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Journal of Cellular Biochemistry 112:1844–1856 (2011)

Journal of Cellular Biochemistry

MicroRNA Expression During Osteogenic Differentiation of Human Multipotent Mesenchymal Stromal Cells From Bone Marrow

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ABSTRACT

MicroRNAs comprise a group of non-coding small RNAs (17–25 nt) involved in post-transcriptional regulation that have been identified in various plants and animals. Studies have demonstrated that miRNAs are associated with stem cell self-renewal and differentiation and play a key role in controlling stem cell activities. However, the identification of specific miRNAs and their regulatory roles in the differentiation of multipotent mesenchymal stromal cells (MSCs) have so far been poorly defined. We isolated and cultured human MSCs and osteo-differentiated MSCs from four individual donors. miRNA expression in MSCs and osteo-differentiated MSCs was investigated using miRNA microarrays. miRNAs that were commonly expressed in all three MSC preparations and miRNAs that were differentially expressed between MSCs and osteo-differentiated MSCs were identified. Four underexpressed (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, and hsa-miR-424) and three novel overexpressed miRNAs (hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b) in osteo-differentiated MSCs were selected and their expression were verified in samples from the fourth individual donors. The putative targets of the miRNAs were predicted using bioinformatic analysis. The four miRNAs that were underexpressed in osteo-differentiated MSCs were predicted to target RUNX2, CBFB, and BMPs, which are involved in bone formation; while putative targets for miRNAs overexpressed in osteo-differentiated MSCs were MSC maker(e.g., CD44, ITGB1, and FLT1), stemness-maintaining factor(e.g., FGF2 and CXCL12), and genes related to cell differentiation(e.g., BMPER, CAMTA1, and GDF6). Finally, hsa-miR-31 was selected for target verification and function analysis. The results of this study provide an experimental basis for further research on miRNA functions during osteogenic differentiation of human MSCs. J. Cell. Biochem. 112: 1844–1856, 2011.

KEY WORDS: MULTIPOTENT MESENCHYMAL STROMAL CELLS; MicroRNA; OSTEOBLAST; DIFFERENTIATION; MICROARRAY

MicroRNAs (miRNAs) comprise a group of non-coding small RNAs (17–25 nt) involved in post-transcriptional regulation that have been identified in various plants and animals [Bartel, 2004]. miRNAs constitute 1% of the genes in the known genome, and regulate >30% of native coding genes in humans. They have been implicated in several biological processes, including development, proliferation, differentiation, apoptosis, and cancer pathogenesis [Bartel, 2004; Berezikov et al., 2005; Hwang and Mendell, 2006]. Several studies have identified populations of miRNAs in embryonic stem cells (ESCs), hematopoietic stem cells, and multipotent mesenchymal stromal cells (MSCs), and in their differentiated progeny. They have demonstrated that miRNAs are associated with stem cell self-renewal and differentiation and play a key role in controlling stem cell activities [Suh et al., 2004; Fazi et al., 2005; Felli et al., 2005; Krichevsky et al., 2006; Zhang et al., 2006].

MSCs are commonly isolated from adult bone marrow and have the potential to differentiate into adipose tissue, bone, cartilage, tendon and muscle, and thus hold great hope for therapeutic applications [Pittenger et al., 1999]. However, the molecular mechanisms governing self-renewal and differentiation remain unclear. Some studies have identified cell-type differentiation-specific miRNA expression patterns, but these are complicated by inter-donor variation [Lakshmipathy and Hart, 2008]. For example,

J. Gao and T. Yang contributed equally to this work.

Grant sponsor: National Science Foundation of China; Grant number: 30672143.

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Received 6 May 2010; Accepted 8 March 2011 • DOI 10.1002/jcb.23106 • © 2011 Wiley-Liss, Inc.
Published online 17 March 2011 in Wiley Online Library (wileyonlinelibrary.com).

hsa-miR-143, hsa-miR-170, and hsa-miR-103 were reported to regulate adipocyte differentiation of human MSCs [Esau et al., 2004; Wilfred et al., 2007]. hsa-miR-130, hsa-miR-206, and hsa-miR-302a were found to be associated with human MSC-derived neuronal cells [Greco and Rameshwar, 2007], hsa-miR-140, hsa-miR-638, and hsa-miR-663 were identified to regulate human MSC differentiation into chondrocytes [Tuddenham et al., 2006; Goff et al., 2008], and hsa-miR-30a-5p, hsa-miR-125b, hsa-miR-21, hsa-miR-24, and hsa-let-7 were associated with osteocyte differentiation [Goff et al., 2008; Mizuno et al., 2008].

These results demonstrate the expression of miRNAs during MSC differentiation. However, variations between MSC isolates from individual donors make generalizations difficult [Goff et al., 2008; Lakshmipathy and Hart, 2008], and the identification of specific miRNAs and their regulatory roles in MSC differentiation have so far been poorly defined.

Osteogenic differentiation of MSCs can be induced in the presence of dexamethasone, β-glycerophosphate, and ascorbate [Pittenger et al., 1999]. To further define the regulatory mechanisms of miRNAs in the osteogenic differentiation of MSCs, we examined the differential expression of miRNAs between undifferentiated MSCs and osteogenically differentiated MSCs from four individual human donors, using miRNA microarrays and real-time reverse transcription-polymerase chain reaction (RT-PCR), four underexpressed miRNAs and three overexpressed miRNAs were identified in osteo-differentiated MSCs relative to undifferentiated MSCs. The putative target genes of these miRNAs were predicted using bioinformatic analysis. hsa-miR-31 that was underexpressed in osteo-differentiated MSCs was selected for target verification and function analysis. The identification of miRNAs controlling the osteogenic differentiation of MSCs provides the basis for determining the function of miRNAs in MSC differentiation.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF HUMAN MSCs

Bone marrow was isolated from the iliac crest of four individual donors undergoing iliac bone graft surgery (A: 28-year-old Chinese male; B: 19-year-old Chinese male; C: 24-year-old Chinese female; D: 26-year-old Chinese male).

Human MSCs were isolated and cultured as previously reported [Pittenger et al., 1999; Jiang et al., 2002]. Bone marrow aspirates of 10–15 ml were placed in a tube containing heparin (100 U/L), mixed with isochoric phosphate-buffered saline (PBS) and mixed by blowing with a suction pipe. The mixture was added to an equal volume of 1.073 g/ml Percoll solution (Sigma) in a 50-ml conical tube and centrifuged at 2,000 rpm for 30 min. Mononuclear cells were collected from the middle layer and interface, diluted with two volumes of PBS, then collected by centrifugation at 1,000 rpm. The cells were resuspended in complete culture medium (Dulbecco's modified Eagle's medium [Gibco]; 10% fetal bovine serum [FBS, Hyclone]; 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine [Sigma]). The cells were seeded at 5,000 cells/cm^2 in 25-cm^2 culture flasks (Falcon) and incubated at 37°C in 5% CO_2 and 95% humidity. After 3 days of culture, the culture medium and nonadherent cells were discarded, adherent cells were washed twice with PBS, and new medium was added. The cells were harvested after 10–14 days of culture using 0.25% trypsin and 1 mM EDTA, and replated at 10^4 cells/cm^2 in 25-cm^2 culture flasks (Falcon). After 14 days, the cells were nearly 80% confluent and were dissociated using 0.25% trypsin and 1 mM EDTA, and then replated at a ratio of 1:3 to expand the cells through successive passages.

PHENOTYPE ANALYSIS

MSCs were suspended in PBS at a concentration of about 10^6 cells/ml and washed twice with PBS. About 5×10^5 cells per 500 μl were incubated and stained with 5 μl mouse anti-human CD34-fluorescein isothiocyanate (FITC), CD45-FITC, CD29-FITC, and CD44-FITC antibodies for 20 min at room temperature, rinsed twice with cleaning solution (PBS + 1% FBS + 0.1% NaN3), resuspended in 500 μl of cleaning solution, and analyzed using a flow cytometer.

OSTEOBLAST DIFFERENTIATION

MSCs were seeded at 10^4 cells/cm^2 in 25-cm^2 culture flasks (for RNA isolation) or in six-well plates with 1 cm × 1 cm glass coverslips in each well (for staining). Cells were grown to 50–70% confluences over 24–48 h in standard growth medium. The medium was replaced with osteogenic differentiation medium (100 nM dexamethasone, 50 mM L-ascorbic acid, 10 mM β-glycerophosphate [Sigma]), and this medium was replaced every third day for 7–21 days. Samples were stained or harvested for RNA isolation at 7, 14, or 21 days of differentiation.

CYTOCHEMICAL STAINING

Alkaline phosphatase (ALP) staining. Cells induced to osteoblast differentiation were washed twice in PBS, fixed in methanol/formalin (9:1) for 30 min, and incubated with ALP substrate solution (5 ml 2% sodium glycerophosphate, 5 ml 2% barbital sodium, 5 ml 2% calcium chloride, 2 ml 2% magnesium sulfate, 1–2 drops chloroform) in 25 ml water for 3 h at 37°C. The cells were then incubated in 2% cobaltous nitrate solution for 2 min, and washed three times in deionized water. Finally, the cells were put in 1% ammonium sulfide solution for 1 min and washed with deionized water. The cells were photographed using an Olympus inverted system microscope.

RNA EXTRACTION

Total RNA was isolated from cultured cells (MSCs and day 14 osteo-differentiated MSCs from the four independent donors) using Trizol reagent (Invitrogen), according to the manufacturer's instructions. The integrity and purity of the total RNA were verified spectrophotometrically and by gel-electrophoresis on formaldehyde denaturation gel.

miRNA MICROARRAY ANALYSIS

A single-channel fluorescence miRNA microarray chip from CapitalBio Corporation (Beijing, China) was used, containing 509 probes in triplicate, corresponding to 435 human (including 122 predicted miRNAs [Xie et al., 2005]), 196 mouse, and 261 rat miRNAs found in the miRNA Registry (http://microrna.sanger.ac.uk; miRBase Release 7.0, accessed October, 2005) or 743 probes in triplicate, corresponding to 576 human (including 122 predicted

miRNAs [Xie et al., 2005]), 238 mouse, and 358 rat miRNAs found in the miRNA Registry (http://microrna.sanger.ac.uk; miRBase Release 8.2, accessed July, 2006). The consistently expressed small nuclear RNA U6 was spotted as an internal control, and all oligonucleotide probes were labeled with Cy3 fluorescent dyes (green color).

Procedures were performed as described previously [Liao et al., 2008]. Briefly, low-molecular weight RNAs were enriched from 100 μg total RNA (samples A, B, C) using mirVana miRNA Isolation Kit (Ambion) and amplified using the NCode miRNA Amplification System (Invitrogen). Amplified RNA was then labeled with 5′-phosphate-cytidyl-uridyl-cy3-3′ (Dharmacon, Lafayette, CO) using 2 U T4 RNA ligase (New England Biolabs, Ipswich, MA) [Thomson et al., 2004]. Labeled miRNAs from sample A were used for hybridization with the miRNA microarray containing 509 probes, and labeled miRNAs from samples B and C were used for hybridization with the miRNA microarray containing 743 probes. The arrays were hybridized at 42°C overnight and washed with two consecutive washing solutions (0.2% sodium dodecyl sulfate, 2 × SSC at 42°C for 5 min, and 0.2% SSC for 5 min at room temperature). Digital signal intensities for each spot were obtained using fluorescence scanning (LuxScan 10K/A, CapitalBio) and image analysis software (LuxScan3.0, CapitalBio). Raw data were normalized to mean array intensity for inter-array comparison and analyzed using the Significance Analysis of Microarrays (SAM, version 2.1, Stanford University, CA, USA) software [He et al., 2005], to determine differential expression of miRNAs between MSCs and day 14 osteo-differentiated MSCs.

TABLE I. Real-Time RT-PCR Oligonucleotide Primers

Name	Primer sequence 5′ → 3′
U6-Forward	CTCGCTTCGGCAGCACA
U6-Reverse	AACGCTTCACGAATTTGCGT
hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC
hsa-miR-30c-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATT-CGCACTGGATACGACGCTGAG
hsa-miR-30c-AS	CGTGTAAACATCCTACACTCTCT
hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA
hsa-miR-15b-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT-GGATACGACAGTAAA
hsa-miR-15b-AS	CGTAGCAGCACATCATGGTTTA
hsa-miR-130b	CAGUGCAAUGAUGAAAGGGCAU
hsa-miR-130b-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC-GCACTGGATACGACATGCCC
hsa-miR-130b-AS	CGCAGTGCAATGATGAAAGG
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU
hsa-miR-31-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC-ACTGGATACGACAGCTAT
hsa-miR-31-AS	CAAGGCAAGATGCTGGCATA
hsa-miR-106a	AAAAGUGCUUACAGUGCAGGUAG
hsa-miR-106a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC-GCACTGGATACGACGCTACC
hsa-miR-106a-AS	GGAAAAGTGCTTACAGTGCAGGT
hsa-miR-148a	UCAGUGCACUACAGAACUUUGU
hsa-miR-148a-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA-CTGGATACGACACAAAG
hsa-miR-148a-AS	CGTCAGTGCACTACAGAACTTTG
has-miR-424	CAGCAGCAAUUCAUGUUUUGAA
has-miR-424-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA-CTGGATACGACTTCAAA
has-miR-424-AS	CCAGCAGCAATTCATGTTTTG

REAL-TIME RT-PCR

All primers were designed according to miRNA sequences, and a universal PCR reverse primer using Primer Express version 5.0 (Applied Biosystems, Foster City, CA) [Chen et al., 2005]. U6 small nuclear RNA gene was used as an internal control [Schmittgen et al., 2004], and oligonucleotide primers were shown in Table I. The analyzed miRNAs included hsa-miR-31, hsa-miR-106a, hsa-miR-148a, hsa-miR-424, hsa-miR-30c, hsa-miR-15b and hsa-miR-130b, and real-time RT-PCR oligonucleotide primers were shown in Table I.

Total RNA from MSCs and day 14 osteo-differentiated MSCs from sample D were assayed by real-time RT-PCR, following a previously described protocol [Schmittgen et al., 2004]. 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 s, and 60°C for 30 s, with a final recording step of 74°C for 3 s to prevent any primer dimer formation, for a total of 40 cycles. Melting curves were acquired at 75–95°C, and samples were also run on a 3% agarose gel to confirm specificity.

miRNA TARGET PREDICTION

miRNA target prediction was determined using the online software, TargetScan (http://genes.mit.edu/targetscan) [Lewis et al., 2003; Rajewsky and Socci, 2004] and PicTar (http://pictar.bio.nyu.edu) [John et al., 2004; Krek et al., 2005]. Four underexpressed miRNAs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, and hsa-miR-424) and three overexpressed miRNAs (hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b) in osteo-differentiated MSCs were selected for target predictions.

FUNCTION ANALYSIS AND TARGET VERIFICATION FOR has-miR-31

We selected hsa-miR-31 for target verification and function analysis. Anti-miR-31 was used to inhibit miR-31 activity. Anti-miR-31(the antisense strand of miR-31) and negative control were designed and synthesized by GenePharma (Shanghai, China). Anti-miR-31: 5′-AGCUAUGCCAGCAUCUUGCCU-3′, negative control: 5′-CAGUACUUUUGUGUAGUACAA-3′. Negative control, a random sequence anti-miRNA molecule based on miRNAs in *C. elegans*, has been extensively compared to all human, mouse and rat genome sequences and microRNA sequence by BLASTN, and validated to not produce identifiable effects on known miRNA function. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen). MSCs were plated in six-well plates with 1 cm × 1 cm glass coverslips to 50% confluence, and then 20 μM anti-miR-31 was mixed with Lipofectamine 2000 according to the manufacturer's instructions. The mixture was added to cells, and the final concentrations of anti-miR-31 and negative control were 50 nM. In such conditions, the survival rate of MSCs after transfection could be up to 90%. For osteogenic differentiation, the medium was replaced with fresh osteogenic differentiation medium (10% fetal bovine serum [FBS, Hyclone], 100 nM dexamethasone, 50 mM L-ascorbic acid, 10 mM β-glycerophosphate [Sigma]) at 4 h after transfection.

At 72 h after transfection, proteins from cells was prepared for Western blot analysis. Proteins were extracted with RIPA buffer (Sigma), then whole cell lysates were separated by SDS–PAGE gel

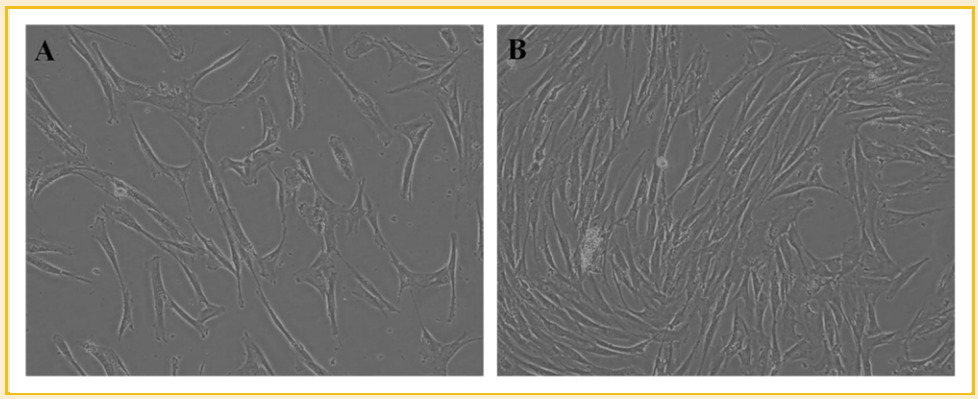

Fig. 1. Human MSCs in monolayer culture. Inverted microscope images (100×) showing MSCs in primary culture at 7 days (A) and 14 days (B).

and transferred onto PVDF membranes. After blocking in 5% skim milk, membranes were incubated with RUNX2 antibody (1 μg/ml, Abcam) and BMPR2 antibody (1:1,000, Abcam), and then incubated with a mouse IgG secondary antibody (1:2,000, Abcam). β-Actin was used as loading control. The immunoreactive proteins were detected using the ChemiDocXRS System (BIO-RAD).

Alkaline phosphatase (ALP) staining and its measurement were performed 7 days later. ALP staining was performed as previously described. ALP activity of monolayers was determined by p-Nitrophenyl Phosphate Liquid Substrate System (Sigma) according to the manufacturer's instructions, and the absorbance at 405 nm was measured using a Multiskan Ascent microplate reader (Thermo).

RESULTS

GROWTH CHARACTERISTICS AND PHENOTYPE OF MSCs

After 3 days of primary culture, we can see that the adherent cells were at an initial rare density, single cells or the colonies. After 5–7 days of primary culture, each of the colonies contained hundreds of cells. The cells were spindle shaped and flat (Fig. 1A). The cells replicated rapidly and reached almost 80% confluence after 14 days of culture (Fig. 1B). The cells could be expanded by successive subculture for about 10 passages. Passages up to passage 5 took 5–7 days each, while passages after passage 5 could take 12–14 days.

MSCs are difficult to identify using simple markers, and we therefore characterized several phenotypes known to be associated with human MSCs by flow cytometric analysis of expressed surface antigens. MSCs of passage 3 from all four donors (samples A, B, C, D) were uniformly positive for CD29 (the percentage of positive cells: 87.7%, 92.1%, 85.7%, 70.9%) and CD44 (the percentage of positive cells: 93.3%, 92.7%, 99.8%, 99.4%), and were negative for hematopoietic lineage markers including CD34 and CD45 [Dominici et al., 2006] (Fig. 2).

OSTEOGENIC DIFFERENTIATION

MSCs at passage 3 were cultured in osteogenic medium for 14–21 days. These cells in the osteoinductive groups stained positively for ALP (Fig. 3A,B).

miRNA EXPRESSION PROFILES IN MSCs AND OSTEO-DIFFERENTIATED MSCs

Based on previously described miRNA microarray technology for miRNA expression, we detected miRNA expression levels in undifferentiated and osteo-differentiated MSCs in triplicate cultures from each of three donors (donors A, B, C) (Fig. 4).

Forty-one miRNAs were identified in undifferentiated MSCs from all three samples (Table II). SAM statistics using two-class unpaired comparisons were used to identify differential expression of miRNAs between MSCs and osteo-differentiated MSCs in each sample. The differentially expressed miRNAs ($P < 0.05$, false discovery rate $<5\%$, fold change >1.5) between the two cell types fell into two groups: (1) miRNAs overexpressed in osteo-differentiated MSCs (36 in sample A, 14 in sample B, 6 in sample C); (2) miRNAs underexpressed in osteo-differentiated MSCs (24 in sample A, 27 in sample B, 20 in sample C) (Tables III–V). Variation in differentially expressed miRNAs among the three independent samples was observed, and we therefore identified differentially expressed miRNAs that were present in at least two samples, including eight miRNAs underexpressed in osteo-differentiated MSCs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, hsa-miR-424, hsa-miR-210, hsa-let-7i, PREDICTED_MIR191, hsa-miR-99a), and five miRNAs overexpressed in osteo-differentiated MSCs (hsa-miR-30a-5p, hsa-miR-30c, hsa-miR-130a, hsa-miR-15b, hsa-miR-130b) (Table VI).

We had reported five miRNAs (hsa-miR-130b, hsa-miR-152, hsa-miR-28, hsa-miR-26b, and hsa-miR-193b) up-regulated in the chondrogenic differentiation of MSCs from three samples (donors B, C, D) previously [Han et al., 2010]. There was no overlap between miRNAs overexpressed in osteo-differentiated MSCs and those overexpressed in chondro-differentiated MSCs, except for hsa-miR-130b.

REAL-TIME RT-PCR OF miRNAs

To confirm the identification of these differentially expressed miRNAs, we used real-time RT-PCR to detect the expression levels of the seven miRNAs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, hsa-miR-424, hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b) in the fourth individual sample (sample D). Real-time RT-PCR showed that

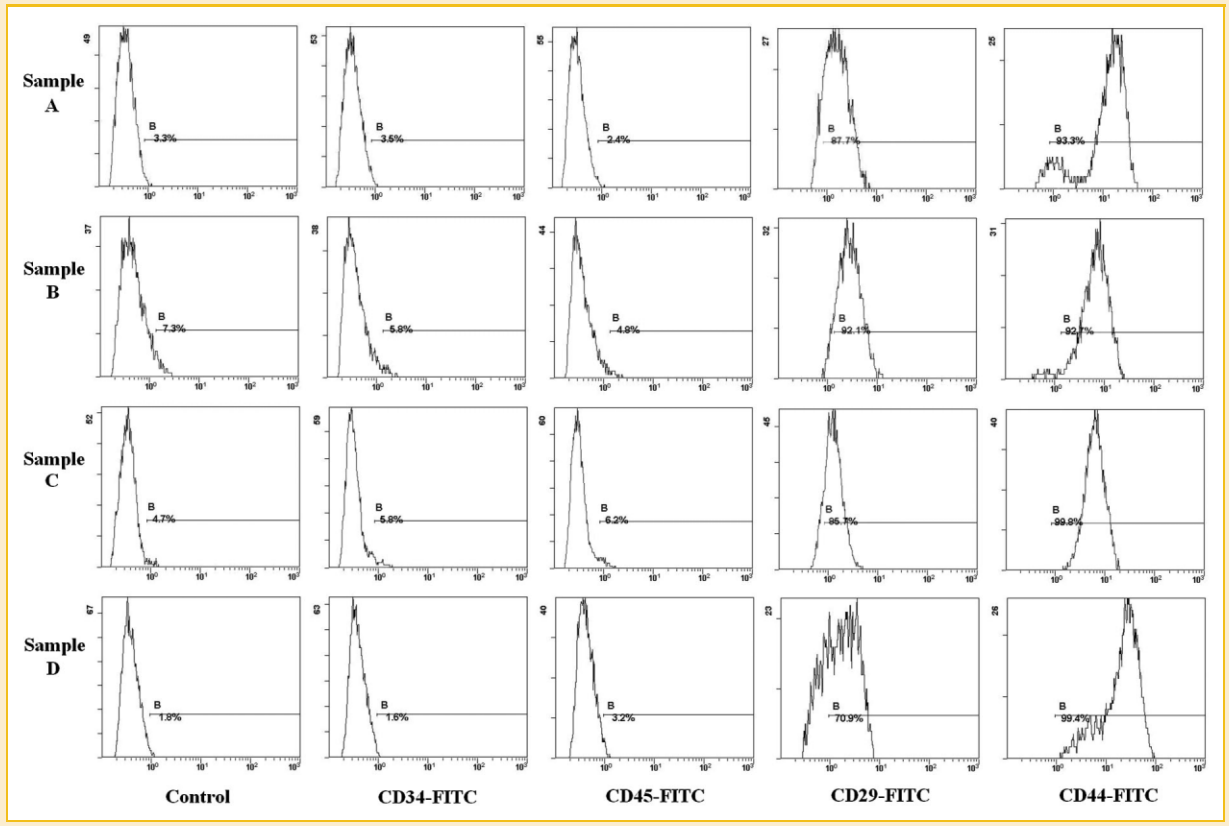

Fig. 2. Phenotype analysis of human MSCs. FITC, fluorescein isothiocyanate.

all seven selected miRNAs were differentially expressed between MSCs and osteo-differentiated MSCs in sample D, in accordance with the microarray results from samples A, B, and C (Fig. 5 and Table VII).

PUTATIVE TARGETS FOR miRNAs

Several potential targets for hsa-miR-31, hsa-miR-106a, hsa-miR-148a, hsa-miR-424, hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b were predicted using online software. These targets related to osteogenic differentiation, maintenance of stemness, adipogenic differentiation, and chondrogenic differentiation. The four miRNAs that were underexpressed in osteo-differentiated MSCs were predicted to target runt-related transcription factor (RUNX) 2, core-binding factor, beta subunit (CBFB) and bone morphogenetic proteins (BMPs), which are involved in bone formation; while putative targets for miRNAs overexpressed in osteo-differentiated MSCs were MSC maker, stemness-maintaining factor, and genes related to cell differentiation (Table VIII).

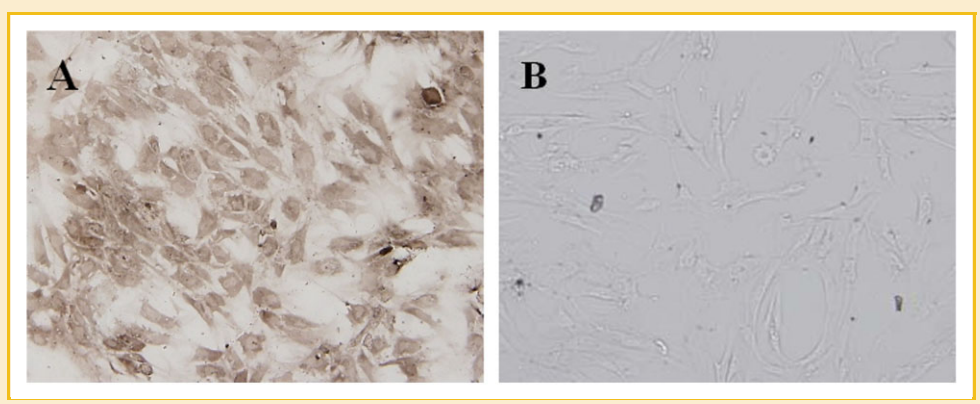

Fig. 3. Cytochemistry of undifferentiated and osteo-differentiated MSCs. Osteo-differentiated MSCs cultured in osteogenic medium for 14–21 days (100×): ALP staining (A). Undifferentiated MSCs cultured in standard growth medium (×100): ALP staining (B).

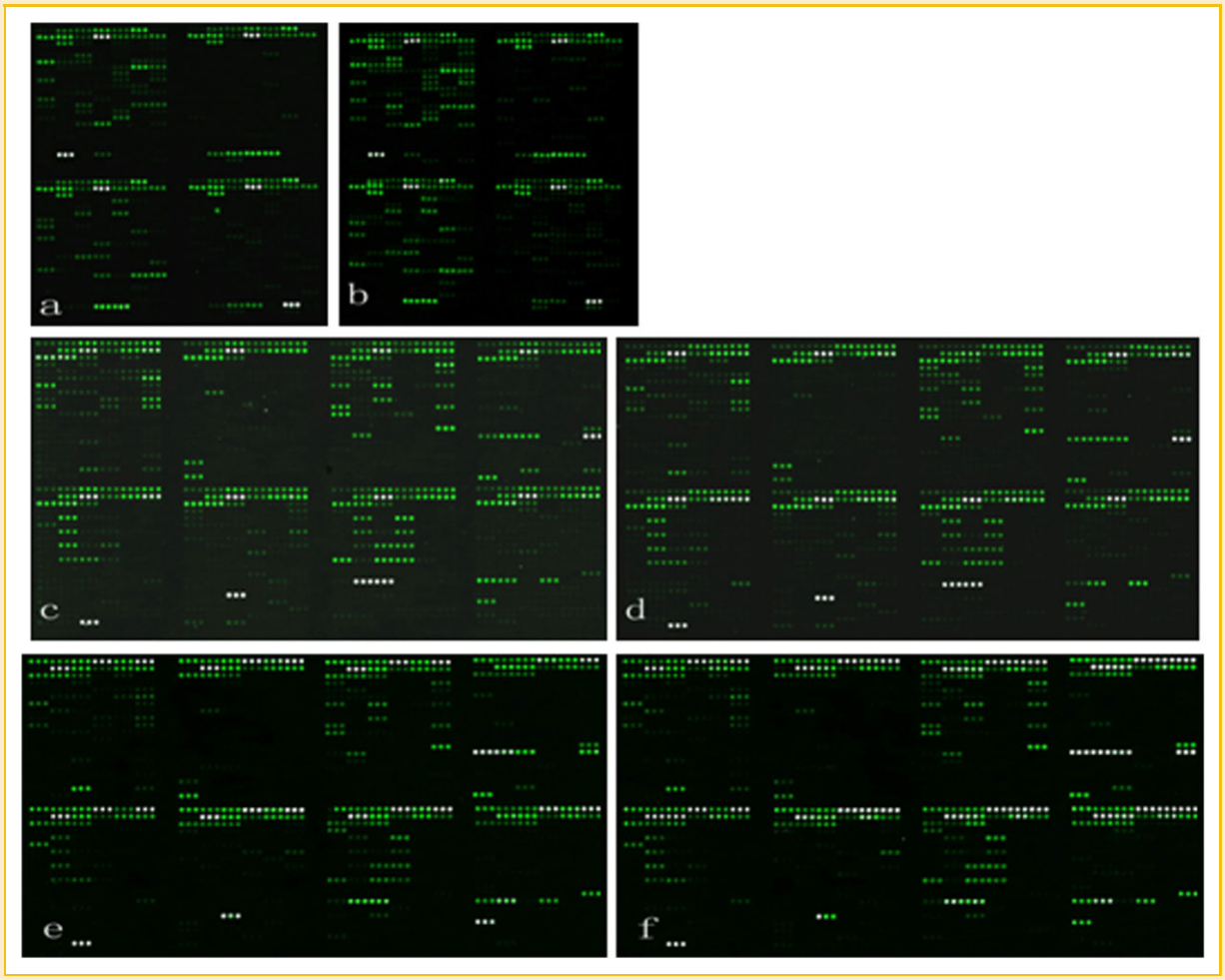

Fig. 4. Microarray analysis of undifferentiated and osteo-differentiated MSCs. miRNA expression profiles of MSCs (a) and osteo-differentiated MSCs (b) in sample A, MSCs (c), and osteodifferentiated MSCs (d) in sample B, MSCs (e), and osteo-differentiated MSCs (f) in sample C.

FUNCTION ANALYSIS AND TARGET VERIFICATION FOR has-miR-31

hsa-miR-31 that was underexpressed in osteo-differentiated MSCs was selected for target verification and function analysis. hsa-miR-31 was predicted to target RUNX2 and BMPR2, which are involved in osteogenic differentiation. To determine whether RUNX2 and BMPR2 were targets for miR-31 and identify whether miR-31 could influence osteoblastic differentiation, anti-miR-31 was transfected into MSCs.

For target verification, we used Western blot analysis to test the protein expression level of RUNX2 and BMPR2. Transfection with anti-miR-31 increased their protein expression compared with transfection with negative control (Fig. 6). These results suggested that miR-31 may target RUNX2 and BMPR2.

For function analysis, miR-31 effects were assessed by observing the ALP staining and activity. ALP expression was increased following anti-miR-31 transfection compared with transfection of negative control (Fig. 7), and ALP activity was also increased following transfection of anti-miR-31 (Fig. 8). These proved that inhibition of miR-31 activity promoted osteoblastic differentiation of MSCs.

DISCUSSION

In this study, we isolated and cultured human MSCs and osteo-differentiated MSCs from four individual donors. miRNA expression in MSCs and osteo-differentiated MSCs was investigated in three donors using miRNA microarrays. miRNAs that were commonly expressed in all three separate MSC preparations and miRNAs that were differentially expressed between MSCs and osteo-differentiated MSCs were identified. Four underexpressed (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, and hsa-miR-424) and three novel overexpressed miRNAs (hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b) in osteo-differentiated MSCs were selected and their expression levels were determined in samples from the fourth individual donors. The putative targets of the miRNAs were predicted using bioinformatic analysis. hsa-miR-31 that was underexpressed in osteo-differentiated MSCs was selected for target verification and function analysis.

Their ease of isolation and culture and their multilineage-differentiation potential make MSCs suitable and appealing cells in tissue engineering [Jiang et al., 2002]. However, the heterogeneity of

TABLE II. miRNAs Expressed in Undifferentiated MSCs From All Three Donors

miRNA	Correlation
hsa-let-7a/7b/7c/7d/7e/7f/7i	1, 2
hsa-let-7g	2
hsa-miR-15b	2
hsa-miR-16	1, 2
hsa-miR-21	1, 2
hsa-miR-22	1, 2
hsa-miR-23a/23b	1, 2
hsa-miR-24	1, 2
hsa-miR-26a	1, 2
hsa-miR-27a/27b	1, 2
hsa-miR-29a	1, 2
hsa-miR-29b	2
hsa-miR-30d	2
hsa-miR-99a	
hsa-miR-100	1, 2
hsa-miR-103	2
hsa-miR-107	
hsa-miR-125a	1
hsa-miR-125b	1, 2
hsa-miR-130a	2
hsa-miR-143	1, 2
hsa-miR-145	1
hsa-miR-181a	
hsa-miR-193a/193b	
hsa-miR-199a	1
hsa-miR-199a*	1, 2
hsa-miR-210	
hsa-miR-214	1, 2
hsa-miR-221	1
hsa-miR-222	1, 2
hsa-miR-320	1, 2
PREDICTED_MIR191	

1: miRNAs also identified by Greco and Rameshwar [2007]; 2: miRNAs also identified by Oskowitz et al. [2008].

the cells and their variability between donors make characterization of MSCs necessary. In this study, MSCs were characterized on the basis of their growth characteristics and phenotypes. Gene expression patterns, including miRNA expression analysis, have recently been used to characterize cells in more detail [Goff et al., 2008; Lakshmipathy and Hart, 2008], but the variability among MSC samples and among different passages has made the analysis of gene expression in MSCs difficult. However, Greco and Rameshwar [2007] identified 32 miRNAs concurrently present in three MSC isolates from different human donors, and Oskowitz et al. [2008] reported 58 miRNAs that were expressed in undifferentiated MSCs of three different human donors. In the current study, 41 miRNAs commonly expressed in all three separate MSC samples were identified, including 24 miRNAs also identified in the previous studies [Greco and Rameshwar, 2007; Oskowitz et al., 2008] (Table II). Interestingly, these miRNAs do not include those highly expressed in human ESCs, such as hsa-miR-200c, hsa-miR-368, hsa-miR-154, hsa-miR-371, hsa-miR-372, and hsa-miR-373 [Suh et al., 2004; Lakshmipathy et al., 2007], and the pattern of miRNA expression in MSCs differs from that in human ESCs [Liu et al., 2009].

Several previous studies have indicated that miRNAs play a key regulatory role in MSC osteogenic differentiation. miR-125b was found to regulate cell proliferation and inhibit osteoblastic differentiation in mouse MSCs [Mizuno et al., 2008]. In another study, microarrays and real-time RT-PCR identified 27 unique miRNAs during MSC differentiation into adipocytes, osteocytes, or chondrocytes in pooled samples of MSCs from four different donors. With the exceptions of miR-143 and miR-145 enrichment in adipocytes, and miR-638 and miR-663 expression exclusively in chondrocytes, several of the regulated miRNAs (hsa-miR-30a-5p, hsa-miR-125b, hsa-miR-21, hsa-miR-24, and hsa-let-7, etc.) were associated with changes during osteocyte differentiation [Goff et al., 2008]. In a third example, expression analysis of miRNAs during hMSC differentiation identified 19 miRNAs that were upregulated during osteogenic differentiation, including hsa-miR-130a, hsa-miR-199a, hsa-miR-346, hsa-miR-21, and hsa-miR-10a [Oskowitz et al., 2008]. The lack of specificity of the miRNAs expressed during MSC differentiation found in these reports was due to the variability among MSC from different individual donors. Accordingly, it is difficult to determine the specific expression patterns of MSCs and their differentiated cells based on information from a few donors. However, biological reproducibility could be used to determine the effects of variations between individual donors, and thus make generalizations easier. We therefore explored the expression of miRNAs in MSCs from four different donors during osteogenesis, and identified miRNAs that were differentially expressed between MSCs and osteo-differentiated MSCs.

Goff et al. [2008] and Oskowitz et al. [2008] reported a set of miRNAs that were up-regulated during osteogenic differentiation, including hsa-miR-30a-5p and hsa-miR-130a. However, few miRNAs downregulated during osteogenic differentiation were mentioned in their studies. We therefore selected four under-expressed miRNAs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a, and hsa-miR-424) and three novel overexpressed miRNAs (hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b) in osteo-differentiated MSCs for study.

hsa-miR-130b, which is overexpressed in osteo-differentiated MSCs, has previously been reported to be upregulated in chondro-differentiated MSCs [Han et al., 2010]. These results demonstrate that hsa-miR-130b plays a part in both osteogenic and chondrogenic differentiation of MSCs, and is not specific to either. However, hsa-miR-130b has been confirmed to play an important role in MSC differentiation.

Increasing evidence supports the importance of miRNA regulation in osteogenic differentiation of MSCs, but the regulatory mechanism has so far been poorly defined. Prediction and identification of the miRNA-targeting genes offers an experimental basis for further research on miRNA regulatory mechanisms. Bioinformatic methods based on sequence similarities between targets and miRNAs were used to predict the potential target genes. By using transfection with anti-miRNA or pre-miRNA for those differentially expressed miRNAs, target verification could be performed by testing the protein expression level of the miRNA-targeting genes. We predicted the putative target genes for the candidate miRNAs using TargetScan 5.1 and PicTar, and obtained several potential targets related to processes including osteogenesis, osteogenic differentiation, maintenance of stemness, and cell differentiation. Further, We selected hsa-miR-31 for target verification, and proved that RUNX2 and BMPR2 were targets for miR-31.

TABLE III. Differentially Expressed miRNAs Between MSCs and Osteo-Differentiated MSCs in Sample A

Gene name	Numerator (r)	Denominator (s + s0)	Score (d)	Fold change
36 miRNAs overexpressed in osteo-differentiated MSCs				
PREDICTED_MIR202	1.8439	0.058897	31.307056	3.588255
hsa-miR-30a-5p	1.9967	0.096035	20.791405	3.985898
hsa-miR-495	2.546367	0.128994	19.740266	5.825729
hsa-miR-148a	1.518433	0.082318	18.446006	2.863486
hsa-miR-130a	0.999033	0.056586	17.655036	1.998906
hsa-miR-127	1.4714	0.084084	17.499192	2.769439
hsa-miR-494	1.088067	0.065103	16.7129415	2.126166
hsa-miR-34a	1.680167	0.10245	16.399921	3.196768
hsa-miR-376a	2.127067	0.136083	15.630643	4.34608
hsa-miR-30d	1.395867	0.095792	14.571831	2.63108
hsa-miR-376b	3.591633	0.250184	14.355971	11.79295
hsa-miR-106b	1.7057	0.119887	14.227601	3.255669
hsa-miR-191	2.096867	0.160193	13.089617	4.261462
rno-miR-93	2.066367	0.162553	12.711944	4.184739
hsa-miR-19b	1.072233	0.084948	12.622282	2.105844
hsa-miR-30c	0.951167	0.078883	12.057934	1.934914
hsa-miR-493	2.473633	0.223485	11.06843	5.507979
hsa-miR-148b	1.194567	0.108643	10.995356	2.289221
hsa-miR-99b	1.2083	0.1194	10.119758	2.302833
hsa-miR-143	0.880067	0.087919	10.009920	1.842793
hsa-miR-17-5p	0.8406	0.090972	9.240231	1.78888
hsa-miR-152	1.738067	0.207754	8.365974	3.291356
hsa-miR-28	1.067733	0.130088	8.207777	2.091643
hsa-miR-130b	1.302933	0.163601	7.964078	2.465882
rno-miR-151*	0.906567	0.117632	7.7068208	1.875663
hsa-miR-368	1.814567	0.23563	7.7009028	3.449372
hsa-miR-15b	0.674	0.0884	7.6244597	1.594709
hsa-miR-106a	0.802967	0.115531	6.9502501	1.742169
hsa-miR-155	0.705733	0.135182	5.2206121	1.627032
hsa-miR-185	0.788267	0.155298	5.0758455	1.72043
hsa-miR-10a	0.667933	0.134168	4.9783511	1.594162
hsa-miR-195	0.826133	0.166757	4.9541230	1.759363
hsa-miR-361	0.873233	0.214784	4.065628	1.816948
rno-miR-140*	0.622667	0.154662	4.0259959	1.529143
hsa-miR-20a	0.756533	0.189508	3.9920837	1.698013
hsa-miR-25	0.7575	0.195688	3.8709516	1.683977
24 miRNAs underexpressed in osteo-differentiated MSCs				
rno-miR-31	−1.7899	0.050577	−35.389527	0.289199
hsa-miR-29a	−2.03327	0.064169	−31.686228	0.24422
PREDICTED_MIR191	−2.92603	0.103098	−28.381191	0.131639
hsa-miR-122a	−2.58623	0.103628	−24.956989	0.166665
hsa-miR-23a	−1.02237	0.059972	−17.047302	0.492098
hsa-miR-181a	−0.9549	0.059537	−16.038883	0.515828
hsa-miR-199a*	−1.0003	0.063467	−15.760877	0.499934
hsa-miR-221	−1.20553	0.078577	−15.34197	0.433616
hsa-miR-31	−1.08847	0.073044	−14.901551	0.469938
hsa-miR-125b	−1.20907	0.098309	−12.298575	0.432156
hsa-miR-24	−0.62347	0.054218	−11.499355	0.649027
hsa-miR-21	−1.28233	0.113949	−11.253599	0.410607
hsa-miR-222	−0.85673	0.079149	−10.824318	0.55166
hsa-miR-29b	−0.7137	0.065969	−10.818734	0.609897
hsa-let-7i	−0.70867	0.066973	−10.581299	0.61161
hsa-miR-210	−0.8851	0.108913	−8.1266956	0.539893
hsa-let-7c	−0.60673	0.076984	−7.881319	0.656505
hsa-miR-100	−0.6334	0.081814	−7.7419280	0.64456
hsa-miR-99a	−0.71187	0.094837	−7.506205	0.611831
hsa-let-7a	−1.08217	0.161709	−6.6920556	0.47395
hsa-miR-16	−1.07037	0.161739	−6.6178802	0.473146
hsa-let-7d	−0.58797	0.090552	−6.4931591	0.664203
hsa-let-7f	−0.817	0.167998	−4.863162	0.572518
hsa-let-7b	−0.60787	0.140372	−4.330396	0.655835

SAM score (d): T-statistic value; numerator: numerator of the T-statistic; denominator (s + s0): denominator of the T-statistic.

The putative target genes of miRNAs underexpressed in osteo-differentiated MSCs include several genes associated with bone formation such as RUNX2, CBFB, and BMPs. The protein encoded by CBFB is the beta subunit of a heterodimeric core-binding transcription factor belonging to the PEBP2/CBF transcription factor family, which master-regulates a host of genes specific to hematopoiesis (e.g., RUNX1) and osteogenesis (e.g., RUNX2). CBFB has also been reported to enhance the osteogenic differentiation of both human and mouse MSCs [Lien et al., 2007]. RUNX2 is essential for osteoblastic differentiation and skeletal morphogenesis, and studies have demonstrated that osteoblastic differentiation is associated primarily with increases in Runx2/Cbfa1 activity in bone marrow stromal cells [Shui et al., 2003; Lien et al., 2007]. BMPs are a family of secreted signaling molecules that can induce ectopic bone growth. BMPs were originally identified through the ability of demineralized bone extract to induce endochondral osteogenesis in

TABLE IV. Differentially Expressed miRNAs Between MSCs and Osteo-Differentiated MSCs in Sample B

Gene name	Numerator (r)	Denominator (s + s0)	Score (d)	Fold change
14 miRNAs overexpressed in osteo-differentiated MSCs				
PREDICTED_MIR189	1.372233	0.082661	16.600799	2.586105
hsa-miR-363*	2.247767	0.181794	12.364333	4.761873
hsa-miR-483	1.7644	0.14945	11.805949	3.378221
hsa-miR-15b	1.4366	0.125842	11.415918	2.704364
hsa-miR-590	1.576767	0.150827	10.454116	2.972025
hsa-miR-663	1.008633	0.099926	10.093753	2.012599
hsa-miR-30a-5p	0.910567	0.116467	7.8182366	1.87568
hsa-miR-30c	1.1568	0.160921	7.1886311	2.218373
hsa-miR-130a	1.546267	0.215541	7.1738938	2.882573
hsa-miR-484	0.783967	0.11564	6.7793752	1.71902
mmu-miR-455-3p	1.009067	0.160089	6.3031518	1.998705
hsa-miR-107	0.841867	0.143627	5.861482	1.78453
mmu-miR-199b	0.5977	0.1063	5.6227758	1.512943
hsa-miR-487b	0.793533	0.152635	5.1989023	1.722213
27 miRNAs underexpressed in osteo-differentiated MSCs				
hsa-miR-210	−2.25007	0.087162	−25.8147	0.209982
mmu-miR-685	−2.31887	0.10388	−22.3226	0.200409
hsa-miR-192	−3.0098	0.170064	−17.698	0.123708
hsa-miR-148a	−0.93047	0.06845	−13.5934	0.524808
hsa-miR-494	−1.0377	0.084089	−12.3405	0.48687
hsa-miR-224	−1.14803	0.112838	−10.1741	0.450111
hsa-miR-128a	−1.37323	0.136091	−10.0906	0.384253
hsa-miR-106a	−1.098	0.115415	−9.51352	0.466242
hsa-miR-138	−0.81877	0.087186	−9.39101	0.566958
hsa-miR-199a	−0.623	0.066624	−9.35095	0.649502
hsa-miR-101	−0.90997	0.101191	−8.99253	0.532051
hsa-miR-424	−0.96423	0.11523	−8.36788	0.511315
mmu-miR-709	−0.85267	0.103315	−8.25307	0.553539
hsa-let-7i	−0.6877	0.083711	−8.21522	0.621137
hsa-miR-31	−0.86643	0.105789	−8.19022	0.548058
mmu-miR-703	−1.31253	0.160877	−8.15861	0.400735
hsa-miR-27a	−0.64177	0.082747	−7.75573	0.641061
PREDICTED_MIR165	−0.7236	0.101913	−7.1002	0.605132
hsa-miR-155	−0.6205	0.091845	−6.75597	0.650177
hsa-miR-181b	−0.60207	0.092878	−6.48232	0.658895
hsa-miR-23b	−0.59407	0.093497	−6.35383	0.662134
hsa-miR-28	−0.517	0.083405	−6.19865	0.698214
hsa-miR-382	−0.86823	0.141025	−6.15659	0.544922
hsa-miR-143	−0.63487	0.111609	−5.68832	0.64332
hsa-miR-21	−0.63183	0.117474	−5.378508	0.644474
rno-miR-31	−0.61567	0.115583	−5.326623	0.653939
mmu-miR-714	−0.84387	0.16487	−5.118373	0.555933

SAM score (d): T-statistic value; Numerator: numerator of the T-statistic; Denominator (s + s0): denominator of the T-statistic.

TABLE V. Differentially Expressed miRNAs Between MSCs and Osteo-Differentiated MSCs in Sample C

Gene name	Numerator (r)	Denominator (s + s0)	Score (d)	Fold change
6 miRNAs overexpressed in osteo-differentiated MSCs				
hsa-miR-29b	1.8688	0.163043	11.46202	3.668929
hsa-miR-130b	1.896067	0.219211	8.649491	3.722204
hsa-miR-221	1.044733	0.136409	7.658835	2.068232
hsa-miR-193a	0.900467	0.137011	6.572213	1.865034
hsa-miR-29a	0.642833	0.10107	6.360307	1.562322
hsa-miR-193b	0.683567	0.203642	3.356713	1.614117
20 miRNAs underexpressed in osteo-differentiated MSCs				
hsa-miR-424	−2.73453	0.174103	−15.7065	0.150957
hsa-miR-34a	−2.54993	0.174725	−14.594	0.171482
hsa-miR-593	−1.5538	0.113974	−13.633	0.340546
mmu-miR-709	−1.387	0.165108	−8.40058	0.382051
mmu-miR-665	−1.00997	0.121614	−8.3047	0.495875
hsa-miR-145	−0.97917	0.136192	−7.18961	0.507437
PREDICTED_MIR189	−0.96537	0.148362	−6.50684	0.511481
hsa-miR-10a	−1.48193	0.228809	−6.47674	0.357661
hsa-miR-148a	−1.03737	0.174428	−5.94724	0.489425
hsa-miR-214	−0.8374	0.176697	−4.73919	0.56265
hsa-miR-491	−0.8719	0.189808	−4.59359	0.547233
PREDICTED_MIR191	−0.78457	0.185551	−4.22832	0.580167
hsa-miR-99a	−0.77257	0.183303	−4.21469	0.587412
hsa-miR-602	−1.56907	0.387043	−4.05399	0.348966
hsa-miR-107	−0.85437	0.221853	−3.85105	0.557477
hsa-miR-103	−0.61617	0.169595	−3.63316	0.655837
hsa-miR-106a	−0.6976	0.193727	−3.60094	0.618178
hsa-miR-557	−0.70073	0.201221	−3.48241	0.610952
hsa-miR-638	−0.70583	0.204377	−3.45359	0.611021
hsa-miR-663	−0.6652	0.199415	−3.33576	0.629543

SAM score (d): T-statistic value; Numerator: numerator of the T-statistic; Denominator (s + s0): denominator of the T-statistic.

TABLE VI. Candidate miRNAs in Samples A, B, and C

miRNA	Sample A fold change	Sample B fold change	Sample C fold change
miRNAs overexpressed in osteo-differentiated MSCs			
hsa-miR-30a-5p	3.985897825	1.875679919	(1.474392956)
hsa-miR-30c	1.934913659	2.218372719	/
hsa-miR-130a	1.998906422	2.882572679	(0.879006585)
hsa-miR-15b	1.594708819	2.704363693	/
hsa-miR-130b	2.465881626	(0.823512829)	3.722204255
miRNAs underexpressed in osteo-differentiated MSCs			
hsa-miR-210	0.539892697	0.209982494	/
hsa-miR-31	0.46993816	0.548058316	/
hsa-let-7i	0.611609564	0.621136704	(0.778777903)
PRE_MIR191	0.131638599	(0.735870862)	0.580166834
hsa-miR-99a	0.611831072	(0.818920213)	0.587412106
hsa-miR-424	/	0.511314802	0.15095666
hsa-miR-148a	(2.86348562)	0.524808168	0.489425203
hsa-miR-106a	(1.742169354)	0.466242261	0.618177597

vivo in an extraskeletal site. Many BMPs, including BMP2, BMP3, and BMP8, are part of the transforming growth factor-beta superfamily, which induce bone and cartilage formation [Wozney, 1998; Tsumaki and Yoshikawa, 2005]. BMP receptors (BMPRs), including BMPR1A, BMPR1B, and BMPR2, are also involved in endochondral bone formation and embryogenesis. miRNAs underexpressed in osteo-differentiated MSCs and associated with these three osteogenesis-related genes, such as hsa-miR-31, hsa-miR-106a, hsa-miR-148a, and hsa-miR-424, can thus be predicted to play important roles in inhibiting the osteogenic differentiation of MSCs.

miRNAs overexpressed in osteo-differentiated MSCs (hsa-miR-130b, hsa-miR-30c, and hsa-miR-15b) were predicted to target genes including MSC maker and stemness-maintaining factor. CD44, integrin β1 (also known as CD29), fms-related tyrosine kinase 1, platelet-derived growth factor (PDGF) receptor, α polypeptide (also known as CD140A), and PDGFA-associated protein 1 [Pittenger et al., 1999; Minguell et al., 2001; Gottschling et al., 2007; Chen et al., 2008; Tokunaga et al., 2008; Wagner et al., 2008], which have either emerged as positive markers of MSCs or have been reportedly expressed in MSCs, were predicted to be possible targets for hsa-miR-130b, hsa-miR-30c and hsa-miR-15b. Chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor 1, SDF1), is a small cytokine that belongs to the intercrine family. SDF1 promotes the growth, survival, and development of human bone marrow stromal stem cells [Kortesidis et al., 2005]. According to the results of the current study, CXCL12 was predicted to be a possible target for hsa-miR-30c. Fibroblast growth factor 2, which is known to enhance the self-renewal capacity of MSCs [Bianchi et al., 2003], was predicted to be a possible target for hsa-miR-15b. Based on these results, we can infer that these three miRNAs may be responsible for maintaining the stemness of MSCs.

In addition, BMP-binding endothelial regulator (BMPER) and growth differentiation factor 6 (GDF6, also known as BMP13), were predicted to be targets of hsa-miR-15b and hsa-miR-130b. BMPER is an inhibitor of BMP function [Binnerts et al., 2004], and GDF6 inhibits osteogenic differentiation of MSCs [Shen et al., 2009]. Calmodulin-binding transcription activator 1, which was predicted to be a possible target of hsa-miR-30c and hsa-miR-130b, is associated with cell differentiation and cell cycle regulation [Nakatani et al., 2004]. Collagens type IV, α1 and type IX, α3, which are associated with cartilage formation [Solovieva et al.,

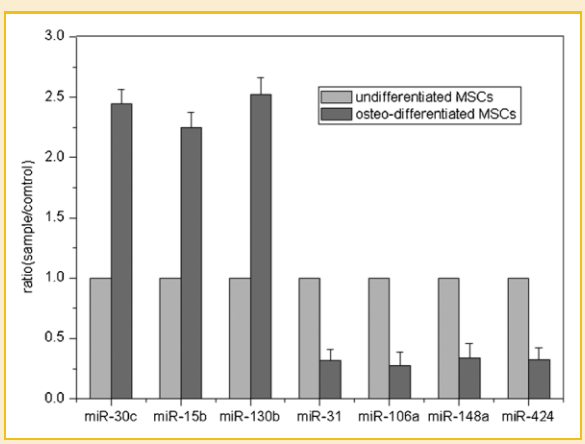

Fig. 5. Histogram of real-time RT-PCR results. hsa-miR-31, hsa-miR-106a, hsa-miR-148a, hsa-miR-424, hsa-miR-30c, hsa-miR-15b, and hsa-miR-130b are listed on the *x*-axis. The *y*-axis refers to the relative expression levels between MSCs (control) and osteo-differentiated MSCs (sample). Control expression is 1. For each miRNA, experiment was performed three times, and significant *t*-test result shows $P < 0.05$.

TABLE VII. miRNAs in MSCs and Osteo-Differentiated MSCs Verified by Real-Time RT-PCR

Name	E	ΔCP	Ratio before normalization	Ratio after normalization
U6	1.841	0.22	1.143699243	
miR-30c	1.865	1.65	2.796533844	2.445165425
miR-15b	1.846	1.54	2.57037692	2.247423819
miR-130b	1.85	1.72	2.880947691	2.518973156
U6	1.841	0.56	1.407440751	
miR-31	1.850	−1.30	0.449445207	0.319335081
miR-106a	1.865	−1.51	0.390188942	0.277232943
miR-148a	1.86	−1.19	0.477836803	0.33950758
miR-424	1.856	−1.28	0.453127266	0.321951219

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

TABLE VIII. Putative Targets of miRNAs

miRNA	Representative possible targets	Software	Function
hsa-miR-31	RUNX2 (runt-related transcription factor 2)	TargetScan	Osteogenic differentiation
	BMPR2 (bone morphogenetic protein receptor, type II)	TargetScan	Osteogenesis
hsa-miR-106a	RUNX2 (runt-related transcription factor 2)	TargetScan	Osteogenic differentiation
	CBFB (core-binding factor, beta subunit)	TargetScan	Osteogenic differentiation
	BMP2 (bone morphogenetic protein 2)	TargetScan	Osteogenesis
	BMP3 (bone morphogenetic protein 3)	TargetScan	Osteogenesis
	BMP8B (bone morphogenetic protein 8b)	TargetScan	Osteogenesis
	BMPR1A (bone morphogenetic protein receptor, type IA)	TargetScan	Osteogenesis
	BMPR1B (bone morphogenetic protein receptor, type IB)	TargetScan	Osteogenesis
	BMPR2 (bone morphogenetic protein receptor, type II)	TargetScan	Osteogenesis
hsa-miR-148a	RUNX2 (runt-related transcription factor 2)	TargetScan	Osteogenic differentiation
	BMP3 (bone morphogenetic protein 3)	TargetScan	Osteogenesis
	BMP8A (bone morphogenetic protein 8b)	TargetScan	Osteogenesis
	BMP8B (bone morphogenetic protein 8b)	TargetScan	Osteogenesis
	BMPR1B (bone morphogenetic protein receptor, type IB)	TargetScan	Osteogenesis
	BMPR2 (bone morphogenetic protein receptor, type II)	TargetScan	Osteogenesis
hsa-miR-424	CBFB (core-binding factor, beta subunit)	TargetScan	Osteogenic differentiation
	BMP8A (bone morphogenetic protein 8b)	TargetScan	Osteogenesis
	BMPR1A (bone morphogenetic protein receptor, type IA)	TargetScan	Osteogenesis
	BMPR2 (bone morphogenetic protein receptor, type II)	TargetScan	Osteogenesis
miR-15b	PDAP1 (PDGFA associated protein 1)	TargetScan	MSC phenotype
	FGF2 (fibroblast growth factor 2)	PicTar	Stemness maintaining
	BMPER (BMP binding endothelial regulator)	TargetScan	BMP inhibitor
	COL4A1 (collagen, type IV, alpha 1)	TargetScan	Cartilage formation
miR-30c	ITGB1 (integrin, beta 1), also known as CD29	TargetScan	MSC marker
	CXCL12 (chemokine of the CXC subfamily, ligand 12)	TargetScan	Stemness maintaining
	FLT1 (fms-related tyrosine kinase 1)	TargetScan	MSC phenotype
	CAMTA1 (calmodulin binding transcription activator 1)	TargetScan PicTar	Cell differentiation
	COL9A3 (collagen, type IX, alpha 3)	TargetScan	Cartilage formation
miR-130b	CD44 (CD44 molecule)	TargetScan	MSC marker
	PDGFRA (CD140A) (platelet-derived growth factor receptor, alpha polypeptide)	TargetScan PicTar	MSC phenotype
	GDF6 (growth differentiation factor 6)	PicTar	Inhibit osteogenesis
	CAMTA1 (calmodulin binding transcription activator 1)	TargetScan PicTar	Cell differentiation

2006; Hopwood et al., 2007], were predicted to be possible targets for hsa-miR-15b and hsa-miR-30c. Based on this bioinformatic analysis, it is possible to infer that miRNAs overexpressed in osteo-differentiated MSCs (hsa-miR-30c and hsa-miR-15b), which were predicted to target BMP inhibitor genes related to cartilage formation, might be involved in promoting osteogenic and inhibiting chondrogenic differentiation of MSCs. hsa-miR-130b, which was predicted to target genes related to the inhibition of cell differentiation, could also promote MSC differentiation.

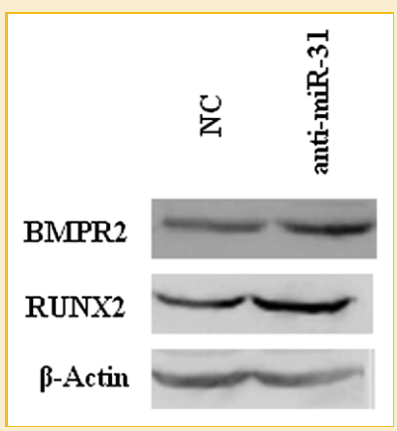

Fig. 6. The protein expression level of RUNX2 and BMPR2. Anti-miR-31 and negative control (NC) were transfected into MSCs and fresh osteogenic differentiation medium were added at 4 h later. Western blot analysis for RUNX2 and BMPR2 were performed after 72 h.

Functional studies of miRNA can provide direct evidence to prove that miRNA could modulate osteogenic differentiation of MSCs. hsa-miR-31 that was underexpressed in osteo-differentiated MSCs was selected for function analysis. By transfecting anti-miR-31 into MSCs, miR-31 effects were assessed by observing the ALP staining and activity. The results showed that inhibition of miR-31 activity promoted osteoblastic differentiation of MSCs.

In this study, we investigated miRNA expression in MSCs and osteo-differentiated MSCs. Forty-one miRNAs were commonly expressed in all three separate MSC preparations, and miRNAs differentially expressed between human MSCs and osteo-differentiated MSCs included four that were underexpressed and three that were overexpressed in osteo-differentiated MSCs. The target genes for these miRNAs were predicted by bioinformatic analysis. hsa-miR-31 that was underexpressed in osteo-differentiated MSCs was selected for target verification and function analysis. Further studies in target verification and function analysis for these miRNAs are needed to provide more conclusive evidence to explain the miRNA regulatory mechanisms. The results of this study provide an experimental basis for further research on miRNA functions during osteogenic differentiation of human MSCs.

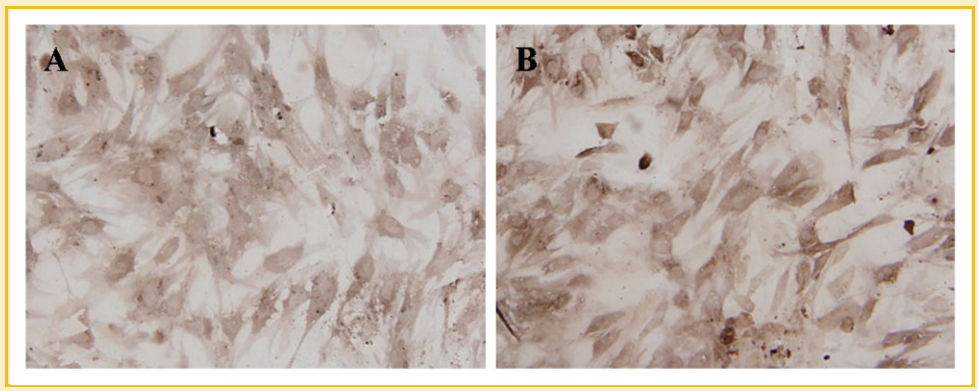

Fig. 7. ALP staining in anti-miR-31-transfected MSCs. ALP staining in NC-transfected MSCs (A) and anti-miR-31-transfected MSCs (B), in which osteoblastic differentiation was induced by osteogenic differentiation medium for 7 days.

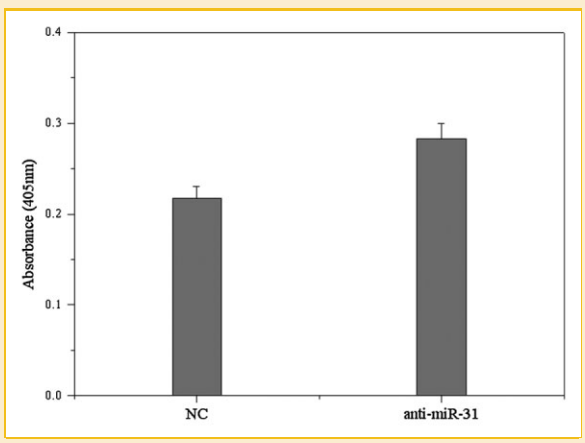

Fig. 8. ALP activity in anti-miR-31-transfected MSCs. Measurement of ALP activity in NC-transfected MSCs and anti-miR-31-transfected MSCs, in which osteoblastic differentiation was induced by osteogenic differentiation medium for 7 days. Each experiment was performed in triplicate wells, and significant *t*-test results show $P < 0.05$.

ACKNOWLEDGMENTS

We thankful to CapitalBio Corporation (Beijing, China) for providing miRNA microarrays and corresponding data analysis.

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