MSCs were induced into chondrogenesis in micromass culture and the expression profiles of key chondrogenic marker genes were observed using semiquantitative PCR (**A**). Among the various genes specifically expressed during chondrogenesis, *LEF-1* was identified as one of the most distinctively expressed genes (**B**), and its increasing expression pattern was observed during chondrogenesis of MSC (**C**). The expression of key chondrogenic marker genes, such as *Sox 9* (**D**), and *AggreCAN* (**E**), were compared against *LEF-1* (**F**) using quantitative real-time PCR. MSC, mesenchymal stem cell; LEF-1, lymphoid enhancer-binding factor-1; PCR, polymerase chain reaction.

if so, to what extent they were capable of regulating the gene expression. To validate such microRNAs targeting *LEF-1*, 5 microRNAs that showed the most distinctive expression pattern against *LEF-1* during differentiation, namely, miR-138, -411, -449a, -550, and -767, were overexpressed in 2 human chondrosarcoma cell lines, SW1353 and JJ, via the

transfection of a microRNA mimic. Their effect on endogenous *LEF-1* expression was monitored through quantitative real-time PCR (Fig. 3). Despite the obvious merit of overexpressing microRNA directly in hBM-MSCs, the use of human chondrosarcoma cell lines was chosen as the best alternative because the hBM-MSCs possessed the innate

TABLE 4. GENES OVEREXPRESSED DURING CHONDROGENESIS

Probe set ID	Gene symbol	Gene description	Fold change
8092169	<i>TNFSF10</i>	Tumor necrosis factor (ligand) superfamily, member 10	14.9
8095886	<i>CXCL13</i>	Chemokine (C-X-C motif) ligand 13	13.8
8107722	<i>MEGF10</i>	Multiple EGF-like domains 10 sema domain, transmembrane	11.5
8113666	<i>SEMA6A</i>	Domain (TM), and cytoplasmic domain, (semaphorin) 6A	10.9
7924461	—	—	10.7
8102232	<i>LEF1</i>	Lymphoid enhancer-binding factor 1	10.4

FIG. 2. Effect of *LEF-1* on chondrogenesis of BM-MSCs. siLEF-1 effectively inhibited *LEF-1* expression up to day 7, which was then shown to be recovered by day 10 (data not shown). Two genes involved in differentiation, *Col2a1* and *Sox 9*, and a gene involved in maturation, *type X collagen*, were monitored.

difficulty of being hard to transfect and to stably maintain introduced microRNA for extended periods of time, along with the highly variant nature of the cells between donors. Among the top 5 candidate microRNAs that were predicted by multiple microRNA search engines to target *LEF-1*, only miR-449a elicited a significant reduction of endogenous *LEF-1* expression in 2 human chondrosarcoma cell lines, SW1353 and JJ. The introduction of a miR-449a mimic resulted in a more than 70% reduction of *LEF-1* expression in the JJ cell line, which displays the full effect miR-449a may have on *LEF-1* expression.

miR-449a directly targets *LEF-1* via binding to the 3' UTR

To better quantify the effect of the sequence-specific regulation of miR-449a on *LEF-1*, the *LEF-1* UTR was cloned into a pGL3-control plasmid (Fig. 4A, B). As expected, the luciferase activity showed a drastic decrease of approxima-

tely 70% in the HeLa cell line upon transfection with miR-449a (Fig. 4C). In addition, this reduction of luciferase activity occurred proportionally in a dose-dependent manner against miR-449a, further strengthening the notion that miR-449a targets *LEF-1* (Fig. 4D).

Following such observations, we then aimed to identify the exact seed site of miR-449a and *LEF-1*. The entire 3' UTR of *LEF-1* and the expected miR-449a-binding seed site were individually cloned into the pGL3 reporter vector, while a construct with a seed sequence-specific mutation was also constructed to determine the sequence-specific effect of miR-449a (Fig. 5A). In both tested HeLa and JJ cell lines, the miR-449a mimic induced an approximately 50% and 60% reduction of luciferase activity, respectively, in constructs containing the entire *LEF-1* UTR and the region immediately flanking the miR-449a-binding site (Fig. 5B, C). In contrast, when the predicted miR-449a-binding site within the 3' UTR of *LEF-1* was mutated, there was a complete recovery of luciferase activity, indicating that miR-449a binds to the predicted seed site.

After validating the dose-dependent and sequence-specific regulation of *LEF-1* by miR-449a, we then aimed to discover the actual change in expression at the level of both mRNA and protein. SW1353 and JJ cell lines were transfected with either microRNA-scramble, miR-449a mimic, or miR-449a antagomir, and the overall change in the expression of *LEF-1* was monitored via quantitative real-time PCR and western blot analysis. As expected, the mRNA levels of *LEF-1* in both SW1353 (Fig. 6A) and JJ (Fig. 6B) cell lines showed a positive correlation with the presence of either miR-449a or miR-449a antagomir. The same expression pattern was also observed at the protein level (Fig. 6C).

Inhibition of *LEF-1* leads to the subsequent reduction of *Sox 9* and the downregulation of proteoglycan synthesis

Sox 9 is a pivotal transcription factor in chondrogenesis that is required for the proper differentiation of hBM-MSCs into chondroblasts as well as for precartilaginous condensation.

FIG. 3. Screening of potential microRNA specifically targeting *LEF-1*. microRNAs highly predicted by both TargetScan and miRanda to target *LEF-1* were selected and were individually transfected into 2 human chondrosarcoma cell lines, SW1353 (A) and JJ (B), and the expression of *LEF-1* was observed using quantitative real-time PCR. Each real-time value was normalized against the control, miR-sc-treated samples, and respective *P* values were obtained to monitor statistical significance. Among the top 5 microRNAs tested, only miR-449a showed distinctive repression of *LEF-1*. *Indicates *P* value < 0.05. miR-sc, miR-scramble.

FIG. 4. miR-449a specifically binds to the seed site within 3' UTR of *LEF-1*. A reporter assay utilizing pGL-3 *LEF-1* UTR vector co-transfected with 50 nM of miR-449a displayed a sequence-specific reduction of *LEF-1* mediated by miR-449a. A schematic diagram of the miR-449a binding to the endogenous *LEF-1* mRNA (A) and the construct made where the entire 1.2-kb of *LEF-1* UTR has been cloned at the 3' end of the luciferase gene on pGL-3 plasmid (B). Up to 80% reduction of luciferase activity has been observed in the presence of miR-449a (C). The reduction via miR-449a occurred in a dose-dependent manner (D). All luciferase assay has been carried out in Hela cell line. *Indicates *P* value < 0.05.

Heterozygous mutation of *Sox 9* coding genes has been reported to cause campomelic dysplasia, which is a subtype of human dwarfism characterized by hypoplasia of all cartilage-derived skeletal elements [29,30]. In addition, a previous report has indicated that the *Sox 9* promoter region contained a binding site for *LEF-1* [31], while another study showed direct regulation of *Sox 9* expression by *LEF-1* [32]. Based on such findings, we then sought to determine whether inhibiting *LEF-1* via miR-449a would in turn translate into the proportional repression of *Sox 9*, and whether the opposite effect would be observed when a miR-449a antagonist was used. To evaluate this hypothesis, we individually

transfected miR-scramble, miR-449a, and Anti-449a into SW1353 cells and observed their effect on *LEF-1* and *Sox 9*. As expected, the expression of *Sox 9* at the levels of both mRNA (Fig. 7A, B) and protein (Fig. 7C) mimicked that of *LEF-1*, indicating the regulatory role of *LEF-1* on *Sox 9*. To further ascertain the effect of such repression of *Sox 9* on the overall progression of chondrogenesis, we then observed the overall level of proteoglycan synthesis by safranin O staining in alignment with our previous data (Fig. 7D). For further confirmation, the degree of safranin O staining was quantified by destaining the cells as previously described, and a matching pattern was found (Fig. 7E).

FIG. 5. Sequence-specific activity of miR-449a on *LEF-1*. The exact seed site of miR-449a on *LEF-1* UTR was identified by producing various forms of *LEF-1* 3' UTR in the pGL3-construct (A). In both JJ (B) and Hela (C) cell lines inhibited the luciferase activity of miR-449a, which was drastically recovered when the predicted seed site was mutated. **Indicates *P* value < 0.01.

FIG. 6. miR-449a modulates expression of *LEF-1* and affects chondrogenesis. Overexpression of miR-449a has led to a significant reduction of endogenous *LEF-1* expression, whereas inhibition of miR-449a has led to an increase of endogenous *LEF-1* expression in 2 separate human chondrosarcoma cell lines, (A) SW1353 and (B) JJ cell lines. Both miR-449a- and anti-449a-treated groups were normalized against the miR-sc-treated group, which acted as a control, and respective *P* values represent statistical significance. (C) A similar pattern was also observed at the level of protein via western blot analysis. **Indicates that the *P* value is statistically significant at <0.01 in all cases.

miR-449a plays a regulatory role in the chondrogenesis of hBM-MSCs

Ultimately, to verify that miR-449a elicited a similar regulatory function in both hBM-MSC differentiation as well as in chondrogenic cell lines, we transfected MSCs derived from 3 separate donors with either miR-449a or a control and monitored the change in expression of *LEF-1* and other downstream chondrogenic marker genes. At day 10, in

alignment with our previous results, we observed the expected increase of *LEF-1*, *Sox 9*, and *type II collagen* compared to the day 0 control (Fig. 8, the data shown are representative figures from experiments carried out using 3 independent donor-derived hBM-MSCs). This, in conjunction with the successful formation of micromass, confirms that successful chondrogenesis was achieved. In addition, as observed in Fig. 8A, it was clearly evident that miR-449a-transfected hBM-MSCs yielded significantly reduced

FIG. 7. *LEF-1* elicits its effect on chondrogenesis via targeting *Sox 9*. Reduced *LEF-1* upon overexpression of miR-449a had resulted in a consequent reduction of critical factors in chondrogenesis. In SW1353 cell line, it was observed that the expression pattern of *LEF-1* upon the introduction of either miR-449a or anti-449a showed positive correlation with expression of *Sox 9* (A, B). Both miR-449a- and anti-449a-treated groups were normalized against the miR-sc-treated group, which acted as a control, and respective *P* values represent statistical significance. Such a pattern was further confirmed in the protein level via western blot analysis (C). Safranin O staining of miR-sc, miR-449a and anti-449a transfected SW1353 cell lines showed distinctive repression of proteoglycan synthesis (D, E). *Indicates *P* value <0.05.

FIG. 8. miR-449a regulates chondrogenesis in hBM-MSC by targeting *LEF-1*. hBM-MSCs transfected with either miR-449a mimic or miR-scramble control were induced for chondrogenesis for 10 days. At day 10, *LEF-1*, *Sox 9*, and *type II collagen* have shown drastic increase compared to the day 0 control (A–C). Furthermore, in presence of miR-449a, *LEF-1* (A), *Sox 9* (B), and *type II collagen* (C) have all shown significant inhibition compared to the miR-sc-treated group. *Indicates P value < 0.05 , **Indicates P value < 0.01 . hBM-MSC, human bone marrow-derived mesenchymal stem cells.

expression of *LEF-1* at differentiation day 10 compared to the cells treated with the scramble control. In alignment with our results obtained from the chondrogenic cell lines SW1353 and JJ, the reduction of *LEF-1* via introduction of miR-449a also led to the noticeable reduction of the downstream key chondrogenic marker gene *Sox 9* (Fig. 8B). Lastly, we also confirmed that *type II collagen* was significantly reduced at day 10 of differentiation upon treatment with miR-449a (Fig. 8C).

Discussion

Previous studies have reported that Wnt signaling plays a critical role in determining the overall progression of chondrogenesis [33]. *Wnt4* and *Wnt9a* are highly expressed in developing joints, while *Wnt7a* and *Wnt8a* are found in developing cartilage. *Wnt5a* and *Wnt11* are expressed specifically in the perichondrium, whereas *Wnt5b* is restricted to prehypertrophic chondrocytes. Although a number of Wnts, such as *Wnt4*, *Wnt8*, and *Wnt11*, play an inhibitory role in stimulating hypertrophy and blocking chondrogenic differentiation, other Wnt family members, such as *Wnt3a*, *Wnt5a*, and *Wnt5b*, are known to promote chondrogenesis [34–40]. In one study, the authors observed a dramatic increase in *Wnt3a* with treatment of a high-density culture of C3H10T1/2 cells with a well-established chondrostimulatory factor, BMP-2. Additionally, when *Wnt-3A* and a kinase-dead GSK-3 β were overexpressed, the total and nuclear levels of both *LEF-1* and β -catenin showed a noticeable increase, which in turn displayed active interaction with BMP-2 during chondrogenesis [41]. These findings indicate that certain members of the Wnt family, such as *Wnt-3a*, play an essential role during the progression of chondrogenesis and that they exert their effect via the well-established β -catenin/*LEF-1* interaction. In perfect alignment with this theory, a study reported that the overexpression of *LEF-1* in 3 separate cell sources, namely C3H10T1/2, ATDC5, and primary chondrocytes, showed induction of a key chondrogenic marker gene, *Sox 9* [32]. However, upon the prolonged presence of *LEF-1* induced by a constitutively active *LEF-1* overexpression vector, hBM-MSCs rapidly went into hypertrophy [42]. Similarly, a study by Tamamura et al. indicated that the transgenic overexpression of *LEF-1* driven by the *type II collagen* promoter led to defective chondrocyte maturation, whereas an overexpression of mutated β -catenin in chick embryos re-

sulted in the inhibition of both cartilage development and hypertrophy, suggesting that the canonical Wnt signaling has the unique effect of both hypertrophy and chondrogenic differentiation depending on the developmental stage [43]. The findings from previous studies indicate that *LEF-1* plays a pivotal role in the proper progression of chondrogenesis of hBM-MSCs, and has a significant involvement in the commencement and regulation of hypertrophy. In parallel, our data, from both the high-throughput screening analysis via microarray analysis and the experimental validation using RT-PCR and quantitative real-time PCR, showed the expected increase in *LEF-1* during the early phase of differentiation (Fig. 1).

To identify the potential microRNA targeting this gene of interest, we were able to narrow down the potential microRNA with the widely used target prediction databases TargetScan and miRanda. By introducing the mimic of each microRNA into human chondrosarcoma cell lines, we were then able to identify one particular microRNA that elicited the most significant reduction of *LEF-1*, miR-449a. In 2 of the human chondrosarcoma cell lines tested, SW1353 and JJ, miR-449a induced a significant reduction in *LEF-1* expression, whereas the inhibition of miR-449a via the transfection of the antagomir resulted in the drastic recovery of expression (Fig. 6). In addition, we also observed a dose-dependent reduction of luciferase activity when the pGL-3-*LEF-1* construct was cotransfected with the miR-449a mimic, further confirming the regulatory effect of miR-449a on *LEF-1* (Fig. 4). Finally, via mutation analysis of the predicted miR-449a-binding site, we also successfully identified the exact sequence of *LEF-1* that miR-449a recognizes, which validates the fact that miR-449a is indeed a negative regulator of *LEF-1* (Fig. 5). Such a result provides solid evidence of the direct repression of *LEF-1* via miR-449a. Furthermore, this reduction of *LEF-1* resulted in the consequent reduction of *Sox 9* (Fig. 7A–C). Established as the master regulator of chondrogenesis, *Sox 9* expression is known to precede the upregulation of 2 critical marker genes of chondrogenesis, *type II collagen* and *aggrecan* [44], and it is thought to activate the 48-bp enhancer located in the first intron of the *type II collagen* gene [45]. Collectively, it is widely accepted that *Sox 9* plays a critical role in the overall progression of chondrogenesis, with a specific focus on precartilaginous condensation

and proper differentiation of hBM-MSCs into chondrocytes. Accordingly, the reduction of LEF-1 expression by miR-449a resulted in the downregulation of *Sox 9*, which in turn affected the overall level of proteoglycan synthesis (Fig. 7D). In addition, upon the suppression of *LEF-1* via siLEF-1 in hBM-MSCs, the key chondrogenic marker gene *type II collagen*, along with *Sox 9*, clearly showed a significant reduction throughout the 7-day differentiation period (Fig. 2), indicating that *LEF-1* is indeed a pivotal factor involved in the regulation of chondrogenesis, which is in perfect alignment with the previous reports that have suggested the critical and positive effect *LEF-1* has on the overall progression of proper chondrogenesis [32–35,37–40].

Upon successful verification of the regulatory role miR-449a elicited on *LEF-1*, and furthermore, on expression of chondrogenic marker genes, such as *Sox 9* and *type II collagen* in the chondrogenic cell lines SW1353 and JJ, we then tested its effect on hBM-MSCs. Using the identical microRNA mimic used for experiments in cell lines, we transfected 3 separate hBM-MSCs derived from individual donors and induced chondrogenesis. In perfect alignment with the chondrogenic cell lines, the introduction of miR-449a resulted in the significant downregulation of *LEF-1* in hBM-MSCs (Fig. 8A). In addition, we also observed a noticeable reduction in the downstream chondrogenic marker genes *Sox 9* (Fig. 8B) and *type II collagen* (Fig. 8C). These data, in combination with the similar results obtained from multiple chondrogenic cell lines, strongly support our initial hypothesis that miR-449a directly regulates the expression of *LEF-1*, which in turn regulates chondrogenesis, resulting in reduced *Sox 9* and *type II collagen* expression.

In summary, this study has successfully demonstrated that (a) *LEF-1* is indeed a critical factor involved in the proper differentiation and maturation of chondrogenesis of human BMSCs and that (b) miR-449a directly binds to the seed sequence of the *LEF-1* 3' UTR, causing effective repression of its expression, which ultimately leads to (c) a subsequent reduction in *Sox 9* gene expression and the overall progression of chondrogenesis. In addition, considering that previous research has confirmed the critical role played by *LEF-1* in various biological processes, not only limited to chondrogenesis, but also in areas, such as tumorigenesis and development, complete experimental validation of endogenous microRNAs targeting *LEF-1* may prove to be an asset in the field of human physiology.

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Author Disclosure Statement

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