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Original Article

Dynamic of miRNA-101a-3p and miRNA-200a during Induction of Osteoblast Differentiation in Adipose-derived Mesenchymal Stem Cells

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miRNAs are known as the cellular phenomena regulators that exert their effects in post-transcriptional level. Recent studies highlight the role of miRNAs in mesenchymal stem cells differentiation into osteoblasts. The purpose of this study was to recognize the pattern of miRNA-101a-3p and miRNA-200a expression during osteoblastic differentiation of human adipose tissue-derived mesenchymal stem cells. The cells were incubated in osteoblastic differentiation medium for a period of 21 days. Alizarin red S staining was performed to confirm the successful differentiation of adipose-derived mesenchymal stem cells into osteoblast cells. The expression levels of miRNA-101a-3p and miRNA-200a were analyzed by real-time PCR during 0, 7, 14, and 21 days after differentiation induction. Data exhibited the increase of extracellular red color deposition which was evident at the end of the incubation period. The expression of miRNA-101a-3p and miRNA-200a was up regulated during adipose-derived mesenchymal stem cells trans-differentiation into osteoblast-like cells. These miRNAs could be potential novel biomarkers for monitoring successful differentiation of mesenchymal stem cells toward osteoblasts.

Key words: Adipose-derived-mesenchymal stem cells, osteoblast-like differentiation, miRNA-101a-3p, miRNA-200a, transcription

The number of people with osteoporosis and bone-related pathologies increases globally. Today, conventional therapies such as drug therapy are commonly used for their remediation (1). In most cases, there are concerns about the long-term application of drugs and safety of therapeutic modalities (2). In this regard, attempts have been made to develop *de novo* treatments focusing on bone tissue replacement and regeneration acceleration (3). For instance, mesenchymal stem

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cells (MSCs) are multipotent progenitors that could differentiate into several cell types such as adipocytes, chondrocytes, and osteoblasts *etc*. upon stimulation with different growth factors, and from distinct niches (4).

Osteoblasts are specialized cells that participate in bone formation by secreting bone matrix proteins. In this process, osteoblast progenitor cells differentiate into preosteoblast, and then these cells become mature. During the last stage of differentiation, osteoblast cells implant in extracellular matrix (ECM) proteins that they secret for mineralization, and convert into mature osteocyte cells. The differentiation of MSCs into various lineages is controlled by different cellular elements notably specific genes. Accordingly, runt-related transcription factor 2 (*RUNX2*), wingless-related integration site (*WNT*), bone morphogenetic proteins (*BMPs*), and transforming growth factor beta (*TGFβ*) along with microRNAs (miRNAs) participate in bone morphogenesis (5). Among them, miRNAs have the potential to regulate 30-70 % of human genes (6). The critical role of miRNAs was previously highlighted in osteoblast differentiation (7-9). In *in vitro* system, miRNAs such as miR-433-3p, miR-129-5p, and miR- 590- 5p promoted cell differentiation while miR-224 activation decreased cell differentiation capacity (10-13). Also, transfection of human MSCs (hMSCs) with nanoparticles harboring miR-199a-5p promoted osteogenic differentiation (8). In addition, miR-148b nanoparticles were shown to induce osteoblast differentiation of human adipose-derived MSCs (hADSCs) in *in vivo* milieu, and improve regeneration of the bone in calvarial defects in the mouse model (9).

Commensurate with these statements, the current experiment investigated the expression of miRNA-101-3p and miRNA-200a genes during osteoblast differentiation of hADSCs over a period of 21 days. This experiment may add data on the dynamics of miRNAs during MSCs differentiation toward osteoblasts.

Materials and methods

Cells expansion

hADSCs (Royan institute; Tehran, Iran) were expanded in low-glucose content Dulbecco's Modified Eagle's Medium (DMEM/LG; Gibco, Germany). To induce cell proliferation, the basal medium was enriched with 10% fetal bovine serum (FBS; Gibco, Germany) and 1% Pen-Strep (Gibco, Germany). Cells were kept at 37 ˚C in 5% CO_2 under humidified atmosphere. Cells at 80% confluence were detached and sub cultured using 0.25% trypsin-EDTA (Gibco, Germany). Cells at passages 3-6 were subjected to different analyzes.

Osteogenic differentiation

To orient cell differentiation into osteogenic phenotype, cells were seeded at an initial density of 1×10^6 per well of 6-well plates (SPL), and allowed to reach 70-80% confluency. Thereafter, the media were replaced by osteogenic medium supplemented with 10% FBS, 0.1 µM dexamethasone (Sigma Aldrich, USA), 50 µM ascorbic acid (Sigma-Aldrich, USA), and 10 mM β-glycerol phosphate (Cat no: G9891; Sigma-Aldrich, USA) (14). This procedure was continued for 21 days. The differentiation medium was replenished every 3-4 days.

Alizarin red S staining

To examine the formation of extracellular minerals, we performed alizarin red staining. To this end, cells were selected 7, 14, and 21 days after osteogenic differentiation. At the respective time points, the medium was discarded and cells were washed three times with phosphate-buffered saline (PBS). Thereafter, cells were fixed by pre-chilled paraformaldehyde (4%) solution for 20 min. Following three washes with PBS, cells were incubated with alizarin red S solution (Sigma-Aldrich, USA) for 30 min. Then, the cells were

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washed again with PBS, and analyzed under an inverted microscope.

Monitoring miRNA200a and miRNA1013-p levels

Total RNA was isolated from ADMSCs on days 7, 14, and 21 days by using Trizol reagent (Cat no: T9424; Sigma-Aldrich, USA) according to the manufacturer's instructions. The integrity and purity of RNA samples were determined using Nanodrop® (Thermo Scientific, USA). To synthesize cDNA, each RNA sample was adjusted to the concentration of 5 ng/µL using nuclease-free water, and cDNA was synthesized using Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark) as follows: samples were incubated at 42˚C for 60 min, then reverse transcriptase was inactivated at 95˚C for 5 min. After the completion of cDNA synthesis, cDNAs were diluted (1:80) with RNase-free water. Real-time PCR were carried out in triplicate using BioMolecular Systems (Model: mic, Australia) according to miRCURY LNA-Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark): 5 µL SYBR Green solution (Batch no: 17D2701; Ampliqon, Denmark), 1 µL locked nucleic acids (LNAs) linear primers (Exiqon, Vedbaek, Denmark) and 4 µl template cDNA (diluted: 1:80) with 10 µl final volume per well. The program of real-time PCR for miRNA-101a-3p consisted of denaturation at 95˚C for 15 min, and 40 amplification cycles of 95 °C for 20 s and 60 °C for 1 min with 0.5 °C per s ramp rate, and for miRNA-200a was denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 10 s and 60 °C for 25 s with 0.5 °C per s ramp rate. Results were normalized with U6 RNA, and analyzed with $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are presented as mean ± SD. Statistical analyzes were performed by using One-Way ANOVA and Tukey post hoc. P values < 0.05 were considered to be statistically significant.

Results

Morphology, appearance, and osteogenic differentiation of ADSCs

Microscopic analysis revealed that ADSCs acquired fibroblast-like morphology at passage three. These cells were uniform with a whirling appearance at the plastic surface (Figure 1). Alizarin red S is used commonly to show the osteogenic capacity of various progenitor cells. In the current experiment, ADSC cells were exposed to the osteogenic induction medium for a period of 21 days. Our data noted a successful differentiation of ADSCs to osteoblast-like cells indicated by red-colored deposition (Figure 1). We found that cells tended to produce extracellular calcium minerals by time. Compared to the control non-treated ADSCs, the deposition of red-colored extracellular minerals reached maximum levels at the end of the experiment (day 21) (Figure 1). These data demonstrated the successful osteogenic orientation of ADSCs over a period of 21 days *in vitro*.

The transcription of miRNAs was modified during osteogenic differentiation

In the current experiment, we monitored the changes in the level of both miRNA-101a-3p and miRNA-200a at days 0, 7, 14, and 21. Data showed that the levels of both miRNAs were increased by the time during ADSCs differentiation into osteoblast-like cells in comparison with the non-treated control (Figure 2). There was a 4.1-fold increase in the transcription of miRNA-101a-3p on day 21 ($P<0.05$). Similar to the changes of miRNA-101a-3p, the transcription of miRNA-200a was induced during ADSC-osteoblast differentiation, and reached maximum level on day 21 (2.5-fold) in comparison with the control ADSCs ($P<0.05$). Interestingly, a decrease was indicated in the expression of both miRNA-101a-3p and miRNA-200a at day 7 (Figure 2). Thereafter, their levels showed an increasing trend. Commensurate with these statements, the dynamics of miRNAs could be modulated during ADSCs differentiation into osteoblasts.

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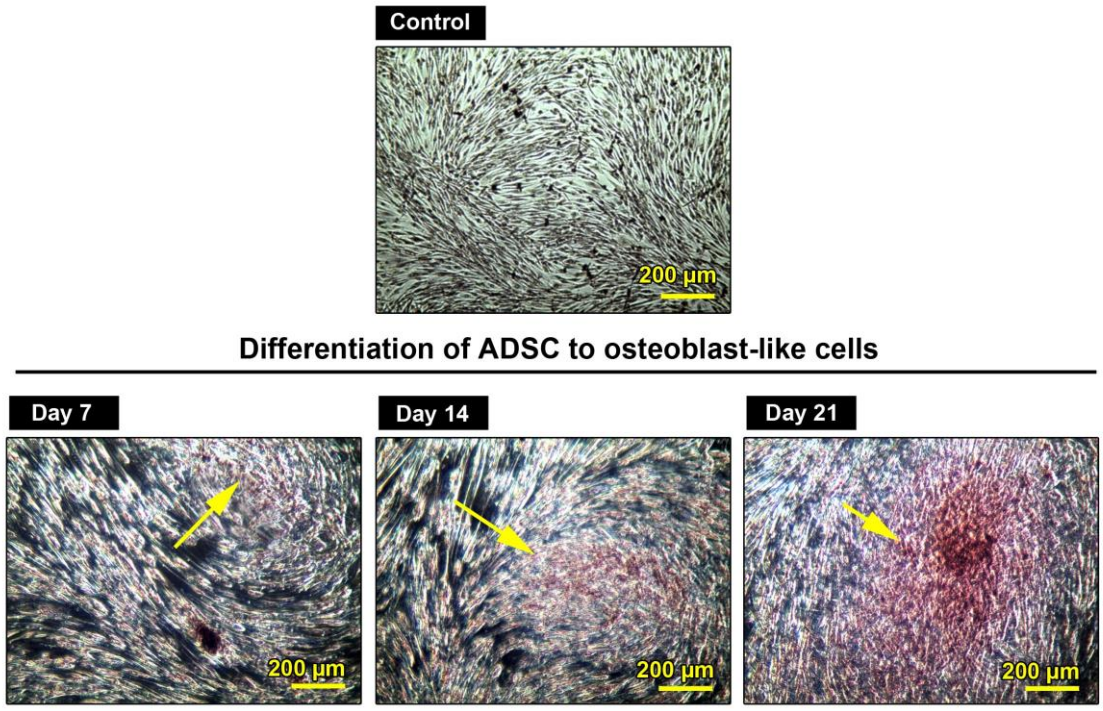

Fig. 1. Alizarin red staining during osteoblastic differentiation of adipose derived stem cells. Osteoblastic differentiation and mineralization of hADSCs were analyzed by alizarin red staining in days 0, 7, 14, and 21. In control (without osteoblastic differentiation medium) red-colored deposition was not observed, while the color intensity increased with time.

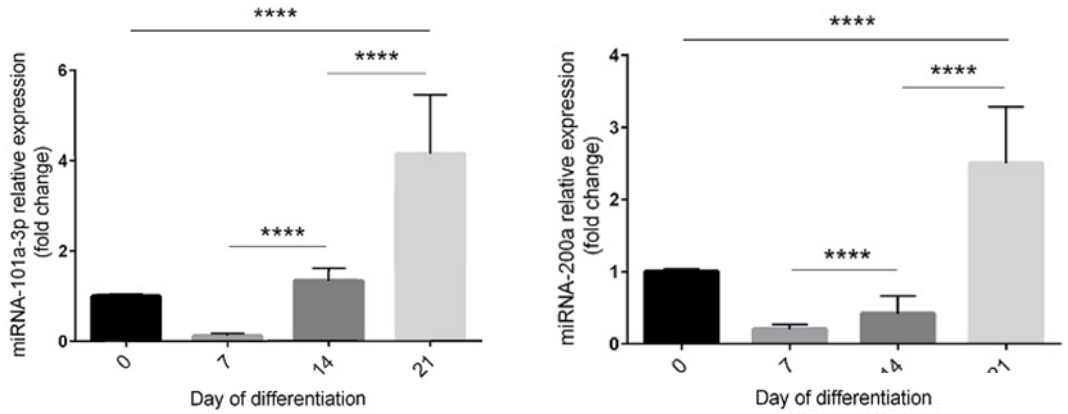

Fig. 2. Expression of miRNA-101a-3p and miRNA-200a during osteoblastic differentiation of adipose derived mesenchymal stem cells. The real-time PCR analysis of miRNA-101a-3p and miRNA-200a expression after 0, 7, 14, and 21 days of adipose-derived mesenchymal stem cells directed osteoblastogenesis. Data are represented as mean ± SD. **** $P < 0.0001$.

Discussion

MSCs have recently been a candidate for the use in stem cell-based therapies. Many factors and clues could affect the therapeutic potential of MSCs (4). In addition to the cell entity and the dynamic growth of factors in surrounding microenvironment, monitoring the gene expression profiles of stem cells, especially miRNAs, during trans-differentiation into the target cell types could help us to precisely forecast the orientation efficiency. As a matter of fact, the identification and measuring of regulatory factors involved in osteoblastic differentiation seems vital in the application of MSCs in stem cell-based therapeutic approaches (5). According to the great body of experiments, the critical role of miRNAs has been proved following

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stem cells orientation toward osteoblasts (7). In this regard, the close relationship between miRNAs i.e. miR-23a/b and osteoblast differentiation was found by using agomir and antagomir system (15). In support of this claim, the transfection of human MSCs and murine osteoblast precursor cell line MC3T3-E1 with nanoparticles carrying miRNA-29b improved osteoblast differentiation, indicating the regulatory role of miRNAs in osteoblast differentiation (16). In addition, osteoblastic differentiation experiments have yielded interesting data. For instance, the expression profile was altered during bone marrow MSCs-osteoblast differentiation (17). We supposed that miRNA-101a-3p and miRNA-200a could be affected during ADSC-osteoblast differentiation, and therefore analyzed the dynamic changes during ADSCs to osteoblast-like cells. Our data was performed to address the dynamic of miRNA-101a-3p and miRNA-200a during ADSCs differentiation into osteoblast-like cells over a period of 21 days. Our results demonstrated that the transcription of miRNA-101a-3p and miRNA-200a was changed during ADSC-osteoblast differentiation, and reached maximum levels at the end of the experiment (day 21).

Documents showed the bioactivity of miRNA-101 under physiological and pathological conditions (18, 19). Previously published data demonstrated that miRNA-101 could act as a tumor suppressor that dictates cancer cell phenotype acquisition (20). Similar to cancerous niche, there are some reports related to the participation of miRNA-101 on osteoblast differentiation of progenitor cells. Consistent with our results, Wang et al. noted that miRNA-101 encouraged BMSCs differentiation toward osteoblast-like cells coincided with the induction of runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase, osteopontin, and osteonectin after 15 days. They also noted the modulatory effect of miRNA-101 via EZH/Wnt/β-catenin signaling axis (18). Like BMSCs, miRNA-101 could also promote osteoblast differentiation of human dental follicle cells, by increasing the transcription of alkaline phosphatase, *RUNX2*, and osterix (21). In line with the changes in the level of miRNA-101, the expression of miRNA-200a was also monitored in the current experiment. In contrast to our data, miRNA-200a was shown to decrease during pre-osteoblast differentiation of MC3T3-E1 cells indicated with the concurrent increase of BMP-2 and enhanced alkaline phosphatase activity during 72 h. One reason would be that the differentiation time monitored was different in comparison with our experiment, as well as the type of cells used for differentiation. Similar to miRNA-101, miRNA-200a targets effectors such which participates in osteoblastic differentiation (22).

In conclusion, we investigated the expression of miRNA-101a-3p and miRNA-200a in the osteoblastic differentiation of hADSCs for the first time. These cells can easily be accessed for therapeutic purposes and should be more considered in future studies. We studied the differentiation process over a 21-day duration that was a longer period than previous studies, and noticed changes in the expressions of miRNA-101a-3p and miRNA-200a during differentiation and mineralization. This study has two main limitations: first, osteoblastic differentiation of hADSCs were not investigated under low-expressed and over-expressed conditions of miRNA-101a-3p and miRNA-200a; and second, the targets of these miRNAs were not examined in the hADSCs. According to our results, it is reasonable to suggest that the dynamics of miRNAs can be helpful during bone morphogenesis. Modulation of miRNAs seems to be helpful in the increase of osteoblastic differentiation of various progenitor cell types. However, more investigations are needed to address the exact modulatory role of miRNAs during bone healing and pathologies.

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Conflict of interest

Authors declare no conflict of interest.

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