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

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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. 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.

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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 viruses, and which were first discovered in C. elegans in 19932-3. miRNAs are generated from endogenous transcripts producing hairpin structures by an RNase 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 cells. 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 suppressed.

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 functions. 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. We have also isolated these cells, confirmed that the isolated cells were indeed hADSCs from human adipose tissue, and proved their pluripotency.

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 study. 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 × 10^5 cells/ml. Droplets (12.5 μl) 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 μl complete chondrogenic medium. The complete chondrogenic medium (Cyagen, USA) contained 194 ml basal medium (Cat. No. HUXMA-03041-194), 20 μl dexamethasone, 600 μl ascorbate, 2 ml ITS + Supplement, 200 μl sodium pyruvate, 200 μl praline, and 2 ml transforming growth factor beta 3 (TGF-β3). 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 μm 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 miRCURYTM 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 miCURY™ 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 miCURY™ LNA Array (v.14.0) (Exiqon) according to array manual. The total 25 μl mixture from Hy3™-labeled samples with 25 μl 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 μg of total RNA. Primers were designed using Primer
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 were 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.

Table I

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Primer sequences used for qRT-PCR of the microarray results</th>
<th>miRNA</th>
<th>Primer sequences used for qRT-PCR of the microarray results</th>
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<td>U6</td>
<td>R(‘5 AAGGCTGCGCATTTTCTGTCCG)</td>
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<tr>
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<td></td>
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<tr>
<td>18s rRNA</td>
<td>F(‘5 CCTGATCCGCTTTTACGTCCG)</td>
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<td>C/EBPβ</td>
<td>R(‘5 GACGGCATCAGAATCGCTCG)</td>
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<td>Runx2</td>
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<tr>
<td>NF-κB2</td>
<td>R(‘5 GACGGCATCAGAATCGCTCG)</td>
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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 chondrogenically differentiated hADSCs, with substantial consistency in the miRNA microarray results (P > 0.05) (Fig. 3).
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\(\beta\)), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (NFKBIA 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.

**miRNA 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\(\beta\), RUNX2, NF-\(\kappa\)B1 and NF-\(\kappa\)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-\(\kappa\)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...
development across a wide range of organisms and tissue types, including bone and cartilage development. 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, miR-199b-3p, miR-210, miR-381, miR-92a, and 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 MSCs, 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. Swingler et al. also reported that miR-455-3p is expressed during chondrogenesis and in adult articular cartilage. It was reported that miR-140 was expressed in cartilage development of zebrafish and mouse, in chondrocyte differentiation and in human articular cartilage. 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 recently, and that miR-140 was involved in the chondrogenesis, 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. Further, these miRNAs that were significantly expressed during chondrogenesis of hADSCs were not significantly expressed during ostogenic differentiation of hADSC cells. 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, and miRNAs, as important regulators in chondrogenesis at post-transcriptional level, 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 MSCs, and miR-455-3p regulated TGFβ signaling, suppressing the SMAD2/3 pathway during chondrogenesis. To explore potential target genes of miRNAs, as reported here, we used miRNA target predicting software, and several chondrogenic-related transcription factors were indicated.

Table II
<table>
<thead>
<tr>
<th>miRNAs up-regulated more than two-folds in all three samples</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Sample 1 fold change</th>
<th>Sample 2 fold change</th>
<th>Sample 3 fold change</th>
<th>Average fold change</th>
<th>STDEV</th>
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<th>miRNAs down-regulated more than two-folds in all three samples</th>
<th>Gene name</th>
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<th>Sample 3 fold change</th>
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Fig. 3. qRT-PCR confirmation of miRNA expression in chondrogenic differentiated hADSC cells. 1 μg 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 miRNA was normalized to U6 miRNA. 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.
including C/EBPβ, NFKBIA, Runx2, SOX5, SOX9, MAPK1, SMAD3, and BMPR2. 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 could enhance the cartilage metabolism as we previously reported. EBPβ30,39. 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 osteoarthritis.32. In hepatocellular carcinoma (HCC) cells and B-cells, C/EBPβ could regulate the cell proliferation with miRNA C/EBPβ30,41. 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).38,42-44. 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 reported.

Runx2 (cbfa1) was up-regulated during chondrogenesis of hADSCs as we have shown previously45, and in the perichondrium,46, implying that it may play a role during chondrogenesis. Runx2 could inhibit chondrocyte proliferation and hypertrophy,46, 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 miRNAs38,40. Moreover, C/EBPβ could enhance the activity of Runx2.50 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 Runx2.25. Therefore, C/EBPβ and Runx2 were targeted genes of the up-regulative 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

### Table III

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target genes</th>
<th>Functions</th>
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</table>

### 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 miRNA was normalized to U6 mRNA. Each bar represents the mean ± 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 < 0.0001).
be involved in the regulation of chondrogenesis of hADSCs. We showed that NF-κB subunits NF-κB1 and NF-κB2 were up-regulated in human ADSCs, but the levels of NF-κB1 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-κB subunits, did not affect the levels of specific canonical NF-κB subunit miRNAs. In contrast, NF-κB p100, which encodes the precursor of the NF-κB p52 subunit, is a direct target of canonical NF-κB heterodimers, and as such the up-regulation of miRNAs that may target NFKBIA/IKBA may be consistent with our NF-κB2 mRNA data. In fact, the activation of NF-κB was associated with inhibition of chondrogenesis of MSCs by both IL-1β and TNFα in a dose-dependent manner. NF-κB has a close relationship with miRNAs during cell regulation, and a positive feedback loop involving NF-κB and miRNA plays an important role in maintaining the epigenetic transformed state.

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 circuit involving miR-223 and two transcriptional factors, NFI-A and C/EBPβ, was involved in cell transformation. From our results just provide the basis for further investigation into the molecular mechanisms of chondrogenesis in hADSCs and hADSC cell differentiation for cartilage engineering.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supplementary data**

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

**References**


**Contributions**

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