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miRNA Expression Profile During Osteogenic Differentiation of Human Adipose-Derived Stem Cells

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ABSTRACT

Human adipose-derived stem cells (hADSC) are capable of differentiating into an osteogenic lineage. It is believed that microRNAs (miRNAs) play important roles in regulating this osteogenic differentiation of human adipose-derived cells, although its molecular mechanism remains unclear. We investigated the miRNA expression profile during osteogenic differentiation of hADSCs, and assessed the roles of involved miRNAs during the osteogenic differentiation. We obtained and cultured human adipose-derived stems cells from donors who underwent elective liposuction or other abdominal surgery at our institution. miRNA expression profiles pre- and post-osteogenic induction were obtained using microarray essay, and differently expressed miRNAs were verified using quantitative real-time polymerase chain reaction (qRT-PCR). The expression of osteogenic proteins was detected using an enzyme-linked immunosorbent assay. Putative targets of the miRNAs were predicted using online software MiRanda, TargetScan, and miRBase. Eight miRNAs were found differently expressed pre- and post-osteogenic induction, among which four miRNAs (miR-17, miR-20a, miR-20b, and miR-106a) were up-regulated and four miRNAs (miR-31, miR-125a-5p, miR-125b, and miR-193a) were down-regulated. qRT-PCR analysis further confirmed the results. Predicted target genes of the differentially expressed miRNAs based on the overlap from three public prediction algorithms: MiRanda, TargetScan, and miRBase Target have the known functions of regulating stem cell osteogenic differentiation, self-renewal, signal transduction, and cell cycle control. We identified a group of miRNAs that may play important roles in regulating hADSC cell differentiation toward an osteoblast lineage. Further study of these miRNAs may elucidate the mechanism of hADSC differentiation into adipose tissue, and thus provide basis for tissue engineering. J. Cell. Biochem. 113: 888–898, 2012.

KEY WORDS: MIRNAS PROFILE; ADIPOSE-DERIVED STEM CELLS; OSTEOGENIC DIFFERENTIATION

In 2001, human adipose-derived stem cells (hADSCs) were first isolated from human adipose tissue by Zuk et al. [2001]. hADSCs are capable of self-renewal and hold the potential of multi-directional differentation. In the presence of lineage-specific induction factors, hADSCs can differentiate into osteogenic, adipogenic, chondrogenic, and myogenic cells. In 2006, Guilak et al. [2006] tested the ability of hADSCs to differentiate into multiple phenotypes of adipogenesis, osteogenesis, chondrogenesis, and neurogenesis in vitro. They confirmed the hypothesis that hADSCs are a type of multipotent adult stem cell and not solely a mixed population of unipotent progenitor cells. Because adipose tissue is abundant, easy to access and culture, and is biologically stable, hADSCs show great potential for tissue engineering and cell-based therapies.

First discovered from *Caenorhabditis elegans* [Lee et al., 1993; Wightman et al., 1993], microRNAs (miRNAs) are a group of regulatory, noncoding, single-stranded, small RNAs (siRNAs) 20–25 nucleotides (nt) in length, which have been identified in various plants, animals, and viruses [Griffiths-Jones et al., 2006; Carthew and Sontheimer, 2009]. miRNAs are generated from endogenous

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transcripts producing hairpin structures by an RNase III-type enzyme. miRNA functions as a regulator in gene silencing by binding to imperfectly complementing sequences in the 3′-untranslated region (UTR) of target mRNAs, leading to translational repression. By silencing various target mRNAs, miRNAs play key roles in various regulatory pathways [Luzi et al., 2008]. miRNAs are involved in regulating a wide range of physiological and pathological processes, including embryonic development [Lü et al., 2005], cell proliferation and apoptosis [Brennecke et al., 2003], lipid metabolism [Xu et al., 2003], and cell differentiation [Dostie et al., 2003; Chen et al., 2004]. Also, miRNAs are involved in regulating some signal transduction pathways such as the Notch signaling pathway [Lai et al., 2005] and the VEGF signaling pathway [Li and Carthew, 2005].

Over the past several years, growing evidence has indicated that several miRNAs are involved in regulating osteogenic differentiation in human hADSCs and human mesenchymal stem cells (hMSCs). Kim et al. [2009] reported that miR-196a was up-regulated during osteogenic differentiation, and its overexpression promoted the osteogenic differentiation and inhibited the proliferation of hADSCs cells. In addition, Luzi et al. [2008] reported that miR-26a regulated the osteogenic differentiation of hADSCs via the bone morphogenetic protein (BMP) signal regulatory protein Smad1 and expression of miR-206 decreased during osteoblast differentiation. MiR-206 overexpression in osteoblasts inhibited osteoblasts differentiation. miR-206 regulated osteoblast differentiation by regulating the expression of its target gene connexin43 [Inose et al., 2009]. Increased expression of miR-2861 down-regulated histone deacetylase 5 (HDAC5) and thus inhibited Runx2 degradation and promoted osteogenic differentiation [Li et al., 2009a, b]. miR-148b, -27a, and -489 were essential for regulating osteogenesis in human hMSCs cells [Schoolmeesters et al., 2009]: miR-27a and miR-489 down-regulate differentiation, while miR-148b up-regulates differentiation. Modulation of these miRNAs induced osteogenesis in the absence of other external differentiation cues and restored osteogenic potential in human mesenchymal cells. miR-204 and its homolog miR-211 were expressed during adipocyte differentiation in mesenchymal progenitor cell lines and bone marrow-derived mesenchymal stem cells (BMSCs), whereas Runx2 protein expression was suppressed. miR-204/211 can act as an important endogenous negative regulator of Runx2 [Huang et al., 2010], which inhibits osteogenesis and promotes adipogenesis of mesenchymal progenitor cells and BMSCs. miR-378 up-regulated nephronectin expression, and enhanced nephronectin glycosylation [Kahai et al., 2009]. Interaction of miR-378 with nephronectin 3′-UTR arrested miR-378 and freed miR-378 target GalNT7. As a consequence, nephronectin glycosylation was enhanced and osteoblast differentiation was promoted. Increased expression of miR-24, miR-125b, and miR-138 inhibited non-osteogenic mRNA expression, thereby promoting osteogenesis [Goff et al., 2008]. miR-199a mediated osteogenesis by regulating HIF1α function, suggesting hypoxic signal is an important regulator of MSC differentiation [Suomi et al., 2008]. miR-29b can down-regulate the expression of known inhibitor of osteogenic differentiation, such as HDAC4, TGF-β3, ACVR2A, CTNNBIP1, and DUSP2, and regulate the expression of type-I collagen, thereby promoting bone formation [Li et al., 2009a, b]. miR-141 and miR-200a significantly regulate BMP-2-induced differentiation of osteoblast progenitor cells by inhibiting the translation of Dlx5, thereby regulating the expression of Osterix-related mRNA [Itoh et al., 2009].

Although such reports have pointed out the importance of miRNAs in regulating osteogenic differentiation in hADSCs and hMSCs, currently studies on the regulatory roles of miRNA during osteogenic differentiation have focused largely on hMSCs derived from bone marrow, and only a few studies have been carried out in hADSCs. We therefore initiated this study. In this report, we used miRNA microarray to screen the miRNAs involving in the osteogenic differentiation and putative target of the interesting miRNAs were also predicted.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF hADSCs

Our experiments were conducted according to institutional guidelines from the ethical committee at Sun Yat-Sen University, Guangzhou, China. The adipose tissue was obtained from donors who underwent elective liposuction or other abdominal surgery with written consent and approval forms. Donors' age ranged from 19–45 years. Donors with malignant tumor and metabolic diseases were excluded from this study. Four samples of adipose tissues from different donors (two males and two females) were included in the study.

A total of 5–10 g of adipose tissue was cut from donors, and the residual fibrous tissue and blood vessels were carefully removed under sterile conditions. Raw adipose tissues were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed tissues were treated with 1:1 (w/w) 0.15% type I collagenase (Sigma, USA) in PBS for 60 min at 37°C with gentle agitation, and subsequently filtered through a 100-μm mesh filter to remove debris. The collagenase was inactivated with 10% fetal bovine serum (FBS; Gibco, USA) in DMEM medium (Gibco, USA) containing 4,500 mg/L (25 mM) D-glucose, 484 mg/L (4 mM) L-glutamine, and 110 mg/ml (1 mM) sodium pyruvate and the infranatant was centrifuged for 5 min at 1,500 rpm. The top lipid layer was carefully discarded and the cellular pellet was resuspended in DMEM/10% FBS and was centrifuged at 1,500 rpm for 5 min. The supernatant was carefully removed. The cell pellet was then resuspended in complete medium containing 10% FBS, 100 u/ml penicillin, and 100 mg/ml streptomycin and plated onto a T25m^2 cell culture flask (Corning, USA) at a density of 1.5×10^5/cm^2. The cells were incubated at 37°C in 5% CO_2 with saturated humidity (Thermo Forma, USA). Medium was first replaced after 24 h propagation to remove cell debris and then replaced every 3 days. After reaching 80% confluence, the cells were harvested and passaged. The third cell generation was used for cell characterization and osteogenic differentiation experiments.

CELL SURFACE MARKER CHARACTERIZATION USING FLOW CYTOMETRY

Human ADSCs were analyzed by flow cytometry for their expression of cell-surface markers CD29, CD44, CD34, CD45, CD105. Briefly,

cells at passage 3 with 85% confluence were disassociated from the culture plate using 0.25% trypsin/EDTA (Gibco, USA) and washed once in maintenance media before counting. Cells were aliquoted into FACS tubes and 3 ml of PBS containing 1% FCS was added to each tube. Subsequently, the cells were centrifuged at 500*g* 4°C for 5 min. A total of 3 μL of fluorescent-labeled mouse anti-CD29, CD44, CD34, CD45, CD105 antibodies (BD Biosciences, Franklin lakes, NJ) were added to each tube and incubated in dark for 30 min with vortexing at 15 min intervals. Then, 500 μL of PBS containing 1% FCS was added to each tube to dilute the samples. Cells were then analyzed using flow cytometer Coulter EPICS®Elite (Beckman Coulter, USA) for surface antigen expression. All samples were measured in triplicate for each sample. Mouse isotype IgG1 (BD Biosciences, Franklin lakes, NJ) were used as isotype controls to determine the positive cell populations. We counted 10,000 events per antibody group. Data were analysed using FlowJo software v7.6.4 (TreeStar, Inc. OR).

INDUCTION OF OSTEOGENIC DIFFERENTIATION

hADSC cells from healthy donors (passage 3) were seeded at 2×10^4 cells/cm^2 in six-well cell culture plates with 1×1 cm glass cover slips in each well. Cells were allowed to grow to 50–70% confluence over 24–48 h in standard growth medium [Moioli et al., 2007]. The medium was replaced with osteoinduction medium (Cyagen, USA) containing 10% fetal calf serum, 1% streptomycin, 10 mM L-glutamine, 50 μM L-ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone. This osteoinduction medium was replaced every 3 days. Osteoinduction was stopped at day 21 and cells were then stained with alizarin red (Cyagen, USA) for microscopic visualization.

TOTAL RNA EXTRACTION

hADSCs (passage 3) were plated at 2×10^4 in 100 mm culture dishes (Corning, USA). When cells attached to dishes, medium was replaced every 3 days with osteoinduction medium as described above. Cells without osteoinduction treatment were used as controls. When osteoinduction was completed after 21 days, cells were harvested and total RNA was extracted using TRIzol® (Invitrogen, USA), following the manufacturer's instructions. The purity of extracted RNA was analysed using the BioPhotometer plus (Eppendorf, Hamberg, Germany) photometer. RNA samples were considered pure if the OD260/280 was >1.8. RNA integrity was examined by 1% agarose gel and 5% urea/acrylamide gel electrophoresis. A ND1000 ultraviolet spectrophotometer (Nanodrop, USA) was used to quantify the extracted RNA.

miRNA MICROARRAY ANALYSIS

Mammalian miRNA chip V3.0 (Capital Bio Co. Beijing, China) covers all the mammalian miRNAs included in the miRBase 10.0 database (Sanger miRNA database, August 2010), which includes 677 human miRNAs, including 122 predicted miRNAs [Xie et al., 2005], 292 rat miRNAs, and 461 mice miRNAs. A total of 924 probes were designed and immobilized on a 75×25 mm, chemically modified glass slides using SmartArray (CapitalBio Co. Beijing, China). The whole array is divided into 8 subarrays, each consisting of 22 rows and 21 columns, with 245-μm point spacing and 150-μm point diameter. The consistently expressed small nuclear RNA U6 was spotted as an internal control.

Extracted RNA samples were first dephosphorylated with alkaline phosphatase (ALP) and then labelled with CU-cy3 and CU-cy5 (Dharmacon, USA) using T4 RNA ligase (New England Biolabs, USA) following the manufacturer's instructions. Labelled and purified RNA samples were then incubated in hybridization buffer containing 15% formamide, 3× saline-sodium citrate (SSC), 2× Denhardt's reagent, and 0.2% SDS, followed by a brief centrifugation. Samples then were denatured at 95°C for 3 min. Hybridization with the miRNA microarray probes was carried out at 46°C in a waterbath overnight. Subsequently, the hybridized chip was washed with washing solution I (0.2% SDS, 2× SSC) at 46°C for 4 min, followed by washing solution II (0.2% SSC) at 46°C for 4 min. Liquid on the surface of the chips was removed by a brief centrifugation.

Digital signal intensities for each spot were obtained and analysed using fluorescence scanning LuxScan 10K-A dual-channel laser confocal chip scanner (CapitalBio Corp. Beijing, China) and image analysis software (SpotData Pro V3.0, CapitalBio Co. Beijing, China). To reduce false-positive and false-negative results, positive and negative controls were included in the microarray experiments. Raw data were normalized to mean array intensity for inter-array comparison and analyzed using the significance analysis of microarrays (SAM; Stanford University, USA) software to determine differential expression of miRNAs before and after induction of differentiation of hADSCs in the three samples.

qRT-PCR ANALYSIS OF miRNA EXPRESSION

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis for osteogenesis related miRNA expression was carried out using 2 μg of total RNA. Primers were designed based on the miRNA sequences using Primer Express Version 5.0 (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table I. A universal PCR reverse transcriptional primer miRNA-R and U6 were used as internal controls. PCR reaction was conducted in 20 μL total volume containing a final concentration of 0.5 μM of each primer, 4 μL ddH_2O, 10 μL of 2 × SYBR Green PCR Master Mix (TOYOBO, Japan), and 5 μL of cDNA sample (1:20 diluted) corresponding to 2 μg of total RNA. Real-time PCR reactions were performed on an

TABLE I. Primer Sequences Used for qRT-PCR of Osteogenic miRNAs. All Primers Were Designed Using Primer Express Version 5.0

miRNAs	Primer sequences
hsa-mir17	5′-ACACTCCAGCTGGGCAAAGTGCTTACAGTGCA
hsa-miR-20a	5′-ACACTCCAGCTGGGTAAAGTGCTTATAGTGCA
hsa-miR-20b	5′-ACACTCCAGCTGGGCAAAGTGCTCATAGTGCA
hsa-mir31	5′-ACACTCCAGCTGGGAGGCAAGATGCTGGCATA
hsa-mir106a	5′-ACACTCCAGCTGGGAAAAGTGCTTACAGTGCAGG
hsa-mir-125a-5p	5′-ACACTCCAGCTGGGTCCCTGAGACCCTTTAACC
hsa-mir-125b	5′-ACACTCCAGCTGGGTCCCTGAGACCCTAACT
hsa-mir-193a-3p	5′-ACACTCCAGCTGGGAACTGGCCTACAAAGTCCCA
hsa-miR-199b-5p	5′-ACACTCCAGCTGGGCCCAGTGTTTAGACTATC
miRNA-R*	5′-CTCAACTGGTGTCGTGGA

*miRNA-R is a universal reverse primer.

iQ5 Real-Time PCR Detection System (BioRad, USA). Reaction conditions were: 95°C for 5 min to denature DNA templates, followed by 40 cycles of 95°C for 15 s denaturation, 65°C for 15 s annealing, and 72°C for 32 s extension. After amplification, a melting curve was obtained by heating at 60–95°C with interval 0.5°C cooling at 30°C for 30 s. The Ct value obtained for the miRNA of interest was normalized to the housekeeping miRNA U6 to obtain the ΔCt values. The ΔΔCt values were then obtained by subtracting the ΔCt values for each gene of interest against ΔCt values for the control sample. Fold difference in miRNA expression of the sample from the reference undifferentiated cells was calculated using the equation $RQ = 2^{-\Delta\Delta Ct}$. Data analysis was performed using iQ5 software (BioRad, USA). All samples were measured in triplicate.

TARGET PREDICTION

Target gene prediction of the screened miRNAs was performed using online miRNA target predicting software miRanda (www.microrna.org), miRBase (microrna.sanger.ac.uk) and Target Scan (www.targetscan.org). Only genes predicted by all three softwares were considered as target genes of the miRNA.

WESTERN BLOT ANALYSIS

Protein samples were prepared for Western blot analysis. Total protein was extracted using RIPA buffer containing 50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% Sodium deoxycholate, and 0.1% SDS, pH was adjusted to 7.4. Whole cell lysate was quantified by BCA assay and 20 μg of protein samples were loaded on SDS–PAGE gel and run under standard conditions. Gel was then transferred onto nitrocellulose membranes. After blocking in 5% skim milk, membranes were incubated with mouse anti-RUNX2(27-K) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Smad5(YY-6) monoclonal antibody (Santa Cruz Biotechnology), and Schnurri-2 HIVEP2 Mouse anti-Human Monoclonal Antibody (LifeSpan Biosciences, Seattle, WA), respectively at 4°C overnight. After washing, membranes were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG antibody (Bioss, Beijing, China). Membranes were reprobed with mouse anti-β-actin Monoclonal Antibody (Santa Cruz Biotechnology) and mouse anti-GAPDH Monoclonal Antibody (Santa Cruz Biotechnology) for control protein expression. Membranes were exposed to X-ray films and then developed.

ELISA DETECTION OF OSTEOGENESIS-RELATED PROTEINS

Media were collected at days 7, 14, and 21 after osteogenic induction. Enzyme-linked immunosorbent assay (ELISA) assays were performed to detect the concentration of secreted protein expression of bone gamma-carboxyglutamic acid containing proteins (BGP), ALP, collagen-I (Col-1) and bone sialoprotein (BSP) using specific ELISA assay kits (Rapidbio, USA) following manufacturer's instructions.

STATISTICAL ANALYSIS

Arithmetic mean and standard deviation were computed for protein expression and pair *t*-test was implemented to examine the difference between basal culture and osteogenic-induced culture cells. Geometric mean and standard deviation were computed for osteogenic/basal miRNA ratio. $P < 0.05$ was defined as statistical significance. All statistics were two-sided and performed by using SPSS statistical software (version 13.0).

RESULTS

ISOLATION AND CHARACTERIZATION OF THE hADSCs USING FLOW CYTOMETRY

hADSCs cells were isolated and grown in DMEM medium containing 10% FCS. After 4 days primary culture, the isolated human ADSC cells were mostly spindle-shaped at passage 0 (initial adherence to culture dishes), morphologically similar to fibroblast cells (Fig. 1A). The cells replicated rapidly and reached almost 80% confluence after 4 days in culture. During subsequent cell passages, some cells showed colony-like growth. Colony center was formed with high-density cells and surrounding cells grew into a swirling or radial shape (Fig. 1B).

To confirm that the isolated cells were hADSCs, we characterized several phenotypes known to be associated with human ADSC by flow cytometric analysis of expressed surface antigens. Isolated

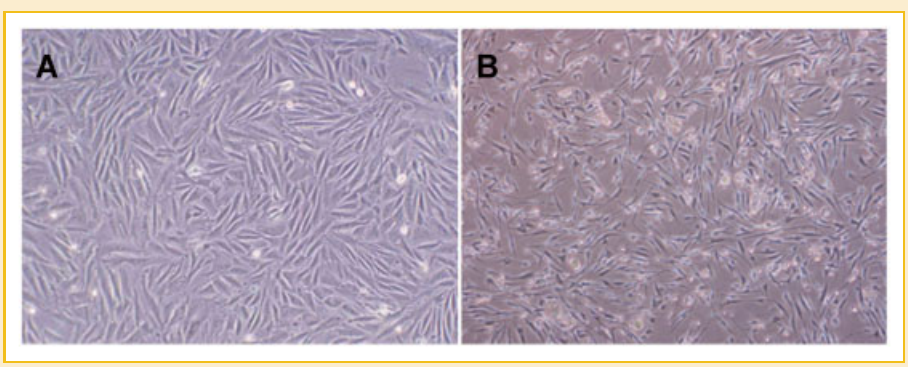

Fig. 1. Morphology of the isolated hADSC cells (×100). A: Isolated cells cultured at day 4. B: Isolated hADSC cells at passage 1. Note the colony-like growth of cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

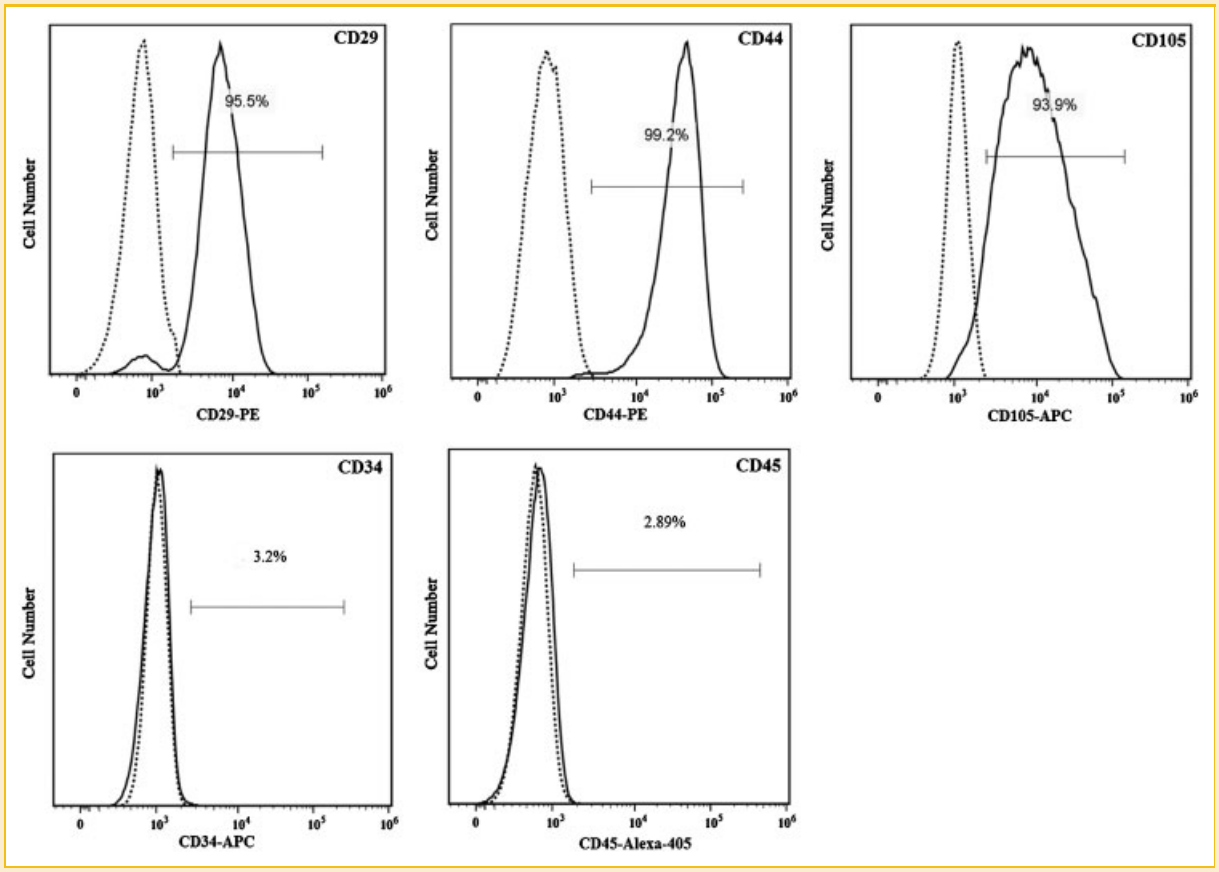

Fig. 2. hADSC cell surface antigen characterization using flow cytometry. Isolated hADSC cells at passage 3 were prepared for flow cytometric analysis. Black lines represent antibody-labeled cells, dashed lines represent antibody isotype control.

hADSCs of passage 3 were positive for CD29, CD44, and CD105 (Fig. 2). CD29+ cells were 95.5%, CD44+ cells were 99.2%, and CD105+ cells were 93.9%; hematopoietic lineage markers CD34 and CD45 were negatively expressed.

OSTEOGENIC DIFFERENTIATION OF hADSC CELLS

hADSC cells at passage 3 were seeded on glass cover slips in six-well cell culture plate and cultured in osteoinducing medium for 21 days. The glass cover slips covered with cells were then stained with Alizarin red. Colony-like growing cells were stained positively with Alizarin red (Fig. 3B), whereas cells without osteoinduction were negatively stained (Fig. 3A), indicating the presence of calcified nodes in the induced cells.

CONFIRMATION OF OSTEOGENIC DIFFERENTIATION BY DETECTION OF REPRESENTATIVE OSTEOGENIC PROTEIN EXPRESSION USING qRT-PCR

The osteogenesis-related protein expression detected by ELISA is shown in Figure 4. Throughout the entire study period, the upper serum levels of BGP, ALP, and Col-1 were significantly higher in the osteoinduced group compared with in basal group. From week 1 after the osteogentic differentiation, all protein expressions increased overall and then peaked at week 2 before slightly decreasingat week 3. From days 7 to 14, the expression of BGP and Col-1 increased from 563.01 ng/L (SD = 21.18) to 785.52 ng/L (SD = 34.34) and from 22.93 μg/L (SD = 1.51) to 30.17 μg/L (SD = 1.26), respectively; the levels of BGP and Col-1 then stabilized until day 21. The ALP expression at day 7 was 70.68 U/L (SD = 2.28) and then increased to 82.28 U/L (SD = 0.22) at day 14 and 75.33 U/L (SD = 1.45) at day 21. Finally, BSP expression was increased prior to day 21, with 5.34 ng/ml (SD = 0.01) at day 7 and 5.87 ng/ml (SD = 0.31) at day 14, then slightly decreased to 5.46 ng/ml (SD = 0.02) at the end of experiment. These results indicated evidence of osteogenic differentiation after the cells were exposed to the osteoinduction buffer.

miRNA EXPRESSION BEFORE AND AFTER OSTEOGENIC INDUCTION USING MICROARRAY ANALYSIS

miRNA expression levels before and after osteogenic differentiation were detected using miRNA microarray chips. SAM statistical software was used to identify differential expression of miRNAs between undifferentiated hADSCs and osteo-differentiated hADSCs samples. In sample 1, there were 16 differentially expressed miRNAs identified, which included 7 up-regulated and 9 down-regulated miRNAs. In sample 2, 37 differentially expressed miRNAs were identified, of which 15 miRNAs were up-regualted and 22 miRNAs were down-regulated. In sample 3, we identified 13 miRNAs with different expression before and after osteogenic induction, of which

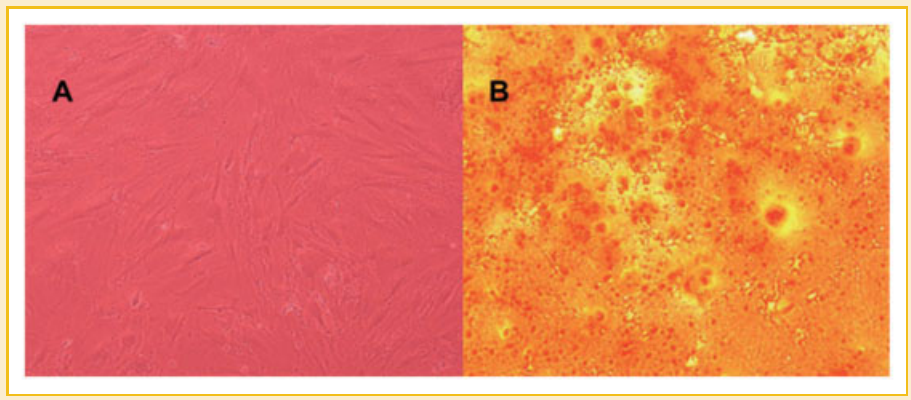

Fig. 3. Morphology of hADSC cells after osteogenic induction (×100). Cells at passage 3 were plated on glass cover slip and induced for osteogenic differentiation using inducing buffer. After 21 days, cells were stained with alizarin red and visualized under microscope. A: Control hADSC cells without induction for osteogenic differentiation. B: hADSC cells after induction for osteogenic differentiation. Note the formation of calcium nodules and the loss of cellular morphology. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

7 were up-regulated and 8 were down-regulated. Results are summarized in Table II. There was a large variation in the number of differentially expressed miRNAs among the three independent samples. miRNAs with consistent differential expression in three samples were therefore identified, which included four up-regulated miRNAs (miR-17, miR-20a, miR-20b, and miR-106a), and four down-regulated miRNAs (miR-31, miR-125a-5p, miR-125b, and miR-193a-3p, Table III).

qRT-PCR VALIDATION OF THE MICROARRAY RESULTS

To confirm the microarray results, we conducted real-time qPCR to detect the expression levels of the eight differentially expressed

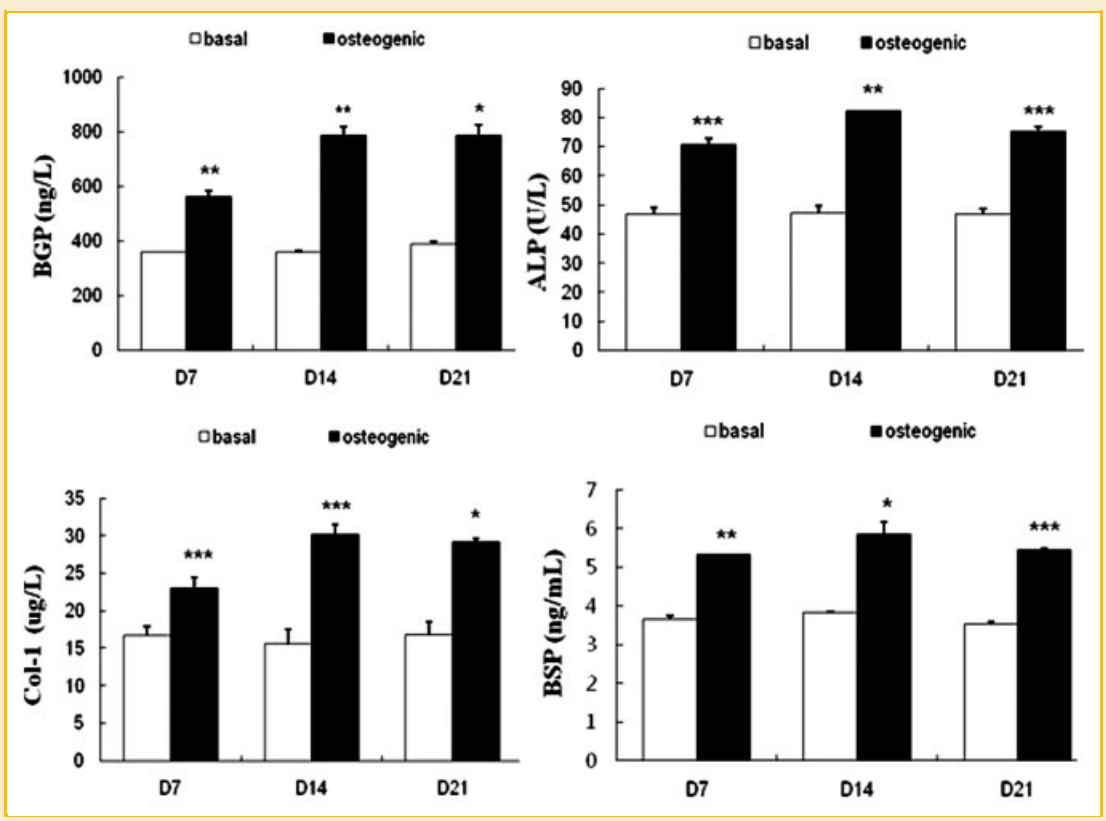

Fig. 4. Determination of osteogenesis-related proteins expression to confirm osteogenic differentiation. hADSC cells were induced for osteogenic differentiation. At days 7, 14, and 21, media was collected and osteogenesis-related protein concentrations of BGP, ALP, Col-1, and BSP were detected using ELISA. Basal level was obtained using parallel growth media without osteogenic induction at same time point. Mean ± SD values (n = 3 cultures) were presented on the graph. Pair *t*-test was implemented for comparisons between osteogenic and basal group at a given time point. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

TABLE II. miRNAs Expression in Osteogenic Differentiaed hADSCs by Microarray

Up-regulation			Down-regulation		
Gene ID	Score(d)	Fold change	Gene ID	Score(d)	Fold change
Sample 1			Sample 1		
hsa-miR-199b-5p	14.66841	2.8470	**hsa-miR-125b**	**−10.9604**	**0.3921**
hsa-miR-20a	**5.476649**	**2.4356**	**hsa-miR-125a-5p**	**−10.6097**	**0.4016**
hsa-miR-106a	**5.384724**	**2.3923**	**hsa-miR-193a-3p**	**−10.0659**	**0.4319**
hsa-miR-17	**4.538275**	**2.2785**	hsa-miR-424	−7.68563	0.4195
hsa-miR-20b	**4.327011**	**2.0471**	**hsa-miR-31**	**−7.52939**	**0.3545**
hsa-miR-138	3.611771	2.0381	hsa-miR-631	−6.60078	0.4573
hsa-miR-107	3.034787	2.1158	mmu-miR-322	−6.27354	0.3898
			hsa-miR-584	−5.23686	0.3579
			PREDICTED_MIR229	−4.75901	0.3492
Sample 2			Sample 2		
mmu-miR-106a	13.18835	3.0590	PREDICTED_MIR191	−18.433	0.4410
hsa-miR-15a	10.08318	2.1888	hsa-miR-940	−18.2741	0.4123
hsa-miR-138	8.46992	2.2027	PREDICTED_MIR105	−18.0155	0.2928
hsa-miR-20b	**8.325785**	**2.5555**	**hsa-miR-125b**	**−16.649**	**0.2918**
hsa-miR-20a	**7.298364**	**3.3895**	**hsa-miR-125a-5p**	**−13.9244**	**0.1916**
hsa-miR-93	6.865872	4.4324	hsa-miR-612	−9.909	0.4216
hsa-miR-199b-5p	6.628222	2.1133	hsa-miR-874	−9.86456	0.3183
hsa-miR-92a	6.26314	2.0921	hsa-miR-100	−9.53034	0.3942
hsa-miR-106a	**5.813082**	**3.1155**	mmu-miR-685	−8.81351	0.4723
mmu-miR-546	5.263613	2.9443	hsa-miR-133a	−8.22326	0.3772
PREDICTED_MIR166	5.249149	2.1178	PREDICTED_MIR229	−7.62151	0.4294
hsa-miR-17	**4.811736**	**2.7226**	hsa-miR-423-5p	−6.75693	0.3169
rno-miR-20b-5p	4.798053	11.0616	rno-miR-290	−6.44703	0.2888
hsa-miR-30a	4.477245	2.1772	hsa-miR-146a	−5.9349	0.2507
hsa-miR-130a	4.241389	2.0474	hsa-miR-365	−5.65964	0.4833
			hsa-miR-584	−5.43339	0.2138
			hsa-miR-744	−5.05181	0.4728
			hsa-miR-31	**−4.76801**	**0.4600**
			hsa-miR-371-5p	−4.46579	0.2921
			PREDICTED_MIR88	−4.12439	0.4731
			mmu-miR-290-5p	−3.67487	0.2849
			hsa-miR-193a-3p	**−2.72621**	**0.4636**
Sample 3			Sample 3		
hsa-miR-106a	**2.531949**	**2.3253**	**hsa-miR-125a-5p**	**−3.29602**	**0.4135**
hsa-miR-138	1.949702	4.2391	**hsa-miR-125b**	**−3.31976**	**0.4906**
hsa-miR-17	**2.320967**	**2.2805**	**hsa-miR-193a-3p**	**−2.00258**	**0.4208**
hsa-miR-199b-5p	6.011284	2.0171	hsa-miR-199b-5p	−3.27012	0.4929
hsa-miR-20a	**2.472256**	**2.3764**	**hsa-miR-31**	**−2.83913**	**0.4742**
hsa-miR-20b	**3.20187**	**2.2787**	PREDICTED_MIR229	−3.96217	0.4242
mmu-miR-106a	2.703705	2.4412	9		

miRNA expression by microarray in three samples. Table shows the fold changes of miRNA expression: ≥2 indicates up-regulation and ≤0.5 indicates down-regulation. miRNAs with consistent differential expression in three samples were bolded.

miRNAs identified by microarray. Parallel-grown hADSCs cells without osteoinduction were used as a control. Real-time RT-PCR showed that all eight selected miRNAs were differentially expressed between undifferentiated hADSCs and osteodifferentiated hADSCs in all three samples. The qRT-PCR results agree with the microarray results (Table III and Fig. 5).

miRNA TARGET PREDICTION

To investigate the specific role miRNAs in regulating osteodifferentiation, we attempted to predict target genes of the differentially expressed miRNAs: hsa-miR17, hsa-miR-20b, hsa-miR-106a, hsa-miR-20b, hsa-miR-125b, and hsa-miR-193a-3p. Putative targets were predicted using online software. Predicted targets of each

TABLE III. Up- and Down-Regulated miRNAs in Differentiated hADSC Cells by Microarray and qPCR

miRNA	Fold change by microarray	Fold change by qRT-PCR	Gene location
hsa-miR17	2.43 ± 0.26	1.93 ± 0.45	13q31.3
hsa-miR-20a	2.73 ± 0.57	1.65 ± 0.33	13q31.3
hsa-miR-20b	2.29 ± 0.25	1.74 ± 0.46	Xq26.2
hsa-miR106a	2.61 ± 0.44	1.92 ± 0.69	Xq26.2
hsa-miR-31	−2.37 ± 0.39	−1.40 ± 0.12	9q21.3
hsa-miR-125a-5p	−3.32 ± 1.65	−2.94 ± 0.33	19q13.33
hsa-miR-125b	−2.67 ± 0.70	−2.24 ± 0.76	11q24.1
hsa-miR-193a-3p	−2.28 ± 0.11	−2.02 ± 0.80	17q11.2

miRNA expression by microarray and qPCR in differentiated hADSCs were normalized to undifferentiated hADSCs. The table shows eight differentially expressed miRNAs identified using microarray in three samples and the fold changes of the 8 miRNA expression (mean ± SD; Sample number = 3). qRT-PCR was performed to validate the microarray results (mean ± SD; Sample number = 3). Fold changes for down-regulated miRNAs were converted to negative values using equation converted fold = −1/original fold.

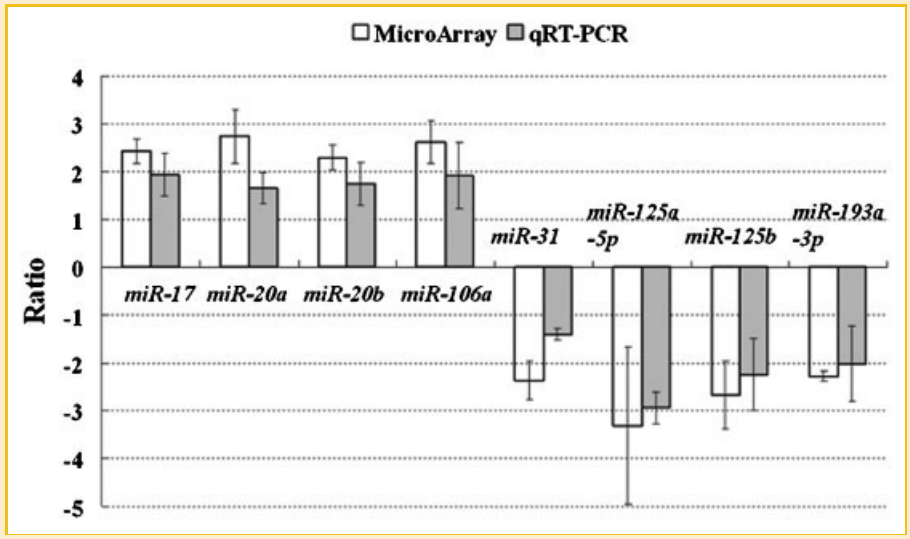

Fig. 5. qRT-PCR confirmation of miRNA expression in osteogenic differentiated hADSC cells. For qRT-PCR analysis, 1 μg of total RNA purified from differentiated hADSCs was used. The RNA samples were reverse transcribed to cDNA, and then amplified. Expression of miRNA was normalized to undifferentiated cell control samples. Figure indicates fold changes of expression relative to control.

miRNA contain a large set of genes potentially involved in tumorigenesis, cell cycle regulation, cell differentiation, transcriptional regulation, and signal transduction. In Table IV, only target genes related to bone formation, osteogenic differentiation and signal transduction are listed. The up-regulated miRNAs (hsa-miR17, hsa-miR-20a, hsa-miR-20b, and hsa-miR106a) in osteo-differentiation of hADSCs were predicted to target genes such as runt-related transcription factor (RUNX) 2, BMP receptor (BMPR), STAT-3 and Myc. Predicted target genes of the down-regulated miRNAs were Osterix, Schnurri-2, and CDK6, which usually regulate osteodifferentiation and cell-cycle progression.

TARGET VALIDATION

To verify the putative targets of the differentially expressed miRNAs, we carried out Western blot analysis (Fig. 6) to examine the protein expression of predicted target. RUNX2, Smad5, and Schnurri-2, which are directly related to osteogenic differentiation, were primarily selected for Western blot analysis. Protein samples were prepared at the same the time when RNA samples were prepared. We found RUNX2 and Schnurri-2 expression in osteo-differentiated ADSC were up-regulated and Smad5 expression in osteo-differentiated ADSC was down-regulated.

DISCUSSION

In this study, we used miRNA microarray technique to profile miRNA expression and screen miRNAs with differential significant expression (>2-fold change) before and after osteogenic induction. We found that miR-17, miR-106a, miR-20a, and miR-20b in the miR-17 family were likely up-regulated. Although it has been reported that miR-26a and miR-196a were up-regulated during osteogenic differentiation of hADSC cells [Luzi et al., 2008; Kim et al., 2009], in our study, miR-26a and miR-196a were not

TABLE IV. Predicted Target Genes of Osteogenic miRNAs

miRNA	Target genes	Function
hsa-miR17	BMPR2	Self-renewal, osteogenic differentiation
	Smad5,7	Osteogenic differentiation
	Myc	Self-renewal, signal transduction
	STAT-3	Pluripotency, self-renewal
	E2F1-3	Pluripotency, self-renewal, cell cycle control
hsa-miR-20a	Osterix	Osteogenic differentiation
	Frizzled-1,4,7	Pluripotency, self-renewal
	Myc	Pluripotency, self-renewal
	BMPR2	Self-renewal, osteogenic differentiation
hsa-miR-106a	BMPR2	Self-renewal, osteogenic differentiation
	RUNX2	Osteogenic differentiation
	LIF	Pluripotency, self-renewal
	STAT-3	Pluripotency, self-renewal
hsa-miR-20b	STAT-3	Pluripotency, self-renewal
hsa-miR-125b	Osterix	Osteogenic differentiation
	Schnurri-2	Osteogenic differentiation
hsa-miR-193a-3p	CDK6 (cyclin dependent kinase 6)	Cell cycle control

Only genes that are related to bone formation and osteogenic differentiation are listed.

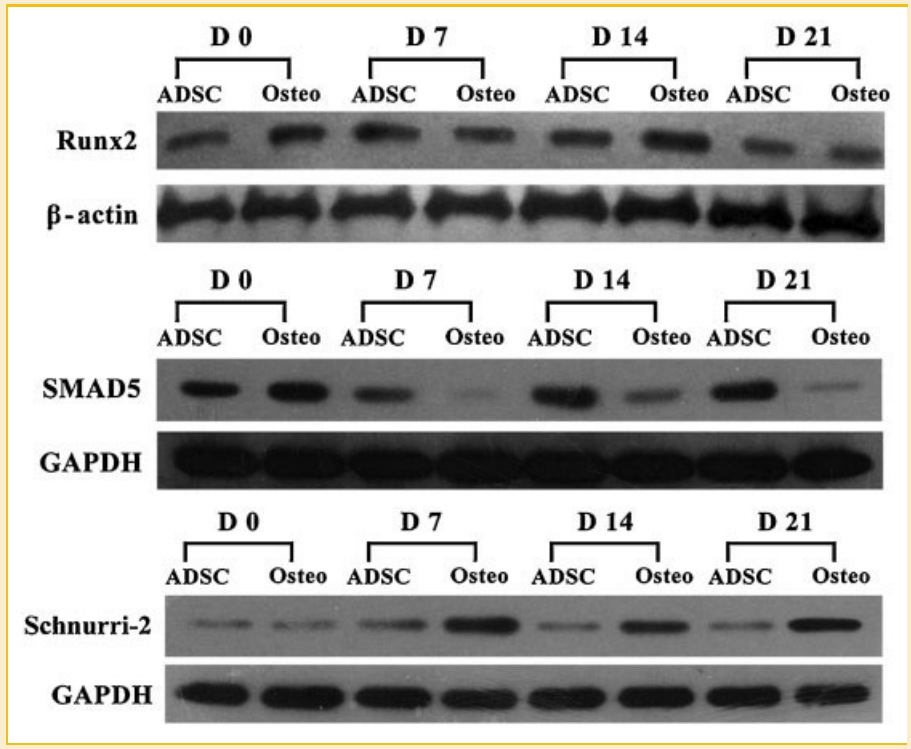

Fig. 6. Protein expression of RUNX2, Smad5, and Schnurri-2. Total protein samples were prepared at days 0, 7, 14, and 21 post-osteogenic induction. Undifferentiated samples were run in parallel as controls. Each lane was loaded with equal quantity of protein sample. Lanes labeled with ADSC indicate hADSC samples without osteogenic induction, whereas lanes labeled with Osteo indicate hADSC samples post-osteogenic induction.

identified with differential expression, likely due to our >2 fold cut-off. We found that miR-26a was consistently up-regulated in all 3 samples with a ~1.6-fold change. However, expression of miR-196a was neither up-regulated nor down-regulated. miR-17 family of miRNA includes miR-17, miR-18, miR-19a, miR-19b, miR-20, miR-25, miR-92, miR-93, miR-106a, and miR-106b etc. They are located on human chromsomes 13, 7, and X, and are homologous to vertebrates such as rats and chimpanzees [Sylvestre et al., 2007]. Our results demonstrated a clustered expression of miR-17 family genes. miRNAs within the same family were all expressed at similar levels. Highly synergistic expression patterns of gene clusters suggests that homologous miRNA gene clusters may share the same cis-regulatory elements and play synergistic regulatory roles.

Understanding the regulatory mechanisms of osteogenic differentiation of stem cells is the basis for treatment of various bone disorders. Recent advances in molecular biology and gene technology have enabled scientists to partially unravel the molecular mechanisms that regulates osteogenic differentiation [Rosen, 2005; Karsenty et al., 2009]. Osteogenic differentiation of stem cells is a cascading mechanism involving expression of multiple genes and functionality [Zou et al., 2008]. Our current knowledge on the regulation of osteogenic differentiation is largely based on the transcription factors. Runx2, Osterix, and β-catenin transcription factors are believed to play key roles in osteogenic differentiation [Komori, 2006]. Other transcription factors also can indirectly regulate cell differentiation by modulating the expression of the key factors [Komori, 2006; Franceschi et al., 2007]. In this study, the predicted target genes of overexpressed miRNAs in the miR-17 family included several transcription factors such as Runx2 and Osterix. Runx2 is essential for osteoblastic differentiation and skeletal morphogenesis, and studies have demonstrated that osteoblastic differentiation is associated primarily with increases in Runx2 activity in bone marrow stromal cells [Wojtowicz et al., 2010]. Osterix, which acts downstream to Runx2, is a zinc-finger-containing transcription factor essential for embryonic osteoblast differentiation and bone formation [Nakashima et al., 2002]. Runx2 and Osterix are predicted targets of overexpressed miRNAs during osteogenic differentiation in hADSCs, and they are likely to participate in differentiation via miRNA regulation.

In addition to the transcription factors that can directly regulate cell differentiation and bone formation, other genes also play important roles in regulating stem cell differentiation and self-renewal. Predicted target genes of overexpressed miRNAs also include genes such as STAT3, Myc, E2F1, and BMPR. BMPRs, including BMPR1A, BMPR1B, and BMPR2, are involved in endochondral bone formation and embryogenesis [Tsumaki and Yoshikawa, 2005]. It is reported [Foshay and Gallicano, 2009] that miRNA-17 family regulate the conversion of self-renewal and differentiation of embryonic stem cells via silencing STAT3. C-Myc is an important gene that controls cell pluripotency and early differentiation, and is one of the four genes that regulates the induction of pluripotent stem cells from a variety of adult cells. Up-regulation of miR-17 family can rapidly down-regulate the expression of c-Myc and Wnt mRNA, and subsequently mediate the stem cell self-renewal and differentiation [Foshay and Gallicano,

2009]. miR-20a modulates the translation of the E2F2 and E2F3 mRNAs and may play a role in regulating cellular proliferation and apoptosis [Sylvestre et al., 2007]. These findings, together with our results, indicate that miRNA-17 family might regulate osteognenic differentiation in hADSCs by regulating their target genes.

Predicted target genes of the down-regulated miRNAs include Osterix, Schnurri-2 and CDK6, which usually regulate osteodifferentiation and cell cycle progression. We anticipated that under-expressed miRNAs may function as negative regulators that inhibit the osteogenic differentiation of hADSCs.

Western blot analysis indicated that Smad5, one predicted target of miR-17, was significantly down-regulated in osteodifferentiated ADSCs, and Schnurri-2, one predicted target of miR-125b, was largely up-regulated in osteo-differentiated ADSCs, which we predictedbut to verify this prediction, we will need to carry out further studies.RUNX2, a predicted target of miR-106a and a member of the runt homology domain transcription factor family essential for osteoblast differentiation was found up-regulated in osteodifferentiated ADSCs by Western blot analysis, which we expected. This is contradictory to our finding that miR-106a was overexpressed in osteodifferentiated ADSCs. First, RUNX2 may not be the real target of miR-106a, which could explain this discrepancy. Second, Runx2 gene expression and protein function are regulated at multiple folds, including transcription, translation, and post-translational modification, another explanation for the discrepancy. miRNA may not be the sole regulator of RUNX2 miR-106a might regulate RUNX2 expression cooperatively with other factors and on adifferent time scale.

Collectively, the up-regulated miRNAs might positively regulate osteogenic differentiation. ALP and BSP were both significantly increased in the osteoinduced group compared on days 7, 14, and 21,which indicates that some miRNAs might promote early-stage osteodifferentiation. Osteogenic diferentiation leads to bone cell mineralization and eventually the formation of calcium nodules. Down-regulated miRNAs in osteogenic differentiation may play a negative regulatory role in this process, and may inhibit calcium nodule formation and expression of collagen type I in late-stage osteogenic differentiation. Thus, the osteogenic differentiation is a complex process that involves a combination of multiple miRNAs and genes. To date, the role of miRNA as a key regulatory element in osteogenic differentiation and bone formation has been increasingly recognized, but the mechanism(s) involved are still poorly defined [Hobert, 2008; Li et al., 2008; Mizuno et al., 2008; Oskowitz et al., 2008; Stefani and Slack, 2008]. Our results have revealed important coordinating roles from a group of miRNAs during osteogenic differentiation, which contibutes to ourunderstanding of stem cell osteogenic differentiation.

Construction of biologically active tissue materials, repairing tissue defects, and restoring organ function are the primary goals of tissue engineering. In choosing between different seed cells, some studies [Sakaguchi et al., 2005; Yoshimura et al., 2007] suggest that the osteoblast capability of BMSCs is greater than capability of ADSCs in vitro because ALP and osteocalcin levels in BMSCs are higher than in ADSCs. Because hADSCs have the advantages of easy access, are abundant, easy to culture, and biologically stable, they have attracted intensive attention and interest in scientific community. To this end, understanding the role of miRNAs played in the hADSCs osteogenic differentiation process will provide insight into the optimization of high-performance hADSC cells, and thereby offer an importantsource of bone engineering and cell-based therapy.

In summary, we have discovered a group of miRNAs that are differentially expressed and work together during osteogenic differentiation in hADSCs. There are many potential targets of miRNAs, indicating that one miRNA might involve multiple signal transducing pathways. To unravel the precise regulatory mechanisms of relevant miRNAs at the transcription factor level during osteogenic differentiation, we will need to carry out further functional studies of the predicted target genes of the interesting miRNAs. We hope our results will facilitate our understanding of the molecular mechanisms of osteogenic differentiation in hADSC cells and subsequently control hADSC cell differentiation and provide high performance seed cells for future bone engineering.

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