

Osteoarthritis and Cartilage



Expression of microRNAs during chondrogenesis of human adipose-derived stem cells

Z. Zhang ^{†a}, Y. Kang ^{†a}, Z. Zhang [†], H. Zhang [‡], X. Duan [†], J. Liu [†], X. Li ^{§**}, W. Liao ^{†*}

[†] Department of Joint Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510080, China

[‡] Department of Orthopaedic Surgery, People's Hospital of Anyang, Anyang, Henan 455000, China

[§] Science Research Center, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, China

ARTICLE INFO

Article history:

Received 30 May 2012

Accepted 23 August 2012

Keywords:

miRNAs

Human adipose-derived stem cells

Chondrogenesis

SUMMARY

Objectives: MicroRNAs (miRNAs) play an important role in the regulation of chondrogenesis of mesenchymal stem cells, but their expression still remains unknown in human adipose-derived stem cells (hADSCs). In this study the miRNA expression profile during chondrogenic differentiation of hADSC and the potential mechanism whereby miRNAs may affect the process of chondrogenesis are considered.

Methods: hADSCs were isolated and cultured. The expression of chondrogenic proteins was detected using enzyme-linked immunosorbent assay (ELISA). miRNA expression profiles before and after chondrogenic induction were obtained using miRNA microarray assay and differently expressed miRNAs were primarily verified using quantitative real-time polymerase chain reaction (qRT-PCR). Putative targets of the miRNAs were predicted using online software programs MiRanda, TargetScan and miRBase.

Results: Twelve miRNAs were found to be differentially expressed pre- and post-chondrogenic induction by over a two-fold change, including eight up-regulated miRNAs (miR-193b, miR-199a-3p/hsa-miR-199b-3p, miR-455-3p, miR-210, miR-381, miR-92a, miR-320c, and miR-136), and four down-regulated miRNAs (miR-490-5p, miR-4287, miR-BART8*, and miR-US25-1*). qRT-PCR analysis further confirmed these results. Predicted target genes of the differentially expressed miRNAs were based on the overlap of at least two online prediction algorithms, with the known functions of regulating chondrogenic differentiation, self-renewal, signal transduction and cell cycle control.

Conclusions: In this study we have identified a group of miRNAs and their target genes, which may play important roles in regulating chondrogenic differentiation of hADSCs. Our results provide the basis for further investigation into the molecular mechanism of chondrogenesis in hADSCs and their differentiation for cartilage engineering.

© 2012 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Introduction

MicroRNAs (miRNAs) are a group of non-coding, single stranded, small RNAs (~22 nt in length) that have been identified in various plants, animals and viruses¹, and which were first discovered in *C. elegans* in 1993^{2,3}. miRNAs are generated from endogenous transcripts producing hairpin structures by an RNase

* Address correspondence and reprint requests to: W. Liao, Department of Orthopaedic Surgery, First Affiliated Hospital of Sun Yat-sen University, #58 Zhongshan 2nd Road, Guangzhou 510080, China. Tel: 86-20-8775-5766; Fax: 86-20-8733-2150.

** Address correspondence and reprint requests to: X. Li, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China. Tel/Fax: 86-20-8733-1918.

E-mail addresses: xuerong2@mail.sysu.edu.cn (X. Li), liaoowmsysu@163.com (W. Liao).

^a Zhiqi Zhang and Yan Kang contributed equally to this study.

III-type enzyme. At transcriptional levels, miRNAs function as regulators in gene silencing by binding to the 3'-untranslated region (3'UTR) of target mRNAs, leading to translational repression. miRNAs are involved in the regulation of a wide range of biological processes including embryonic development, cell proliferation, apoptosis and cell differentiation⁴⁻⁷.

The important regulatory roles of miRNAs during chondrogenesis were identified recently. *Dicer* is an essential component for biogenesis of miRNAs, and *Dicer*-null growth plates showed a progressive reduction in the proliferating pool of chondrocytes, leading to severe skeletal growth defects and premature death in mice⁸. When mouse mesenchymal stem cells (MSCs) were compared with mature mouse chondrocytes, miR-29a and miR-29b were revealed to directly target 3' UTR of Col2a1 encoding type II collagen, and their activity was under the regulation of Sox9⁹. miR-199a(*), a bone morphogenic protein 2-responsive miRNA, significantly inhibited early chondrogenesis, as revealed by the reduced

expression of early marker genes for chondrogenesis such as cartilage oligomeric matrix protein (COMP), type II collagen, and Sox9, whereas anti-miR-199a(*) increased the expression of these chondrogenic marker genes *via* direct targeting to SMAD1 in C3H10T1/2 stem cells¹⁰. Differentiation of mouse MSCs resulted in up- or down-regulation of several miRNAs, with miR-199a expression being over 10-fold higher in chondroblasts than in undifferentiated MSCs. In addition, miR-124a was strongly up-regulated during chondrogenesis while the expression of miR-96 was substantially suppressed¹¹.

Most studies on the regulatory roles of miRNA during chondrogenesis have focused on MSCs from bone marrow (BMSCs). BMSCs and adipose-derived stem cells (ADSCs) have similar biological functions¹². Since adipose tissue is abundant and easy to access and culture, ADSCs may be a promising therapeutic alternative to BMSCs. Human ADSCs (hADSCs) are capable of self-renewal, are pluripotent, and can differentiate into osteogenic, chondrogenic, adipogenic, and myogenic cells^{13,14}. We have also isolated these cells, confirmed that the isolated cells were indeed hADSCs from human adipose tissue, and proved their pluripotency¹⁵.

However, only a few studies have been carried out about miRNA expression during chondrogenic differentiation in hADSCs. Here, we profiled the expression of miRNAs during chondrogenesis in hADSCs by miRNA microarray, and we also predicted the putative targets of the interesting miRNAs.

Materials and methods

Isolation and culture of hADSCs

The adipose tissue was obtained with approval of 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 (IRB No. 2011011). Donors' age ranged from 19 to 45 years. Donors with malignant tumors and metabolic diseases were excluded from this study. Three samples of adipose tissues from three different donors (one male and two females) were used. These are the same samples which were used in our previously osteogenic study¹⁵. Third generation cells were used for initial characterization and chondrogenesis experiments.

Induction of chondrogenic differentiation and histology

Healthy hADSCs (Passage 3) were harvested and resuspended in incomplete chondrogenic medium at 2×10^7 cells/ml. Droplets (12.5 μ l) were carefully placed in each well of a 24-well plate. hADSCs were allowed to adhere at 37°C for 90 min, followed by the addition of 500 μ l complete chondrogenic medium¹⁶. The complete chondrogenic medium (Cyagen, USA) contained 194 ml basal medium (Cat. No. HUXMA-03041-194), 20 μ l dexamethasone, 600 μ l ascorbate, 2 ml ITS + Supplement, 200 μ l sodium pyruvate, 200 μ l praline, and 2 ml transforming growth factor beta 3 (TGF- β 3). After 24 h, the cell droplets coalesced and became spherical. Complete medium was changed every 3 days, and micromasses were harvested on day 21. At 21 days, micromasses were fixed in 4% paraformaldehyde for 3 h, and then dehydrated with ethanol, washed with xylene and embedded in paraffin. Sections at 5 μ m were cut and mounted on glass slides. Alcian blue staining for proteoglycans was performed for visualization under microscopy.

ELISA detection of chondrogenesis-related proteins

Culture media were collected at days 0, 6, 10 and 14 after chondrogenic induction. ELISA was performed to detect the

concentration of secreted protein expression of Col2A1, aggrecan, Col10A1, and chondroitin using specific ELISA assay kits (Abcam, USA) following the manufacturer's instructions.

Total RNA extraction

Total RNA was extracted using Trizol (Invitrogen, USA) from hADSCs with and without (control) chondrogenic induction. The RNA was further purified using miRNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The purity of extracted RNA was analyzed using Eppendorf BioPhotometer Plus (Eppendorf, Hamberg, Germany) and the integrity of the RNA was examined using agarose gel electrophoresis. An ND1000 ultraviolet spectrophotometer (Nanodrop, USA) was used to quantify the extracted RNA.

miRNA microarray analysis

The fifth generation of miRCURY™ LNA Array (v.14.0) (Exiqon) contains more than 1891 capture probes, covering all human, mouse and rat miRNAs annotated in miRBase 14.0. After RNA isolation from all six samples (three paired comparison, with and without chondrogenic induction from three donors, which were shown as Sample 1 undifferentiated and Sample 1 differentiated, Sample 2 undifferentiated and Sample 2 differentiated, Sample 3 undifferentiated and Sample 3 differentiated), the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guidelines for miRNA labeling. After stopping the labeling procedure, the Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array (v.14.0) (Exiqon) according to array manual. The total 25 μ l mixture from Hy3™-labeled samples with 25 μ l hybridization buffer were first denatured for 2 min at 95°C, incubated on ice for 2 min and then hybridized to the microarray for 16–20 h at 56°C in a 12-Bay Hybridization Systems (Hybridization System – Nimblegen Systems, Inc., Madison, WI, USA), which provided an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signal. Following hybridization, the slides were washed several times using wash buffer kit (Exiqon), and dried by centrifugation for 5 min at 400 rpm. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA).

Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities >50 in all samples were chosen for calculating Median normalization factor. Expressed miRNA data was normalized using the Median normalization and chosen for differentially expressed miRNAs screening. After normalization, the distributions of log₂-ratios across every sample were nearly the same (Supplemental Fig. 1). Correlation Matrix and scatter plot showed good correlation among experiments and reproducibility between chips (Supplemental Table 1 and Supplemental Fig. 2). Hierarchical clustering was performed using MEV software (v4.6, TIGR). To identify differentially expressed miRNAs with statistical significance differences, we performed a fold change filtering to determine differential expression of miRNAs before and after induction of differentiation of hADSCs in all three paired samples. The threshold we used to screen Up- or Down-regulated miRNAs was fold change ≥ 2.0 .

qRT-PCR analysis of miRNA expression

qRT-PCR analysis for the expression of chondrogenesis-related miRNA and predicted target transcriptional factors was carried out using 1 μ g of total RNA. Primers were designed 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 μ l total volume containing a final concentration of 0.5 μ M of each primer, 4 μ l ddH₂O, 10 μ l of 2 \times SYBR Green PCR Master Mix (TOYOBO, Japan) and 5 μ l of cDNA sample (1:20 diluted) corresponding to 1 μ g of total RNA. Real-time PCR reactions were performed on an ABI PRISM[®] 7500 Sequence Detection System. Reaction conditions were as follows: 95°C for 5 min to denature DNA templates, then followed by 40 cycles of 95°C for 15 s denaturation, and 65°C for 15 s annealing, and 72°C for 32 s extension. After amplification, a melting curve was obtained by heating from 60°C to 95°C with interval 0.5°C and then cooling at 30°C for 30 s. Results were normalized to U6 to obtain the Δ Ct values. $\Delta\Delta$ Ct values were then obtained by subtracting the Δ Ct values for each gene of interest against Δ Ct values for the control sample. Fold difference in gene expression of the sample from the reference undifferentiated cells was calculated using the equation $\chi = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta$ Induction – Δ C and Δ Induction = $C_{\text{induction}} - C_{\text{U6}}$; $\Delta C = C_{\text{control}} - C_{\text{U6}}$. 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.microna.org), miRBase (microna.sanger.ac.uk) and TargetScan (www.targetscan.org). Only genes predicted by at least two software programs were considered as target genes of the miRNA.

Statistical analysis

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

Results

Chondrogenic differentiation of hADSCs

hADSC cells at passage 3 were cultured in chondrogenic induction medium for 21 days. The micromasses were then fixed

Table I
Primer sequences used for qRT-PCR of chondrogenic miRNAs and potential target transcription factors

U6	F (5' CTCGCTTCGGCAGCAC)
U6	R (5' AACGCTTCACGAATTTGCGT)
miRNA	R (5' CTCAACTGGGTCTGTGGA)
hsa-miR-193b	F (5' AACTCCAGCTGGGAACCTGGCCCTCAAAGTCCCG)
hsa-miR-199a-3p	F (5' AACTCCAGCTGGGACAGTAGTCTGCACATTG)
hsa-miR-490-5p	F (5' AACTCCAGCTGGGACATGGATCTCCAGGTG)
18s rRNA	F (5' CCTGGATACCCGAGCTAGGA)
	R (5' GCGGCGCAATACGAATGCCCC)
C/EBP β	F (5' GACGAGTACAAGATCCGGCC)
	R (5' TTCTGCAGCCGCTCGTTTCTC)
Runx2	F (5' TCTAAATCGCAGGCTTCAT)
	R (5' GAGGACCTACTCCAAAGGA)
NF- κ B1	F (5' ACTGGCTGAGCGGATGCATC)
	R (5' TGCTGTGGTCAGAAGGAATG)
NF- κ B2	F (5' GAACAGCCTTGCATCTAGCC)
	R (5' CAGAGTCCGAGTCCGTATCA)

and stained with Alcian blue, where blue staining indicated synthesis of proteoglycans by chondrocytes. The micromasses with chondrogenic ADSCs were stained positively with Alcian blue [Fig. 1(B)], whereas cells without chondrogenic induction were negatively stained [Fig. 1(A)].

Confirmation of chondrogenic differentiation by detection of representative chondrogenic protein expression

In order to ascertain if hADSCs were chondrogenically differentiated with chondrogenic induction, the chondrogenesis-related protein expression detected by ELISA was illustrated (Fig. 2). Throughout the whole study period, the upper serum levels of Col2A1, Col10A1, aggrecan and chondroitin in the chondrogenic-induced group were significantly higher than those in basal group. Overall, from day 6 after the chondrogenic differentiation, protein expressions were gradually and steadily increased, not even reaching peak expression by the end of the 2-week observation period. These results indicated evidence of chondrogenic differentiation after the cells were exposed to the chondrogenic induction buffer.

miRNA expression before and after chondrogenic induction using microarray

miRNA expression levels before and after chondrogenic differentiation were detected using miRNA microarray chips. SAM statistical software was used to identify differential expression of miRNAs between undifferentiated hADSCs and chondro-differentiated hADSCs samples. The whole differential expression of miRNAs before and after induction of differentiation of hADSCs in all three paired samples was shown in Supplemental file (Supplemental miRNA Expression Data). In Sample 1, there were 256 differentially expressed miRNAs identified, which include 141 up-regulated and 114 down-regulated miRNAs over two-fold. In Sample 2, a total of 78 differentially expressed miRNAs were identified, of which 27 miRNAs were up-regulated and 51 miRNAs were down-regulated over two-fold. In Sample 3, a total of 231 miRNAs with different expression before and after chondrogenic induction were identified, of which 144 were up-regulated and 87 were down-regulated over two-fold. Results are summarized in detail in supplemental data files. We noticed there was a big variation regarding the number of differentially expressed miRNAs among the three independent samples. miRNAs with consistent differential expression in three paired samples were therefore identified, which include eight up-regulated miRNAs (miR-193b, miR-199a-3p/hsa-miR-199b-3p, miR-455-3p, miR-210, miR-381, miR-92a, miR-320c, and miR-136), and four down-regulated miRNAs (miR-490-5p, miR-4287, miR-BART8*, and miR-US25-1*) as shown in Table II.

qRT-PCR validation of the microarray results

To confirm the microarray results, we conducted real-time qRT-PCR to detect the expression levels of the three representative differentially expressed miRNAs identified by microarray, including two up-regulated miRNAs (miR-193b and miR-199a-3p) and one down-regulated miRNA (miR-490-5p). Parallel-grown hADSCs without chondrogenic induction were used as control. The qRT-PCR showed that all detected miRNAs were differentially expressed between undifferentiated hADSCs and chondrogenic differentiated hADSCs, with substantial consistency in the miRNA microarray results ($P > 0.05$) (Fig. 3).

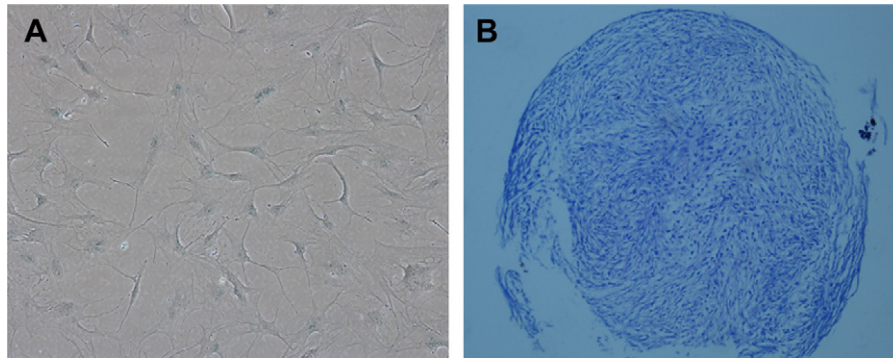


Fig. 1. Morphology of hADSC cells after chondrogenic induction (100 \times). Cells at passage 3 were cultured as micromasses and induced for chondrogenic differentiation using inducing buffer. After 21 days, the micromasses with hADSCs were stained with Alcian blue and visualized under microscope. (A) Control hADSC cells without induction for chondrogenic differentiation at day 6. (B) hADSC cells after induction for chondrogenic differentiation by micromass culture at day 21.

miRNA target prediction

In order to investigate the specific role miRNAs play in the regulation of chondrogenic differentiation, we attempted to predict potential target genes of the differentially expressed miRNAs namely hsa-miR-193b, hsa-miR-199a-3p/hsa-miR-199b-3p, hsa-miR-455-3p, hsa-miR-210, hsa-miR-381, hsa-miR-92a, and hsa-miR-490-5p. Putative targets were predicted using online software programs. Predicted targets of each miRNA contain a large set of genes potentially involved in cell proliferation, cell differentiation, cell cycle regulation, transcriptional regulation and signal transduction. In Table III, only potential target genes related to chondrogenic differentiation, cartilage formation and signal transduction are listed. The up-regulated miRNAs (hsa-miR-193b, hsa-miR-199a-3p/hsa-miR-199b-3p, hsa-miR-455-3p, hsa-miR-210, hsa-miR-381, and hsa-miR-92a) in chondrogenic differentiation of hADSCs were predicted to target genes such as CCAAT/enhancer binding protein beta (C/EBP β), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- α (NFKB1A or IKBA), sex determining region Y (SRY)-box 5 (SOX5), mitogen

activated kinase-like protein 1 (MAPK1), runt-related transcription factor 2 (RUNX2), and bone morphogenetic protein receptor 2 (BMPR2). Predicted target genes of the down-regulated miRNA were SOX4, SMAD4, SMAD5, MAPK1, and BMPR2.

mRNA expression of the predicted target genes before and after chondrogenic induction

To verify the putative targets of the differentially expressed miRNAs, we carried out qPCR analysis (Fig. 4) to examine the mRNA expression of predicted targets C/EBP β , RUNX2, NF- κ B1 and NF- κ B2, which are related to chondrogenic differentiation. We found the expression of these detected potential target genes in chondro-differentiated hADSC was significantly up-regulated, except for NF- κ B1 ($P > 0.05$).

Discussion

miRNAs, a class of short (~22 nt) non-coding, single-stranded RNA molecules, play an important role in differentiation and

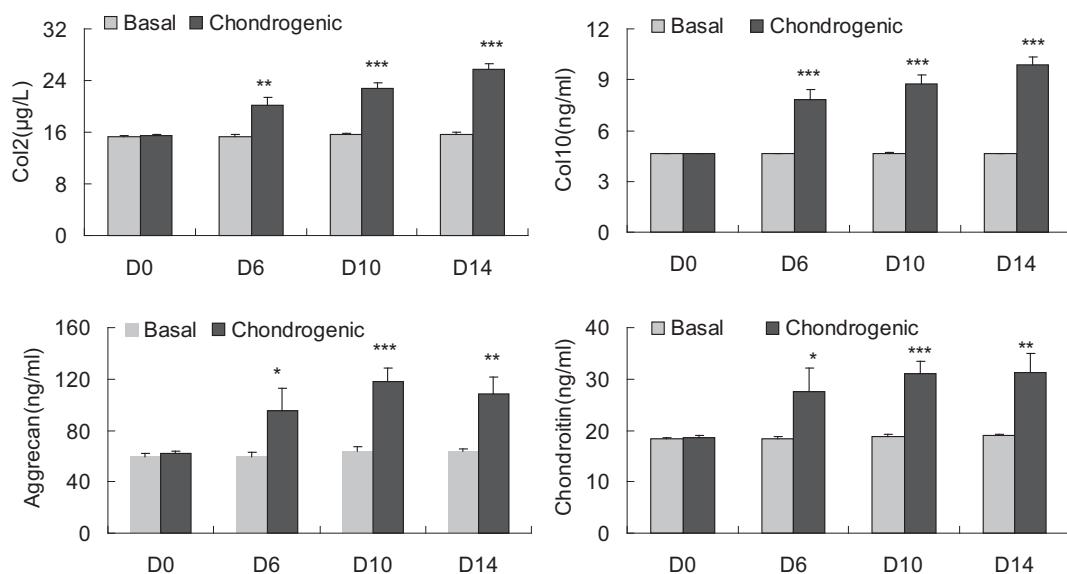


Fig. 2. Determination of chondrogenesis-related proteins expression to confirm chondrogenic differentiation. hADSC cells were induced for chondrogenic differentiation. At day 0, 6, 10 and 14, media were collected and chondrogenesis-related protein concentrations of Col2a1, Col10a1, aggrecan and chondroitin were detected using ELISA. Basal level was obtained using parallel growth media without chondrogenic induction at same time point. Mean \pm S.D. values ($n = 3$ cultures) were presented on the graph. Pair t -test was implemented for comparisons between chondrogenic and basal group at a given time point (* indicates $P < 0.05$; ** means $P < 0.005$; *** denotes $P < 0.001$).

Table II
miRNAs expression in chondrogenic differentiated hADSCs by microarray

Gene name	Gene ID	Sample 1 fold change	Sample 2 fold change	Sample 3 fold change	Average fold change	STDEV
miRNAs up-regulated more than two-folds in all three samples						
hsa-miR-193b	10,987	3.1075056	2.87459181	2.27071	2.750936	0.431885
hsa-miR-199a-3p/hsa-miR-199b-3p	10,995	3.609247053	4.422484486	2.1424689	3.3914	1.155513
hsa-miR-455-3p	28,950	4.06924905	2.43942769	2.41203	2.973569	0.948986
hsa-miR-210	145,852	4.89686732	7.47132223	20.3964	10.92153	8.305831
hsa-miR-381	145,832	3.938098859	3.001146438	2.5195419	3.152929	0.721356
hsa-miR-92a	145,693	6.32213505	4.70770313	2.16024	4.396693	2.098306
hsa-miR-320c	46,228	5.36642876	2.83912774	2.10609	3.437216	1.710478
hsa-miR-136	10,943	2.895205	2.417214	7.076207524	4.129542	2.563054
miRNAs down-regulated more than two-folds in all three samples						
hsa-miR-490-5p	17,822	-3.671	-2.627754	-4.833253	-3.710669	-1.103285
hsa-miR-4287	147,938	-2.409416	-9.195081	-6.896076	-6.166858	-3.451106
ebv-miR-BART8*	17,328	-2.702788	-3.757458	-2.159921	-2.873389	-0.812318
hcmv-miR-US25-1*	42,458	-7.12345	-2.600192	-6.400819	-5.374821	-2.429912

development across a wide range of organisms and tissue types^{17,18}, including bone and cartilage development^{8,15,19–22}. To provide a view of the specific involvement of miRNAs on chondrogenesis of hADSCs, we used miRNA microarray technique to profile miRNA expression and screen miRNAs with differential significant expression (greater than two-fold change) before and after chondrogenic induction. We found that miR-193b, miR-199a-3p/hsa-miR-199b-3p, miR-455-3p, miR-210, miR-381, miR-92a, miR-320c, and miR-136 were up-regulated, and miR-490-5p, miR-4287, miR-BART8*, and miR-US25-1* were down-regulated in all samples tested. Considering the important roles of miRNAs during chondrogenesis of MSC^{9,21,23}, these consistently overexpressed miRNAs could potentially alter the chondrogenic differentiation of hADSCs.

The miRNAs highly up-regulated during chondrogenic differentiation have not been previously shown in human ADSCs. miR-193b, miR-199a and miR-199a* have been identified in MSCs and regulated chondrogenesis^{10,11,23,24}. Swinger *et al.* also reported that miR-455-3p is expressed during chondrogenesis and in adult articular cartilage²¹. It was reported that miR-140 was expressed in cartilage development of zebrafish and mouse^{25,26}, in chondrocyte differentiation and in human articular cartilage²⁷. In our study, miR-140-5p was up-regulated in two samples with a fold change over 2. It was reported that the expression of miR-140-5p was increased in

human osteoarthritic cartilage recently²¹, and that miR-140 was involved in the chondrogenesis^{20,28}, indicating that miR-140 is important in cartilage development. However, it only showed significant over-expression in two of our three paired samples, not over two-fold up-regulated in all three paired samples as has been shown in the other up-regulated miRNAs in this study. Furthermore, these miRNAs that were significantly expressed during chondrogenesis of hADSCs were not significantly expressed during osteogenic differentiation of hADSC cells¹⁵. Therefore, the expression of miRNAs with over two-fold change during chondrogenesis of hADSCs should be considered for a role in the cartilage development.

The metabolism of chondrocytes was regulated at both transcriptional and post-transcriptional levels^{29,30}, and miRNAs, as important regulators in chondrogenesis at post-transcriptional level^{8,11,19}, play the regulatory role by binding target genes, largely based on the transcription factors. For example, miR-199a*, a BMP2-responsive miRNA, regulated chondrogenesis via direct targeting to SMAD1 in murine MSCs¹⁰, and miR-455-3p regulated TGF β signaling, suppressing the SMAD2/3 pathway during chondrogenesis²¹. To explore potential target genes of miRNAs, as reported here, we used miRNA target predicting software¹⁵, and several chondrogenic-related transcription factors were indicated,

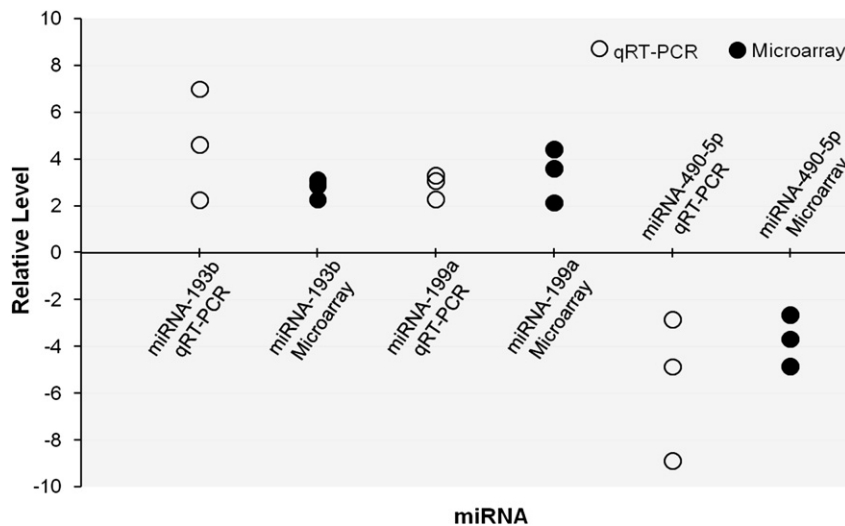


Fig. 3. qRT-PCR confirmation of miRNA expression in chondrogenic differentiated hADSC cells. 1 μ g of total RNA purified from undifferentiated and differentiated hADSCs were used for qRT-PCR analysis. The RNA samples were reverse transcribed to cDNA, and then amplified. Quantitative real-time PCR was done with indicated primers and fold-change of mRNA was normalized to U6 mRNA. Each point represents the mean value of fold change compared with undifferentiated cell control samples (set as 1) from three patients, and each was repeated three times.

Table III

Predicted target genes of chondrogenic miRNAs. Only genes that are related to cartilage formation and chondrogenic differentiation are listed here

miRNA	Target genes	Functions
hsa-miR-193b	RUNX2	Chondrogenesis
	SOX4	Chondrogenesis, apoptosis
	SMAD-3, 4, 5	Chondrogenesis
	MAPK-1, 10, 12	Chondrogenesis, cell cycle control, apoptosis
hsa-miR-199a-3p/ hsa-miR-199b-3	BMPR2, BMP-2, 6, 7	Chondrogenesis
	MAPK-1, 10	Chondrogenesis, cell cycle control, apoptosis
	SMAD-1, 4, 5	Chondrogenesis
	BMPR2, BMP2	Chondrogenesis
hsa-miR-455-3p	TGFB1	Chondrogenesis, cell cycle control, apoptosis
	MMP26	Collagen metabolic/catabolic
	NFKBIA	Self-renewal and multipotency, cell proliferation and differentiation, cell cycle control, apoptosis
	CEBPβ	Chondrogenesis
	RUNX2	Chondrogenesis
	SMAD-3, 4, 5	Chondrogenesis
	SOX-4, 5, 6, 9	Chondrogenesis, apoptosis
	MAPK-1, 12	Chondrogenesis, cell cycle control, apoptosis
	BMPR2, BMP-6, 7	Chondrogenesis
	TGFB1	Chondrogenesis, cell cycle control, apoptosis
hsa-miR-210	CEBPβ	Chondrogenesis
	RUNX2	Chondrogenesis
	SAMD-4, 5	Chondrogenesis
	SOX2	Chondrogenesis
	MAPK1	Chondrogenesis, apoptosis
hsa-miR-381	BMPR2, BMP6	Chondrogenesis
	NFKBIA	Self-renewal and multipotency, cell proliferation and differentiation, cell cycle control, apoptosis
	CEBPβ	Chondrogenesis
	RUNX2	Chondrogenesis
	SOX-2, 4, 5, 6, 9	Chondrogenesis, apoptosis
	SMAD-3, 4, 5	Chondrogenesis
	MAPK-1, 9	Chondrogenesis, cell cycle control, apoptosis
	BMPR2, BMP6	Intracellular signaling cascade, cell proliferation
	MMP7	Collagen metabolic/catabolic, cell proliferation
	hsa-miR-92a	CEBPβ
RUNX2		Chondrogenesis
SOX-4, 9		Chondrogenesis, apoptosis
SMAD-3, 4, 5		Chondrogenesis
MAPK1		Chondrogenesis, apoptosis
BMPR2, BMP7		Chondrogenesis
hsa-miR-490-5p	SOX-2, 4	Chondrogenesis, apoptosis
	SMAD-4, 5	Chondrogenesis
	MAPK-1, 7, 9, 12	Chondrogenesis, cell cycle control, apoptosis
	BMPR2, BMP-7, 9	Chondrogenesis

including C/EBPβ, NFKBIA, Runx2, SOX5, SOX9, MAPK1, SMAD3, and BMPR2^{24,31–38}. In addition, these predicted target genes were up-regulated during chondrogenesis of hADSCs. However, some predicted target genes were supposed to be down-regulated as the potential direct target gene of some up-regulated miRNAs, such as C/EBPβ and Runx2.

We have previously proved that C/EBPβ could enhance the degradation of COL2A1 and aggrecan in human articular chondrocytes, and Interleukin 1 beta (IL-1β) and the adipokine resistin induced the expression of pro-inflammatory cytokines and chemokines *via* post-transcription and transcription, including C/

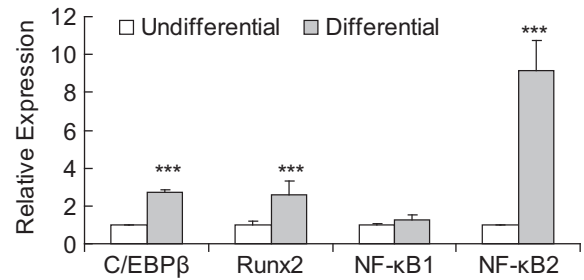


Fig. 4. The expression of targeting transcription factors in chondrogenic differentiated hADSC cells. RNAs from undifferentiated and differentiated hADSCs were tested. Quantitative real-time PCR was done with indicated primers and fold-change of mRNA was normalized to U6 mRNA. Each bar represents the mean \pm S.D. of fold change compared with undifferentiated cell control samples (set as 1) from three patients, and each was repeated three times (***) indicates $P < 0.001$.

EBPβ^{30,39}. It was also reported that C/EBPβ promoted transition from proliferation to hypertrophic differentiation of chondrocytes, and the removal of C/EBPβ could retard the development of osteoarthritis³². In hepatocellular carcinoma (HCC) cells and B-cells, C/EBPβ could regulate the cell proliferation with miRNAs^{40,41}. Here, although C/EBPβ was the candidate target gene of several up-regulated miRNAs during chondrogenesis of hADSCs, it was still a bit increased compared to pre-chondrogenesis. One reason was that other factors which increased the degradation of ADSC during chondrogenesis could increase the C/EBPβ. Moreover, multiple C/EBPβ isoforms with stimulatory or inhibitory activity can be translated from a single mRNA by use of alternative translation initiation sites within the same open reading frame, including liver-enriched transcriptional activator proteins (LAP) and liver-enriched inhibitory protein (LIP)^{39,42–44}. Therefore, C/EBPβ was an interesting potential target factor for miRNAs during chondrogenesis in the balance of chondrogenesis and degradation, and we should pay more attention to C/EBPβ in cartilage metabolism as we previously reported³⁹.

Runx2 (cbfa1) was up-regulated during chondrogenesis of hADSCs as we have shown previously⁴⁵, and in the perichondrium⁴⁶, implying that it may play a role during chondrogenesis. Runx2 could inhibit chondrocyte proliferation and hypertrophy⁴⁶, and its direct transcriptional target was Type X collagen during chondrogenesis⁴⁷. Analysis of the growth plates in transgenic mice and in chick limbs showed that Runx2 promoted chondrogenesis either by maintaining or by initiating early chondrocyte differentiation³⁴. Furthermore, Runx2 regulated differentiation of MSCs as the target of miRNAs^{48,49}. Moreover, C/EBPβ could enhance the activity of Runx2⁵⁰. Runx2 was also an important target gene of miRNAs in osteogenesis as we reported in hADSCs¹⁵. Osteogenic and chondrogenic miRNAs could co-regulate the chondrogenesis of MSCs by some co-target genes. Stein *et al.* have reported that 11 Runx2-targeting miRNAs (miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218, and miR-338) were expressed in a lineage-related pattern in mesenchymal cell types, and during both osteogenic and chondrogenic differentiation, these miRNAs, in general, were inversely expressed relative to Runx2⁵¹. Therefore, C/EBPβ and Runx2 were targeted genes of the up-regulatory miRNAs during chondrogenesis, but they were not shown simply down-regulation as we considered. For the transcriptional factors such as C/EBPβ and Runx2, which widely cooperated with others transcriptional factors and miRNAs in cartilage metabolism, the regulatory circuit should be considered, not just the one-to-one direct regulation.

Another predicted candidate target gene, NFKBIA (IKBA), binds NF-κB and inhibits its function⁵². The NFKBIA was the potential regulator of chondrogenesis in hADSCs, which means NF-κB could

be involved in the regulation of chondrogenesis of hADSCs. We showed that NF- κ B subunits NF- κ B1 and NF- κ B2 were up-regulated in human ADSCs, but the levels of NF- κ B1 mRNA were not significantly affected in cells undergoing chondrogenesis ($P > 0.05$). Moreover, IKBA, which blocks the signal-induced, post-translational activation of canonical NF- κ B subunits, did not affect the levels of specific canonical NF- κ B subunit mRNAs⁵³. In contrast, NF- κ B p100, which encodes the precursor of the NF- κ B p52 subunit, is a direct target of canonical NF- κ B heterodimers⁵⁴, and as such the up-regulation of miRNAs that may target NFKBIA/IKBA may be consistent with our NF- κ B2 mRNA data. In fact, the activation of NF- κ B was associated with inhibition of chondrogenesis of MSCs by both IL-1 β and TNF α in a dose-dependent manner³⁵. NF- κ B has a close relationship with miRNAs during cell regulation, and a positive feedback loop involving NF- κ B and miRNA plays an important role in maintaining the epigenetic transformed state⁵⁵.

Recently, more and more studies have indicated that cell differentiation is regulated by a regulatory circuit at both the transcriptional and post-transcriptional levels. Human granulocytic differentiation was controlled by a regulatory circuitry involving miR-223 and two transcriptional factors, NFI-A and C/EBP α ⁵⁶. A regulatory circuit comprised NF- κ B, Lin28, Let-7 miRNA, STAT3 and IL-6, and a minicircuitry of miR-124, IL-6R, STAT3, miR-24, miR-629 and HNF4 α , was involved in cell transformation^{55,57}. From our predicted targets of miRNAs, it seems up-regulated and down-regulated miRNAs comprised some of minicircuitry during chondrogenesis of hADSCs by co-regulating some of the transcriptional factors. Therefore, it can be expected that the regulatory loop comprised these overexpressed miRNAs and their targeted transcriptional factors have a significant impact on the chondrogenic differentiation of hADSCs, and should be considered in the treatment of cartilage defects and the retardation of the degradation of tissue-engineered cartilage.

In summary, we have discovered a group of miRNAs that are differentially expressed during the chondrogenic differentiation process in hADSCs. Multiple miRNAs are known to work in a coordination to regulate chondrogenic differentiation in these cells. There are multiple potential targets of a single miRNA, and one potential target gene may be linked with several identified miRNAs, indicating complex regulatory circuit during chondrogenesis of hADSCs. To provide the precise circulating regulatory mechanism of these overexpressed miRNAs and transcriptional factors during chondrogenic differentiation, further studies are required. Our results just provide the basis for further investigation into the molecular mechanisms of chondrogenesis in hADSCs and hADSC cell differentiation for cartilage engineering.

Contributions

- Conception and experiment design: Zhiqi Zhang, Weiming Liao.
- Analysis and interpretation of the data: Zhiqi Zhang, Weiming Liao, Yan Kang, Xuerong Li, Ziji Zhang, Hao Zhang, Xin Duan, Jin Liu.
- Drafting of the article: Zhiqi Zhang, Yan Kang.
- Critical revision of the article for important intellectual content: Zhiqi Zhang, Weiming Liao.
- Final approval of the article: Zhiqi Zhang, Yan Kang, Xuerong Li, Weiming Liao.
- Collection and assembly of data: Zhiqi Zhang, Yan Kang, Ziji Zhang, Hao Zhang, Xin Duan, Jin Liu, Xuerong Li.

Funding source

These studies were funded by the National Natural Science Foundation of China (No. 30872615 and 81171709).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We are grateful to Drs M.F. Rai from Washington University School of Medicine, and Gretchen Hensley from University of Texas Southwestern for the critical reading of this paper. The authors would like to thank Drs Aishan he and Ming Fu for adipose tissues from patients. The authors would also like to thank Drs Guangxing Huang and Jianen Huang at the Sun Yat-sen University for valuable assistance.

Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.joca.2012.08.024>.

References

1. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell* 2009;136:642–55.
2. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 1993;75:843–54.
3. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* 1993;75:855–62.
4. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* 2003;113:25–36.
5. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004;303:83–6.
6. Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G. Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA* 2003;9:180–6.
7. Lu J, Qian J, Chen F, Tang X, Li C, Cardoso WV. Differential expression of components of the microRNA machinery during mouse organogenesis. *Biochem Biophys Res Commun* 2005;334:319–23.
8. Kobayashi T, Lu J, Cobb BS, Rodda SJ, McMahon AP, Schipani E, et al. Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc Natl Acad Sci USA* 2008;105:1949–54.
9. Yan C, Wang Y, Shen XY, Yang G, Jian J, Wang HS, et al. MicroRNA regulation associated chondrogenesis of mouse MSCs grown on polyhydroxyalkanoates. *Biomaterials* 2011;32:6435–44.
10. Lin EA, Kong L, Bai XH, Luan Y, Liu CJ. miR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1. *J Biol Chem* 2009;284:11326–35.
11. Suomi S, Taipaleenmaki H, Seppanen A, Ripatti T, Vaananen K, Hentunen T, et al. MicroRNAs regulate osteogenesis and chondrogenesis of mouse bone marrow stromal cells. *Gene Regul Syst Bio* 2008;2:177–91.
12. Nakao N, Nakayama T, Yahata T, Muguruma Y, Saito S, Miyata Y, et al. Adipose tissue-derived mesenchymal stem cells facilitate hematopoiesis in vitro and in vivo: advantages over bone marrow-derived mesenchymal stem cells. *Am J Pathol* 2010;177:547–54.
13. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, et al. Clonal analysis of the differentiation potential of human

- adipose-derived adult stem cells. *J Cell Physiol* 2006;206:229–37.
14. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;7:211–28.
 15. Zhang ZJ, Zhang H, Kang Y, Sheng PY, Ma YC, Yang ZB, et al. miRNA expression profile during osteogenic differentiation of human adipose-derived stem cells. *J Cell Biochem* 2012;113:888–98.
 16. Zhang L, Su P, Xu C, Yang J, Yu W, Huang D. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Bio-technol Lett* 2010;32:1339–46.
 17. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* 2005;120:21–4.
 18. O'Hara SP, Splinter PL, Gajdos GB, Trussoni CE, Fernandez-Zapico ME, Chen XM, et al. NFkappaB p50-CCAAT/enhancer-binding protein beta (C/EBPbeta)-mediated transcriptional repression of microRNA let-7i following microbial infection. *J Biol Chem* 2010;285:216–25.
 19. Dong S, Yang B, Guo H, Kang F. MicroRNAs regulate osteogenesis and chondrogenesis. *Biochem Biophys Res Commun* 2012;418:587–91.
 20. Miyaki S, Nakasa T, Otsuki S, Grogan SP, Higashiyama R, Inoue A, et al. MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. *Arthritis Rheum* 2009;60:2723–30.
 21. Swingler TE, Wheeler G, Carmont V, Elliott HR, Barter MJ, Abu-Elmagd M, et al. The expression and function of microRNAs in chondrogenesis and osteoarthritis. *Arthritis Rheum* 2012;64:1909–19.
 22. Yamasaki K, Nakasa T, Miyaki S, Ishikawa M, Deie M, Adachi N, et al. Expression of MicroRNA-146a in osteoarthritis cartilage. *Arthritis Rheum* 2009;60:1035–41.
 23. Han J, Yang T, Gao J, Wu J, Qiu X, Fan Q, et al. Specific microRNA expression during chondrogenesis of human mesenchymal stem cells. *Int J Mol Med* 2010;25:377–84.
 24. Laine SK, Alm JJ, Virtanen SP, Aro HT, Laitala-Leinonen TK. MicroRNAs miR-96, miR-124, and miR-199a regulate gene expression in human bone marrow-derived mesenchymal stem cells. *J Cell Biochem* 2011;113:2687–95.
 25. Tuddenham L, Wheeler G, Ntounia-Fousara S, Waters J, Hajihosseini MK, Clark I, et al. The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. *FEBS Lett* 2006;580:4214–7.
 26. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, et al. MicroRNA expression in zebrafish embryonic development. *Science* 2005;309:310–1.
 27. Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One* 2008;3:e3740.
 28. Yang J, Qin S, Yi C, Ma G, Zhu H, Zhou W, et al. MiR-140 is co-expressed with Wwp2-C transcript and activated by Sox9 to target Sp1 in maintaining the chondrocyte proliferation. *FEBS Lett* 2011;585:2992–7.
 29. Tebo JM, Datta S, Kishore R, Kolosov M, Major JA, Ohmori Y, et al. Interleukin-1-mediated stabilization of mouse KC mRNA depends on sequences in both 5'- and 3'-untranslated regions. *J Biol Chem* 2000;275:12987–93.
 30. Zhang Z, Xing X, Hensley G, Chang LW, Liao W, Abu-Amer Y, et al. Resistin induces expression of proinflammatory cytokines and chemokines in human articular chondrocytes via transcription and messenger RNA stabilization. *Arthritis Rheum* 2010;62:1993–2003.
 31. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. *J Biol Chem* 2005;280:8343–50.
 32. Hirata M, Kugimiya F, Fukai A, Ohba S, Kawamura N, Ogasawara T, et al. C/EBPbeta promotes transition from proliferation to hypertrophic differentiation of chondrocytes through transactivation of p57. *PLoS One* 2009;4:e4543.
 33. Retting KN, Song B, Yoon BS, Lyons KM. BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. *Development* 2009;136:1093–104.
 34. Stricker S, Fundele R, Vortkamp A, Mundlos S. Role of Runx genes in chondrocyte differentiation. *Dev Biol* 2002;245:95–108.
 35. Wehling N, Palmer GD, Pilapil C, Liu F, Wells JW, Muller PE, et al. Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways. *Arthritis Rheum* 2009;60:801–12.
 36. Xu J, Kang Y, Liao WM, Yu L. MiR-194 regulates chondrogenic differentiation of human adipose-derived stem cells by targeting Sox5. *PLoS One* 2012;7:e31861.
 37. Yamane S, Reddi AH. Induction of chondrogenesis and superficial zone protein accumulation in synovial side population cells by BMP-7 and TGF-beta1. *J Orthop Res* 2008;26:485–92.
 38. Yang B, Guo H, Zhang Y, Chen L, Ying D, Dong S. MicroRNA-145 regulates chondrogenic differentiation of mesenchymal stem cells by targeting Sox9. *PLoS One* 2011;6:e21679.
 39. Zhang Z, Bryan JL, DeLassus E, Chang LW, Liao W, Sandell LJ. CCAAT/Enhancer-binding protein {beta} and NF-{kappa}B mediate high level expression of chemokine genes CCL3 and CCL4 by human chondrocytes in response to IL-1{beta}. *J Biol Chem* 2010;285:33092–103.
 40. Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of E-micro-MiR-155 transgenic mice. *Blood* 2009;114:1374–82.
 41. Wang B, Majumder S, Nuovo G, Kutay H, Volinia S, Patel T, et al. Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. *Hepatology* 2009;50:1152–61.
 42. Descombes P, Schibler U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 1991;67:569–79.
 43. Poli V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem* 1998;273:29279–82.
 44. Imamura T, Imamura C, Iwamoto Y, Sandell LJ. Transcriptional Co-activators CREB-binding protein/p300 increase chondrocyte Cd-rap gene expression by multiple mechanisms including sequestration of the repressor CCAAT/enhancer-binding protein. *J Biol Chem* 2005;280:16625–34.
 45. Rich JT, Rosova I, Nolta JA, Myckatyn TM, Sandell LJ, McAlinden A. Upregulation of Runx2 and Osterix during in vitro chondrogenesis of human adipose-derived stromal cells. *Biochem Biophys Res Commun* 2008;372:230–5.
 46. Hinoi E, Bialek P, Chen YT, Rached MT, Groner Y, Behringer RR, et al. Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium. *Genes Dev* 2006;20:2937–42.
 47. Zheng Q, Zhou G, Morello R, Chen Y, Garcia-Rojas X, Lee B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. *J Cell Biol* 2003;162:833–42.
 48. Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ, et al. A program of microRNAs controls osteogenic lineage

- progression by targeting transcription factor Runx2. *Proc Natl Acad Sci USA* 2011;108:9863–8.
49. Huang J, Zhao L, Xing L, Chen D. MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. *Stem Cells* 2010;28:357–64.
 50. Tominaga H, Maeda S, Hayashi M, Takeda S, Akira S, Komiya S, *et al.* CCAAT/enhancer-binding protein beta promotes osteoblast differentiation by enhancing Runx2 activity with ATF4. *Mol Biol Cell* 2008;19:5373–86.
 51. Zhang Y, Xie RL, Croce CM, Stein JL, Lian JB, van Wijnen AJ, *et al.* A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc Natl Acad Sci USA* 2011;108:9863–8.
 52. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive I κ B kinase that activates the transcription factor NF- κ B. *Nature* 1997;388:548–54.
 53. Israel A. The IKK complex, a central regulator of NF- κ B activation. *Cold Spring Harb Perspect Biol* 2010;2:a000158.
 54. Huxford T, Ghosh G. A structural guide to proteins of the NF- κ B signaling module. *Cold Spring Harb Perspect Biol* 2009;1:a000075.
 55. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF- κ B, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell* 2009;139:693–706.
 56. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, *et al.* A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBP α regulates human granulopoiesis. *Cell* 2005;123:819–31.
 57. Hatzia Apostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, *et al.* An HNF4 α -miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. *Cell* 2011;147:1233–47.