

Specific microRNA expression during chondrogenesis of human mesenchymal stem cells

JIANWEI HAN*, TONGTAO YANG*, JIE GAO*, JIACHANG WU,
XIUCHUN QIU, QINGYU FAN and BAOAN MA

Department of Orthopedic Surgery, Tangdu Hospital, Fourth Military Medical University,
Xi'an, Shaanxi 710038, P.R. China

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Abstract. Mesenchymal stem cells (MSCs) have the capacity to self-renew and differentiate into multiple cell types, but little is known about the precise mechanism of this procedure. Recent studies show that a group of short noncoding RNAs called microRNAs (miRNAs) play a key role in this procedure. However, little work has been done to determine the miRNAs which specifically regulate the differentiation of MSCs. In this study, we cultured human MSCs and chondrogenic differentiation MSCs of 3 donors, and investigated the miRNA expression profiles of MSCs and chondrogenic differentiated MSCs from 2 donors by using miRNA microarrays. We found 5 miRNAs were significantly overexpressed in chondrogenic differentiated MSCs in each sample, and 4 were further confirmed by real-time RT-PCR assay in the sample from the third independent donor. We also predicted the confirmed miRNAs corresponding to putative target genes by online software. The results indicated that the overexpressed miRNAs in chondrogenic differentiated MSCs may play a role in the procedure of MSC chondrogenesis, which offers us guidance for further studies.

Introduction

Mesenchymal stem cells (MSCs), also called mesenchymal stromal cells, present the characteristic of long-term self-renewal and multi-lineage differentiation potential. Given appropriate cultures, MSCs are able to differentiate into different mesodermal cell lineages, including osteocytes, chondrocytes, and adipocytes (1,2). With this characteristic,

MSCs offer great hope for a variety of therapeutic applications (3,4). However, MSCs are heterogeneous populations of cells and MSCs isolated from different donors present a high degree of variability (5), which makes it difficult to determine the precise mechanism of its self-renewal and multi-lineage differentiation.

MicroRNAs (miRNAs) are noncoding, short single-stranded RNAs of 18-25 nucleotides and play important roles in several biological processes post-transcriptionally (6,7). After the first miRNA (lin-4) was discovered by Lee *et al* (8), hundreds of miRNAs have been identified in plants and animals, and many of them are highly conserved across species and tissue-specific (9,10). Previous studies show that miRNAs play a key role in stem cell self-renewal, cell division and differentiation (11-15). A unique group of miRNAs have been reported to be expressed in human and mouse embryonic stem cells (ESCs), which suggest they are involved in ESC development (16-20). Due to heterogeneity of MSCs, it is difficult to determine the specific expression patterns of MSCs and their differentiated cells. Therefore, compared with ESCs, only a few studies have identified the specific miRNAs during MSC differentiation. For example, miR-143 has been reported to be involved in adipocyte differentiation through the target gene ERK5 (12), miR-125b inhibits osteoblastic differentiation of mouse mesenchymal stem cells by down-regulation of cell proliferation through targeting ErbB2/3 genes (21). Another specific miRNA, miR-140, was found to be expressed during mouse cartilage development and may function by inhibiting HDAC4, a likely co-repressor of RUNX2 (22).

In this study, we employed microarray technique to explore the expression of miRNAs during chondrogenesis of MSCs. The differentially expressed miRNAs between MSCs and chondrogenic differentiation MSCs were verified by real-time RT-PCR. We obtained 4 miRNAs up-regulated in chondrogenic differentiated MSCs. Then, we predicted their putative target genes with bioinformatic analysis, which may offer us guidance for further study.

Materials and methods

Isolation and culture of human MSCs. The three human bone marrow samples in our study were collected from patients without hematologic diseases after obtaining informed

Correspondence to: Dr Baoan Ma, Department of Orthopedic Surgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038, P.R. China
E-mail: gukemba@fmmu.edu.cn

*Contributed equally

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consent. These three samples were not collected at the same time (A: 19 years old, Chinese, male; B: 24 years old, Chinese, female; C: 26 years old, Chinese, male).

Immediately after the sample being collected, monocytes were separated from the samples by 1.073 g/ml Percoll (Sigma) with density gradient centrifugation method. Monocytes were incubated at 5000 cells per cm² in 25-cm² culture flasks (Falcon) consisting the medium of Low Glucose Dulbecco's modified Eagle's medium (L-DMEM, Gibco) and 15% fetal bovine serum [FBS, (Hyclone)]. The cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. Culture medium changes were performed at day 5 and then twice a week. After 10-14 days of cultivation, the cells were trypsinized and re-seeded at 5x10⁴ cells/cm² in new flasks. When 80% confluent, the cells were harvested and replanted with 1:3 proportion to expand the cells.

Phenotype analysis of MSCs. Cells at the fourth passage with 80% confluence were collected and suspended in phosphate buffered saline (PBS) at a concentration of 1x10⁶ cells/ml. Mouse anti-human antibody (CD34-FITC, CD45-FITC, CD29-FITC, CD44-FITC) (5 µl) was added into 500 µl suspension containing 5x10⁵ cells and the mixture was incubated for 20 min at room temperature. Then the cells were harvested and rinsed twice with cleaning solution (PBS +1% FBS +0.1% NaN₃). The cells were suspended in 500 µl of cleaning solution, which were analyzed using a flow cytometer.

Chondrogenic differentiation of MSCs. MSCs at fourth passage were detached by trypsin and washed twice with PBS. The collecting cells were re-suspended in a 15 ml polypropylene tube (Orange) containing 1 ml chondrogenic differentiation medium at a density of 4x10⁵/ml, centrifuged at 500 x g for 15 min into cell mass. The cell mass was cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide for 21 days with the medium changes twice a week. The chondrogenic medium contained high glucose Dulbecco's modified Eagle's medium (H-DMEM, Gibco), supplemented with 1 ng/ml TGF-β₁ (Sigma), 10⁻⁷/l dexamethasone (Gibco), 50 mg/l vitamin C (Gibco), 6.25 ng/l insulin (Sigma), 6.25 µg/ml transferrin (Sigma), 1.25 µg/ml bovine serum albumin (BSA, Amresco). After being induced for 21 days, the cell mass was fixed with 4% paraformaldehyde at room temperature for staining and RNA isolation.

Cytochemical staining. The harvested cell mass was fixed in neutral-buffered formalin, embedded in paraffin, and sectioned (10-µm thick). The cross-sections were stained with toluidine blue and s-100 protein antigen.

RNA extraction. We used TRIzol reagent (Invitrogen) to isolate RNAs from culture cells (MSCs and chondrogenic differentiated MSCs from independent donor A, B, C) according to the manufacturer's instructions. The integrity and purity of total RNA was verified spectro-photometrically and by gel-electrophoresis on a formaldehyde denaturation gel.

miRNA microarray analysis. We examine global miRNA expression by miRNA microarray single-channel fluorescence

chip (CapitalBio Corp. Beijing, China) that contains 743 probes in triplicate corresponding to current release of Sanger miRNA database (miRBase8.2, July 2006). The consistently expressed small nuclear RNA U6 was spotted as an internal control.

Procedures were performed as described previously (23,24). Briefly, low-molecular-weight RNAs were enriched from 100 µg total RNA [sample A, B, C (25)] with mirVana™ miRNA isolation kit (Ambion) and amplified with the NCode™ miRNA amplification system (Invitrogen). Then, the amplified RNA was labeled with 5'-phosphate-cytidyl-uridyl-cy3-3' (Dharmacon, Lafayette, CO) with 2 units T4 RNA ligase (New England Biolabs, Ipswich, MA). The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% SDS, 4SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). Then, using fluorescence scanning (LuxScan 10K/A, CapitalBio) and image analysis software (LuxScan 3.0, CapitalBio), digital signal intensities for each spot were obtained. Raw data were normalized on mean array intensity for inter-array comparison and analyzed using the Significance Analysis of Microarray (SAM, version 2.1, Stanford University, CA, USA) software (26), to determine differential expression of miRNAs between MSCs and chondrogenic differentiated MSCs.

Real-time quantitative PCR. The primers were designed according to miRNA sequences, and a universal PCR reverse primer (27). U6 small nuclear RNA gene was used as an internal control (28), and oligonucleotide primers were 5'-ctc gcttcgcgcagcaca-3' and 5'-aacgcttcacgaatttcgct-3'.

Four miRNAs including hsa-miR-130b, hsa-miR-152, hsa-miR-26b, and hsa-miR-28 selected by SAM in last step were subjected to real-time quantitative PCR. Their RT-PCR oligonucleotide primers were hsa-miR-130b: 5'-GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACATGCCC-3' and 5'-CGCAGTGCAATGATGAA AGG-3'; hsa-miR-152: 5'-GTCGTATCCAGTGCAGGGT CCGAGGTATTCGCACTGGATACGACCCCAAG-3' and 5'-GTCGTTCAGTGCATGACAGAACTT-3'; hsa-miR-26b: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACACCTAT-3' and 5'-GCCGCTTCAAGTAA TTCAGGATA-3'; hsa-miR-28: 5'-GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACCTCAAT-3' and 5'-CGGCAAGGAGCTCACAGTCTAT-3'. All these miRNA primers and U6 primer were designed using Primer Express version 5.0 (Applied Biosystems, Foster City, CA).

Following a previously described protocol (28), total RNA of MSCs and chondrogenic-differentiated MSCs from the third individual donor (sample C) were assayed with real-time RT-PCR. Briefly, procedures were conducted using Light Cycler PCR 1.2 (Roche), and LightCycler FastStart DNA Master SYBR Green I (Roche). Cycling parameters were 95°C for 10 min to denature DNA templates, then 95°C for 10 sec, and 60°C for 30 sec, with a final recording step of 74°C for 3 sec to prevent any primer, 40 cycles in all. Melting curves were performed at 75-95°C and samples were also run on a 3% agarose gel to confirm specificity.

Target prediction. Three softwares available online were used for target prediction. They were targetScan (<http://genes.mit.edu>).

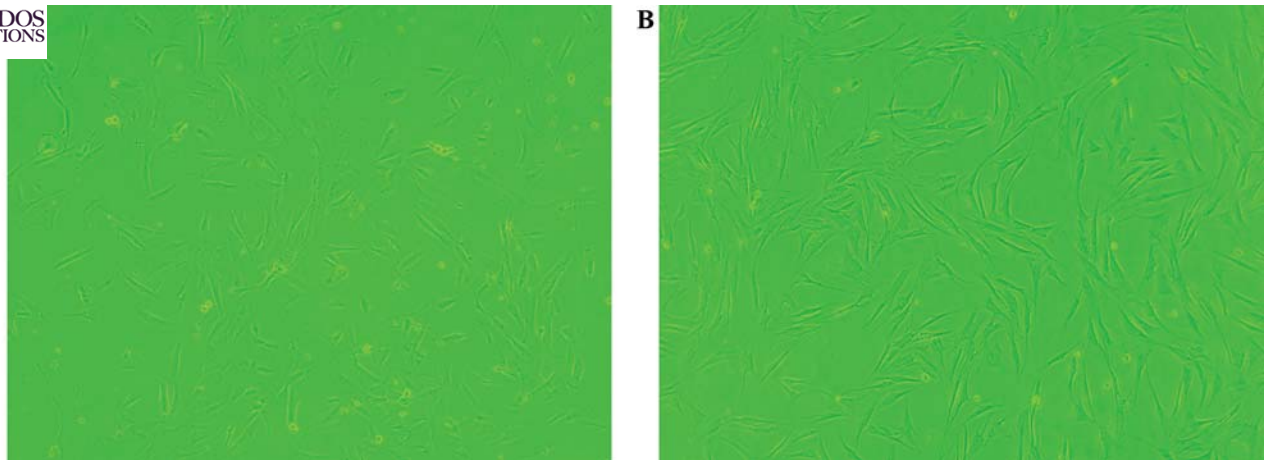


Figure 1. MSCs under inverted microscope (x100). (A) MSCs in primary culture at day 7, (B) MSCs in primary culture at day 14.

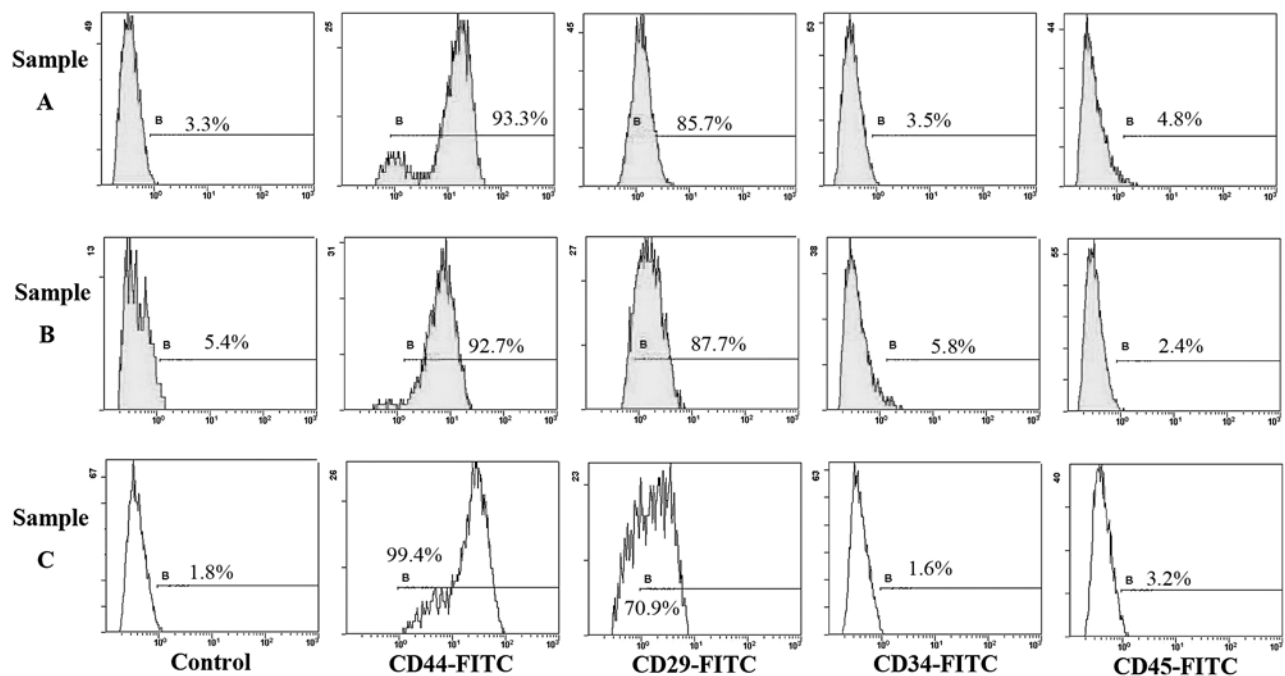


Figure 2. Surface marker analysis of MSCs.

edu/targetscan) (9,29), PicTar (<http://pictar.bio.nyu.edu>) (30), and miRanda (<http://microrna.sanger.ac.uk/targets>) (31). Candidate miRNAs up-regulated in chondrogenic differentiated MSCs were selected for target prediction.

Results

Characterization and phenotype of MSCs. Due to the lack of specific makers, MSC are usually defined by their plastic adherent growth, immunophenotype and differentiation potential (32). After seeded in flasks for 24 h, most of the cells adhered to the flask and began to grow. After 3 or 5 days, the cells obtained a spindle shape and expanded (Fig. 1). We tested the surface antigens of MSCs by using flow cytometry, and the results demonstrate that these three MSC samples were all negative for the hematopoietic lineage markers CD34

and CD45, positive for CD29 and CD44 as expected (32-34) (Fig. 2).

Cytochemical staining of chondrogenic differentiated MSCs. The fixed cell masses prepared previously were stained by Toluidine blue and s-100 protein. The staining of s-100 protein (Fig. 3A) and Toluidine blue (Fig. 3B) were positive.

miRNA microarray analysis. RNA gel electrophoresis confirmed that the quality of the RNA of MSCs and chondrogenic differentiated MSCs was good. Using miRNA microarray technology, we detected the miRNA expression of MSCs and chondrogenic differentiated MSCs from sample A and B (Fig. 4).

SAM and unpaired comparison were performed to identify the differences in miRNA expression between MSCs and

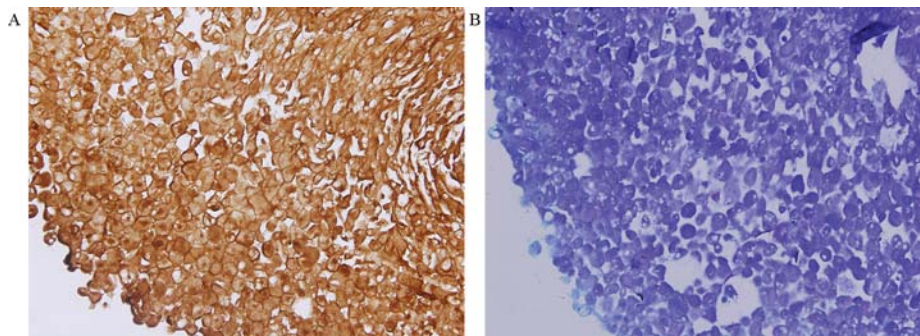


Figure 3. Staining of chondrogenic differentiated MSCs (x100). (A) Staining of Toluidine blue, (B) immunostaining of s-100 protein.

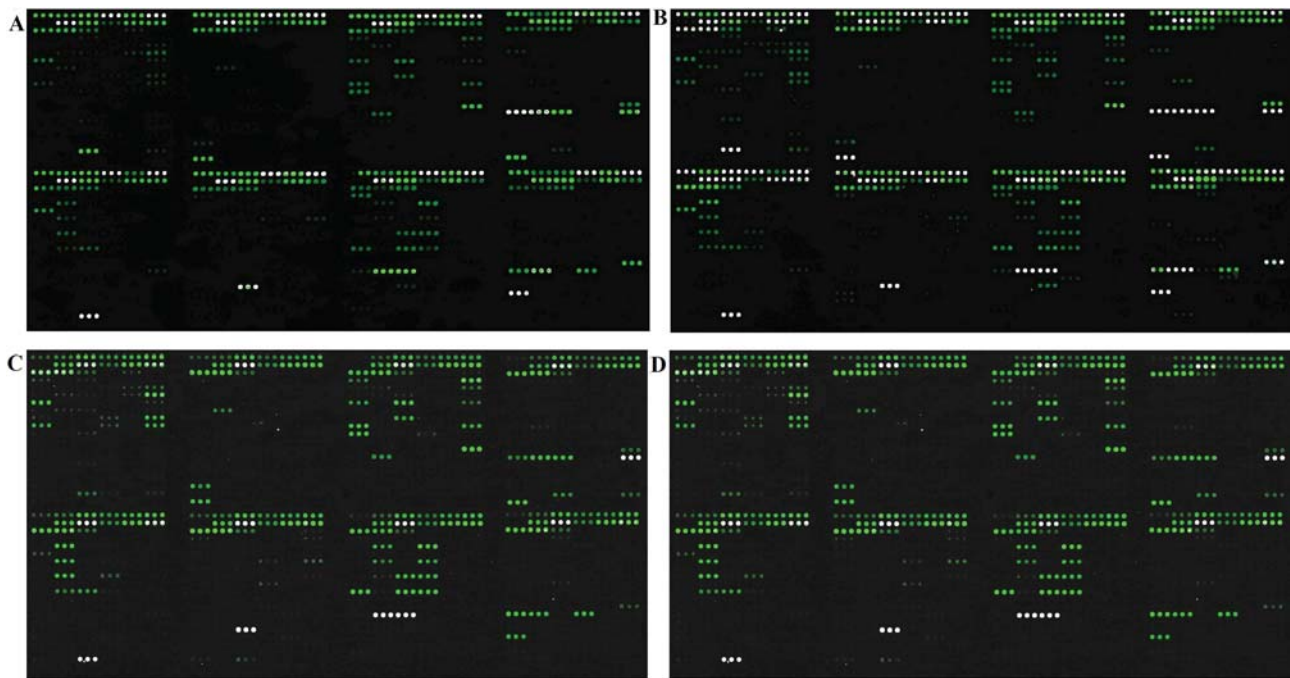


Figure 4. Microarray hybridization signals of MSCs and chondrogenic differentiated MSCs. The microarrays demonstrated miRNA expression profiles of MSCs (A) and chondrogenic differentiated MSCs (B) in sample A, MSCs (C) and chondrogenic differentiated MSCs (D) in sample B.

chondrogenic differentiated MSCs in each sample ($FDR \leq 5\%$, fold change ≥ 2). Comparing the two cell types, analysis of the microarray data showed that 26 miRNAs were over-expressed and 1 underexpressed in sample A, 7 miRNAs overexpressed and 2 underexpressed in sample B (Tables I and II). Comparing these two samples, we observed that 5 human miRNAs over-expressed chondrogenic differentiated MSCs in both samples. They were hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b and hsa-miR-193b. However, none of the miRNAs in chondrogenic differentiated MSCs underexpressed in both samples (Table III).

Real-time PCR of miRNAs. To add reliability to these differentially-expressed miRNAs, we used real-time RT-PCR to verify the expression levels of the miRNAs (hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b) in the third individual sample (sample C).

The results showed that the expression levels for the 4 miRNAs were higher in chondrogenic differentiated MSCs

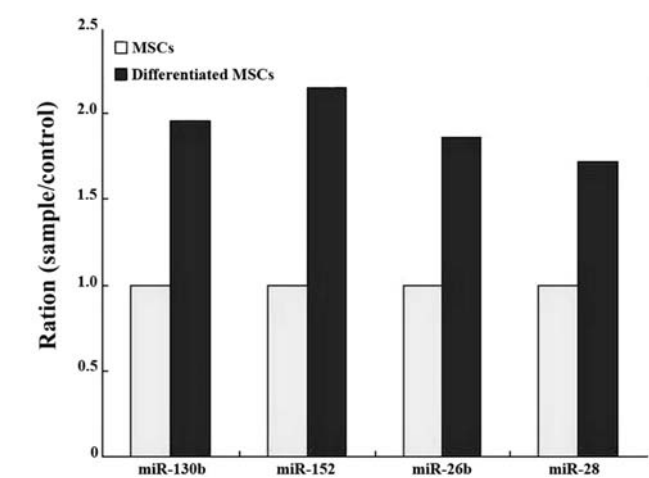


Figure 5. The histogram of real-time PCR results. miR-130b, miR-152, miR-26b and miR-28 are listed on the X-axis; the Y-axis refers to the relative expression levels between MSCs (control) and chondrogenic differentiated MSCs (sample); and control expression is 1.



Gene name	Score (d)	Fold change	q-value (%)
Overexpressed miRNAs in chondrogenic differentiated MSCs			
PREDICTED_MIR191	61.90756	10.22930984	0
hsa-miR-494	50.94587	9.384187345	0
PREDICTED_MIR189	44.50401	17.30665355	0
hsa-miR-23b	42.56505	3.855811988	0
hsa-miR-24	41.70688	4.221921479	0
hsa-miR-101	37.33963	12.52892679	0
hsa-miR-27b	35.93786	9.12200175	0
hsa-miR-143	29.75591	10.12670531	0
hsa-let-7f	29.14949	8.489466293	0
hsa-miR-21	27.18485	4.774973191	0
hsa-miR-19a	26.77491	8.773178739	0
hsa-miR-28	25.04472	7.731432434	0
hsa-let-7d	24.7563	6.814207291	0
hsa-miR-196a	24.39329	17.38690456	0
hsa-miR-153	23.58271	13.33122809	0
hsa-let-7e	23.31158	5.197571623	0
hsa-miR-10b	22.11271	13.2733231	0
hsa-let-7a	21.6041	5.431683232	0
hsa-miR-296	21.3643	4.491348558	0
hsa-miR-34a	21.32207	23.89235671	0
hsa-let-7b	21.28517	5.42947065	0
hsa-miR-125b	21.17034	2.633324849	0
hsa-miR-26b	20.76998	7.953414823	0
hsa-miR-34b	20.73275	24.57993313	0
hsa-miR-127	20.6463	4.961294298	0
hsa-miR-125a	20.55137	6.950404646	0
Underexpressed miRNAs in chondrogenic differentiated MSCs			
hsa-miR-122a	-74.2364	0.004226096	0

Underexpressed miRNAs are in bold.

than MSCs, demonstrating that the PCR results were in concordance with the microarray analysis (Table IV and Fig. 5).

Putative target prediction for miRNAs. With the online software described previously, we predicted the putative targets for hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b, and we obtained several possible targets for these four miRNAs. Many are related to bone and cartilage formation, sulfation of chondroitin, and signal transduction. We listed some representative possible targets in Table V.

Discussion

There are a variety of techniques to detect the expression of miRNAs in tissues. Among these techniques, microarray assay provides a high-flux method for detecting miRNA expression simultaneously (35). While, for its false discovery rate (FDR),

the microarray results should be validated by using Northern blots or real-time PCR. In this study, we obtained miRNA expression maps of MSCs and chondrogenic differentiated MSCs using microarrays, 5 miRNAs (hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b, hsa-miR-193b) were expressed significantly differently between them by SAM assay. Of these 5 different miRNAs, 4 miRNAs (hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b) were isolated from the third donor and validated by real-time PCR, which added credibility to the microarray results. In this step, we observed the variability between the two donors. For example, we detected 78 human miRNAs up-regulated in sample A, but 7 miRNAs in sample B, and the overlap between them was 5. The down-regulated human miRNAs was 1 in sample A, 2 in sample B, and there was no overlap. These differences could be caused by the variability of MSCs (5,36). Another example probably for the variability is that some miRNAs which have been reported to be related to

Table II. Differentially-expressed miRNAs between MSCs and chondrogenic differentiated MSCs in sample B.

miRNA	Score (d)	Fold change	q-value (%)
Overexpressed miRNAs in chondrogenic differentiated MSCs			
hsa-miR-572	13.15153	3.093737687	0
hsa-miR-28	9.093153	2.38822041	0
hsa-miR-193b	8.196323	2.587382493	0
hsa-miR-130b	7.976752	2.950776614	0
hsa-miR-152	6.313401	2.247640546	0
hsa-miR-26b	5.359322	2.021033713	0
hsa-miR-560	4.491367	2.079951506	0
Underexpressed miRNAs in chondrogenic differentiated MSCs			
PREDICTED_MIR189	-15.4503	0.315047598	0
hsa-miR-424	-9.75853	0.229614492	0

Underexpressed miRNAs are in bold.

Table III. miRNAs overexpressed in chondrogenic differentiated MSCs in both sample A and sample B.

Gene name	Fold change	
	Sample A	Sample B
hsa-miR-130b	2.429655338	2.950776614
hsa-miR-152	10.65679325	2.247640546
hsa-miR-26b	7.953414823	2.021033713
hsa-miR-193b	4.111352285	2.587382493
hsa-miR-28	7.731432434	2.38822041

cartilage did not demonstrate differential expression in this study (22,37,38).

miRNAs play the regulatory roles by binding target sites and modulating the translation of specific mRNA. Therefore, bioinformatic methods based on sequence similarity between targets and miRNAs can be used to predict the potential target genes of miRNAs, which can provide guidance for further analysis of miRNA functions (39). Herein we predicted some potential genes of the validated miRNAs by using TargetScan 5.1. Among the predicted genes, there are several genes associated with collagen proteins such as COL2A1, COL4A1, and COL6A1. These genes encode their correspondent type collagen, and their mutations usually result in pathologic skeletal disorders, especially chondrocytes. For example, COL2A1 predicted by hsa-miR-152 encodes type II collagen which is the main component of articular cartilage (40), and the gene mutations are associated with achondrogenesis, chondrodysplasia, and early onset familial osteoarthritis.

The gene COL10A1 predicted by hsa-miR-26b encodes the type X collagen, a short chain collagen specifically expressed by hypertrophic chondrocytes during endochondral ossification (41). Type X collagen plays an important role during endochondral ossification, and loss of its function caused impairment of endochondral ossification and skeletal

Table IV. miRNAs in MSCs and chondrogenic differentiated MSCs verified by real-time RT-PCR.

Name	E	^a CP	Ratio before normalization	Ratio after normalization
U6	1.866	-0.33	0.813952683	
hsa-miR-130b	1.863	0.75	1.594628938	1.959117492
hsa-miR-152	1.875	0.89	1.749730379	2.149670879
hsa-miR-26b	1.88	0.66	1.516855395	1.863567044
hsa-miR-28	1.844	0.55	1.400130461	1.720161983

Control, MSCs; sample, osteo-differentiated MSCs. ^aCP, (control-sample) crossing point; ratio = sample/control.

growth in mice (42-44). Therefore, the mutations of COL10A1 can cause a severe skeletal disorder called Schmid-type metaphyseal chondrodysplasia (45,46). Upon these findings, we can see these two genes have a close relationship with the cartilage development, therefore we predict that the miRNAs associated with these putative collagen-related genes, such as hsa-miR-152 and hsa-miR-26b, probably play some roles in the procedure of endochondral ossification and skeletal growth.

In addition, there are a group of BMP (bone morphogenetic protein) related genes such as BMP3 (bone morphogenetic protein type 3), BMPR1B (bone morphogenetic protein receptor type IB), BMPR2 (bone morphogenetic protein receptor type II), which are predicted by hsa-miR-152 and hsa-miR-130b. Of those genes, BMP3 belongs to the transforming growth factor- β (TGFB) superfamily. BMPs are involved in endochondral bone formation and embryogenesis (46-48). Genes of BMPR1B and BMPR2 encode the bone morphogenetic protein (BMP) receptors. CHST3 (chondroitin sulfotransferase 3) and CHSY1 (chondroitin sulfate synthase 1) predicted by hsa-miR-26b are associated with chondroitin synthesis.



Gene name	Representative possible targets	Function
hsa-miR-130b	BMPR2 (bone morphogenetic protein receptor, type II)	Bone formation
	BMP3 (bone morphogenetic protein, type III)	Bone formation
	BMPR1B (bone morphogenetic protein receptor, type IB)	Bone formation
	COL19A1 (collagen, type XIX, $\alpha 1$)	Collagens fibril-forming
hsa-miR-152	BMP3 (bone morphogenetic protein, type III)	Bone formation
	COL4A1 (collagen, type IV, $\alpha 1$)	Cartilage formation
	COL2A1 (collagen, type II, $\alpha 1$)	Cartilage formation
	COL6A1 (collagen, type IV, $\alpha 1$)	Cartilage formation
hsa-miR-26b	COL10A1 (collagen, type X, $\alpha 1$)	Endochondral ossification
	CHST3 (chondroitin 6 sulfotransferase 3)	Sulfation of chondroitin
	CHSY1 (chondroitin sulfate synthase 1)	Sulfation of chondroitin
	CILP (cartilage intermediate layer protein)	Cartilage formation
	IGF1 (insulin-like growth factor 1)	Growth and development
	SMAD1 (mothers against DPP homolog 1)	Signal transduction
hsa-miR-28	IGF2BP1 (insulin-like growth factor 2 mRNA binding protein 1)	Regulating IGF2 translation

CHST3 encodes an enzyme which catalyzes the sulfation of chondroitin, a proteoglycan involved in cell migration and differentiation, and mutations in this gene are associated with spondylepiphyseal dysplasia and humerospinal dysostosis (49,50). CHSY1 synthesizes chondroitin sulfate (51). Chondroitin sulfate and dermatan sulfate chains play structural roles in cartilage bone, and other connective tissues (52-54). Another predicted gene by hsa-miR-26b is CILP (cartilage intermediate layer protein), and it encodes the cartilage intermediate layer protein (CILP), which increases in early osteo-arthritis cartilage (55). IGF-1 (insulin-like growth factor I) predicted by hsa-miR-26b was reported to promote the differentiation of MSCs into chondrocytes in combination with TGF- β (56).

In conclusion, we applied microarrays to detect the miRNAs in human MSCs and their chondrogenic differentiated cells and obtained 4 specific miRNA in the latter, which were validated by real-time PCR. Due to lack of bone marrow donors, only two samples were tested on microarrays, but we employed a third sample to verify the results obtained from two previous samples by real-time PCR, and the PCR results were consistent with the SAM results. For further confirmation of the obtained results, we will enlarge the sample size of MSCs and continue to study the differently expressed miRNA functions.

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