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Platelet-Rich Plasma Accelerates Bone Differentiation in Human Adipose-Derived Mesenchymal Stromal Cells: An Experimental Study

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Abstract

Background: Mesenchymal stromal cells (MSCs) have high differentiation potential into different cell lines like osteoblasts. Osteogenic differentiation can be regulated through various and complex mechanisms, such as changing the microRNAs expression level. Although platelet-rich plasma (PRP) has been used in MSCs' osteogenic differentiation process, the molecular mechanisms underlying the effect of PRP induction of osteogenesis by microRNAs is not well understood.
Objectives: We evaluated the effect of PRP on the expression of miR-29a, miR-29b, and miR-155 in the PRP-based osteogenic differentiation of human MSCs.
Methods: This experimental study was conducted on healthy individuals referred to Taleghani Hospital in Ahvaz, Iran, for abdominoplasty from September 2017 to April 2018. Stromal cells were isolated from human adipose tissue and differentiated into osteoblasts. Effect of 10% PRP on osteoblasts differentiation was monitored by the measurement of alkaline phosphatase activity and calcium deposition. We also evaluated gene expression of the *Runx2* and the *OPN* along with the expression of miRNAs. All tests were performed in triplicate.
Results: Treatment of MSCs with 10% PRP resulted in induction of osteogenic differentiation by a significant upregulation of the miR-29a/b (miR-29a: 5.27 (0.77), $P < 0.01$ (day 3) and 3.76 (0.124), $P < 0.01$ (day 14); miR-29b: 3.11 (0.35), $P < 0.001$ (day 3) and 4.25 (0.304), $P < 0.01$ (day 14)) and a significant downregulation in the miR-155 expression (0.62 (0.006), $P < 0.001$ (day 3) and 0.55 (0.114), $P < 0.05$ (day 14)).
Conclusions: The remarkable rise in the expression of important osteoblast genes, alkaline phosphatase activity, and calcium deposition verified accelerated differentiation. The present study showed that microRNAs such as miR-29a/b and miR-155 play an active role in the process of bone differentiation during PRP treatment, which in turn, affects mesenchymal cells signaling pathways.

Keywords: Bone, Differentiation, Gene Expression, Mesenchymal Stromal Cells, MicroRNAs, Osteoblasts, Platelet-Rich Plasma

1. Background

Mesenchymal stromal cells (MSCs) are multipotent cells that differentiate into several classes of cell types, including adipocyte, osteoblast, chondrocyte, and endothelial cells (1, 2). Advantages of MSCs are their immunomodulatory properties, low graft-versus-host disease-responses, and simple and easy isolation (3). The International Society of Cellular Therapy (ISCT) recommends the following minimum criteria for MSC definition: (I) the adhesion ability to the plastic surface, (II) expression of surface markers such as CD37, CD90, and CD105 and non-expression of hematopoietic markers such as CD34, CD14, CD31, and CD45, and (III) the ability to differentiate into different cell types (4, 5).

MSCs can differentiate into chondrocytes and osteoblasts after recruitment to the injury site. They play a key role in bone repair and regeneration after injury and fracture (6, 7). Differentiation of stem cells results from highly regulated gene expression at the transcription or post-transcriptional levels, especially by microRNAs (8). MicroRNAs are transcribed either intergenic with an independent promoter or intragenic with the host promoter and play their inhibitory role generally by pairing with the 3'-end, or less commonly, with the 5'-end of mRNA (9). Bone-regulating miRNAs known as osteomiRs are expressed in the osteoblast cells that regulate bone formation by direct inhibition of osteoblast differentiation inhibitor genes or by responding to osteogenic signals such as bone morphogenetic proteins (BMP). To date, some microRNAs like let-7 family, miR-21, miR-16, miR-29 family, miR-155, miR-322, and miR-30 family have been reported

to be involved in osteoblasts differentiation by regulating the expression of bone differentiation genes, such as osteocalcin (*OCN*), osteopontin (*OPN*), runt-related protein 2 (RUNX-2), collagen 1A (*COL 1A*), and alkaline phosphatase (*ALP*) (10-13).

MSCs are used for cell therapy in regenerative medicine, and direct injections of MSCs or cell-seeded scaffolds are administered to heal bone fractures, but the use of biological catalysts that accelerate this process is also improved (14). These catalysts should induce MSCs differentiation and proliferation while being cheap and biocompatible with no effects on the cellular and biological structure of mesenchymal cells (15). One of the most widely used catalysts is platelet-rich plasma (PRP), which was first described in 2007 as a plasma containing large amounts of growth factors (16). Recent reports refer to the presence of about 600 growth factors in PRP responsible for differentiation, tissue regeneration, and wound healing (17). These growth factors are released when platelets are activated by collagen, thrombin, serotonin, calcium, magnesium, thromboxane, or mechanical factors such as freezing cycles. Recently, PRP has been used in bone reconstruction and orthopedic surgery (15-18). Many studies have been undertaken on PRP and its role in facilitating bone differentiation and repair (19, 20), but how microRNAs' profile is altered under its influence is poorly understood.

The present study aimed to investigate the expression of some miRNAs during differentiation of PRP-treated MSCs to better understand the molecular mechanisms of PRP. Since the changing of microRNAs profile on bone differentiation has been reported in several studies (15, 21, 22), the fact that how PRP alter the miRNA profile during bone differentiation is not well studied. Despite the large number of studies on animal models, there are limited studies on the application of PRP in human disease treatment. However, PRP treatment is still ongoing, and future research can fill the existing gaps.

2. Objectives

In this study, we aimed to address some of the ambiguities regarding PRP application in bone repair. The results of this study could be used to manipulate microRNAs to improve bone damages in the future.

3. Methods

3.1. Isolation and Activation of Platelet-Rich Plasma

This experimental study was conducted from September 2017 to April 2018 in Biochemistry Department of Jundishapur University of Ahvaz and Shahid Chamran University of Ahvaz, Iran. All the experiments were performed in three biological replications by calibrated instruments in three groups (i.e., Control, Osteogenic medium, and 10% PRP).

All the tests were in accordance with regulatory guidance and the Ethics Committee of Shahid Chamran University of Ahvaz (Ethical approval number: EE/96.24.3.88367/.scu.ac.ir).

Blood samples were collected from healthy volunteers in tubes containing anticoagulants, and then they were centrifuged at 1340 rpm in sterile conditions for 10 minutes. The layer containing the platelet was transferred to a new tube and centrifuged at 3000 rpm for 15 minutes. The platelet sediments were dissolved in platelet-poor plasma (PPP) and activated using 20% $CaCl_2$ and incubating at -70°C for two hours. Finally, the samples were centrifuged at 3000 rpm for 15 minutes and preserved at -70°C until use.

3.2. Cell Isolation, Expansion, and Characterization

Adipose tissue samples were collected from healthy individuals undergoing abdominoplasty after obtaining their written consent according to the ethics committee of Shahid Chamran University of Ahvaz (Ahvaz, Iran). Cells isolation was carried out according to the previously published procedure (23). Briefly, the samples were washed to remove the blood cells, 1 mg/mL collagenase IV (Sigma) was added and the samples were incubated for 40 minutes at 37°C. Collagenase was removed by centrifugation at 1200 rpm for 5 min. The isolated cells were cultured in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% pen/strep, and 1% amphotericin (Gibco). To confirm the differentiation potential of the isolated cells, adipogenic differentiation medium (0.5 mM IBMX, 10^{-7} mM dexamethasone, 66 nM insulin, and 0.2 mM indomethacin) and osteogenic medium (10^{-7} mM dexamethasone, 10 mM beta glycerol phosphate, and 50 μg/mL ascorbic acid bi-phosphate) were used, and then Oil Red staining (for lipids) and Alizarin Red staining (for osteoblasts) were carried out. Moreover, the expression levels of CD90, CD105, CD34, and CD45 was determined using flow cytometry.

3.3. Osteogenic Differentiation

Confluent cells between passages three and six were seeded in 6-well culture plates and provided with osteogenic medium (OM: 10^{-7} mM dexamethasone, 10 mM beta-glycerol phosphate, 50 μg/mL ascorbic acid bi-phosphate, in DMEM), 10% PRP group (10% of activated PRP in DMEM), and control group (10% FBS in DMEM), separately. The culture plates were incubated at 37°C in 5% CO_2 for 21 days.

3.4. Alkaline Phosphatase (ALP) Assay

Differentiated cells were lysed with RIPA buffer (cell signal) on days 3, 7 and 14 of osteogenic differentiation. The samples were incubated with an alkaline buffer solution and a phosphatase substrate solution (Pars Azmun, Iran) at 37°C for 60 min. *ALP* activity was measured at 405 nm and normalized against total protein content. Protein content was measured by Lowry Protein Assay (BCA, Life Technologies).

3.5. Calcium Deposition Assay

For calcium deposition assay, differentiated cells were treated with 0.6 N HCl on days 3, 7 and 14 of bone differentiation. The amount of deposited calcium was determined using the Calcium Assay Kit (Pars Azmun, Iran) according to the manufacturers' instruction. The results were normalized to total protein content.

3.6. miRNA and Total RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA and miRNA were extracted using TRIzol (Invitrogen) and Qiagen RNeasy Plus Mini kit, respectively. Then, cDNA was synthesized using TAKARA cDNA synthesis kit, and miRNA first strand cDNA was synthesized by BONmiR kit (BONmiR, Iran) according to the manufacturer's instruction. Finally, the expression of miRNAs and osteogenic genes in all the groups was calculated versus the control group with the comparative CT method ($2^{-\Delta\Delta CT}$) with U6 small nuclear RNA (snRNA) as the internal control for miRNAs and β2M for other genes. The specialty of signals was confirmed by melting curves. Primer sequences are shown in Table 1. Target genes of miRNAs were predicted by PicTar online software (https://pictar.mdc-berlin.de/; Table 2).

3.7. Statistical Analysis

Statistical analyses were carried out by one-way analysis of variance (ANOVA) using SPSS Software, and P value less than 0.05 was considered significant. All the tests were run in triplicate. Data are shown as the mean ± standard deviation (± SD).

Table 1. Sequences of Human Primers Used for Quantitative Polymerase Chain Reaction

Gene Name	Primer Sequence
B2M	
F	ATG CCT GCC GTG TGA AC
R	ATC TTC AAA CCT CCA TGA TG
OPN	
F	GACCTGACATCCAGTACCC
R	GTTTCAGCACTCTGGTCATC
RUNX2	
F	GCC TTC AAG GTG GTA GCC C
R	CGT TAC CCG CCA TGA CAG TA
SNORD	
F	ATC ACT GTA AAA CCG TTC CG
R	Universal reverse primer under BONmiR patent
***miR*-29a**	
F	CAT AGC ACC ATC TGA AAT CT
R	Universal reverse primer under BONmiR patent
***miR*-155**	
F	ACTTGGCTAATCGTGATAGT
R	Universal reverse primer under BONmiR patent
***miR*-29b**	
F	TCACCGTTTGAAATCAGTGC
R	Universal reverse primer under BONmiR patent

4. Results

The isolated cells showed fibroblast-like morphology (Figure 1) and could differentiate into two different lineages. Figure 2 shows Oil Red and Alizarin Red staining for adipocytes and osteoblasts, respectively.

While the expression of CD90 and CD105 was positive in the isolated cells (94.5% and 93.4%, respectively), the expression levels of CD34 and CD45 as hematopoietic markers were negligible (1.3% and 1.49%, respectively). These results confirmed the stemness properties of the isolated cells.

The activity of *ALP* and deposition of calcium were measured according to methods. *ALP* activity increased in OM and 10% PRP groups compared to controls, but the activity of *ALP* enzyme in 10% PRP was significantly higher than in OM at all the time points (Figure 3). Both PRP and OM groups led to deposition of calcium in the extracellular matrix and PRP had a significantly higher stimulatory effect on mineralization (Figure 4).

In all the studied groups, expressions of *RUNX2* and *OPN* were up-regulated on the 14th day of differentiation, and 10% PRP had a higher stimulatory effect on *OPN* and *RUNX2* expression than OM (Figure 3).

PRP alters miR-29a/b and miR-155 expression levels. miR-29a/b expression was increased in the 10% PRP and OM groups during osteoblast differentiation; however, the increased expression observed in the PRP group was significantly higher than in the OM group at different time points (days 3 and 14). The expression of miR-155 was down-regulated in both 10% PRP and OM groups on days 3 and 14 (Figure 5).

Table 2. Predicted Target Genes

miRNAs	Predicted Target Genes
miR-29a	COL1A1, COL4A5, COL4A5, COL4A5, SPARC, PCDHA11, SATB1, SPOCK2 (osteonectin)
miR-29b	CXXC6, C1QTNF6, COL5A3, COL5A2, COL11A1, C1QTNF6, COL4A5, TRIB2, HAS3, TRAF4, SP1, COL4A3, BMP1, HDAC4, TGFB3, DUSP2, CTNNBIP1, PTEN
miR-155	SOCS1, SATB1, ZNF537, BACH1, IKBKE,

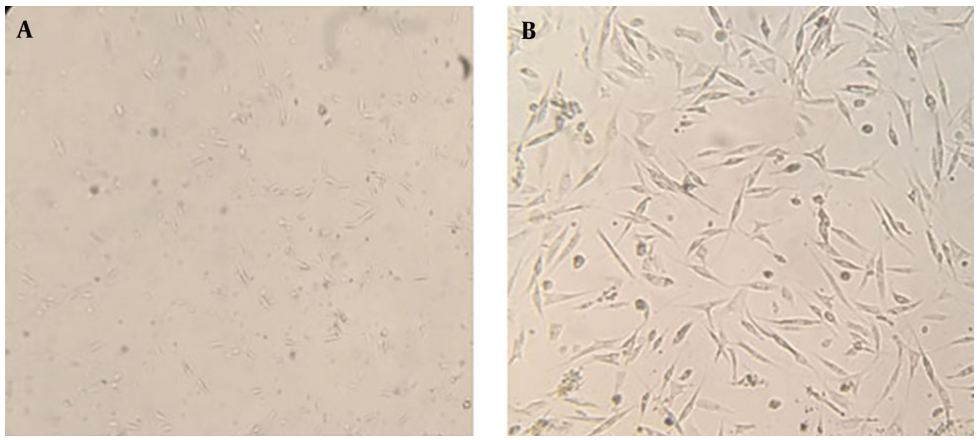

Figure 1. Cells isolated from human adipose tissue on the first day (A), and seventh day (B) of primary culture (× 10)

5. Discussion

Recently, PRP has been widely used as a therapeutic agent for some diseases and injuries like osteoarthritis and bone fractures. While the effects of PRP on bone marrow and adipose-derived MSCs have been extensively investigated (19, 20), the expression profile of miRNAs and other regulatory mechanisms of this substance have not been identified for bone differentiation. In this study for the first time, we aimed to investigate the expression of some important osteogenic microRNAs in PRP-based osteoblast differentiation.

Although some studies have used PRP along with the standard bone differentiation medium (OM) to induce differentiation (18, 24), we found that in the absence of the standard bone differentiation medium, PRP alone could induce bone differentiation. *ALP* activity and calcium deposition in the PRP group increased in the early days of differentiation compared to the OM group, which may be a marker of early differentiation. The higher mineralization and *ALP* activity in the 10% PRP group on the 7th and 14th days may be due to improved bone differentiation in comparison to the OM group. The expression of *RUNX2* and *OPN* genes in the 10% PRP group increased by 2.9 and 4.2 times compared to the OM group, respectively. The results of alkaline phosphatase activity assay, the rate of calcification, and gene expression can be used to verify that activated PRP has a significant effect on the osteogenic differentiation of mesenchymal cells in vitro (Figures 3 and 4).

Different microRNAs can positively or negatively regulate bone differentiation by modifying signaling pathways (25). Wnt signaling pathways, which are the key pathways of bone development, induce miR29a expression, followed by inhibition of Wnt inhibitors (SFRP2, Kermen2, and DKK1) (26). The observed upregulation in miR-29a expression is probably related to the control of the Wnt inhibitor genes.

miR-155 is the negative regulator of bone morphogenetic proteins (BMP) signaling (27), and the results of the present study showed that miR-155 was significantly downregulated in both OM and PRP groups on the 3rd and 14th days of differentiation, which confirms the inhibitory role of this miRNA in MSCs differentiation into osteoblasts.

The expression of bone differentiation inhibitor genes (*HDAC4*, *TGFβ3*, *ACVR2A*, *CTNNBIP1*, and *DUSP2*) is inhibited during the differentiation of MSCs (28). Our predictive analysis showed that these genes are targets for miR-29b, therefore, we suggest that the increased expression of miR-29b may inhibit these genes.

Mineral extracellular matrix (ECM) production is a late-onset event in bone differentiation, and the expression of collagen necessary genes such as *COL1A1*, *COL3A1*, and osteonectin is reduced during this stage to facilitate mineralization (28, 29). Due to the fact that these genes are predicted as the target genes for miR-29a and miR-29b and the expression of these miRNAs increases during days 3 to 14, the observed changes in the expression of this microRNA

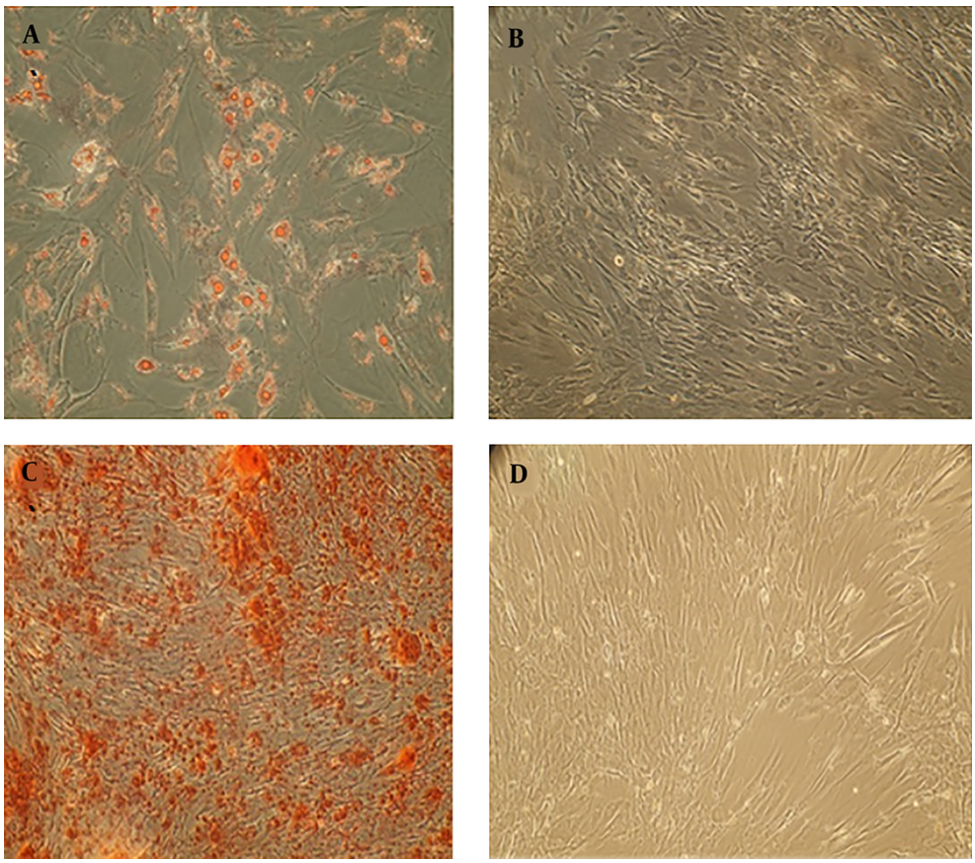

Figure 2. Adipogenic differentiation performed by Oil Red staining (A), osteogenic differentiation performed by Alizarin Red staining (C), and negative control (B and D).

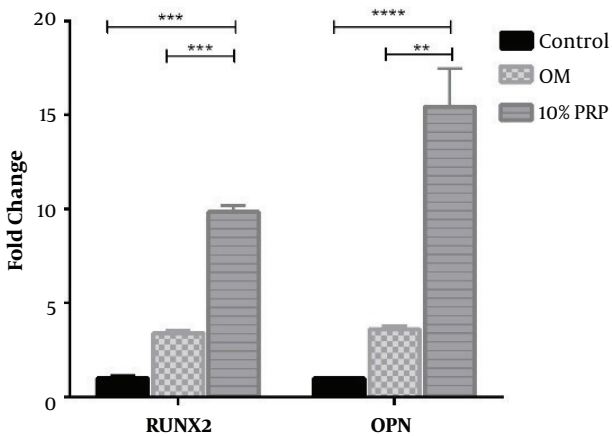

Figure 3. Expression of *RUNX2* and *OPN* on the 14th day of osteoblast differentiation. All the differentiation groups showed significant differences with the control group ($P \leq 0.05$). Abbreviations: OM, osteogenic medium; PRP, platelet-rich plasma.

may cause inhibiting collagen genes and facilitating mineralization.

The limitations of this study were the assessment of cell differentiation only in two cell lines and only studying the expression of four stemness-related surface markers.

In sum, it can be suggested that PRP can improve the process of bone differentiation and facilitate mineralization due to changing the expression of different miRNAs in MSCs. Our subsequent studies will examine further miRNAs in order to clarify microRNAs expression profile during bone differentiation in PRP-treated MSCs.

Acknowledgments

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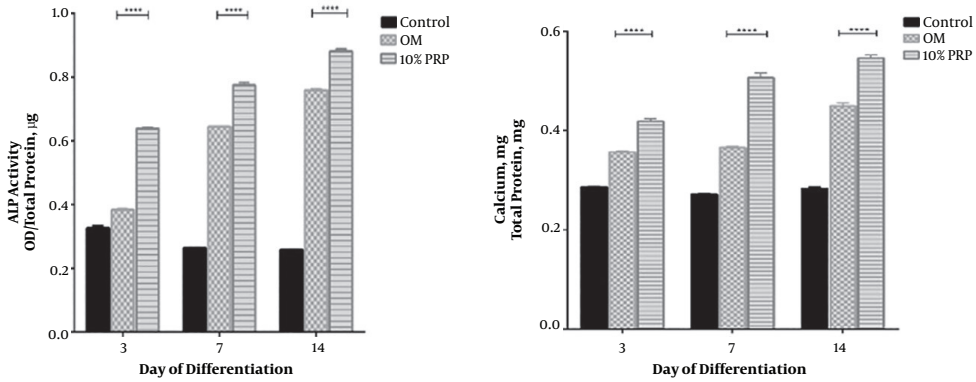

Figure 4. Characteristics of the alkaline phosphatase activity and calcium deposition in differentiation groups. All differentiation groups showed significant differences with the control group ($P \leq 0.05$). Abbreviations: OM, osteogenic medium; PRP, platelet-rich plasma.

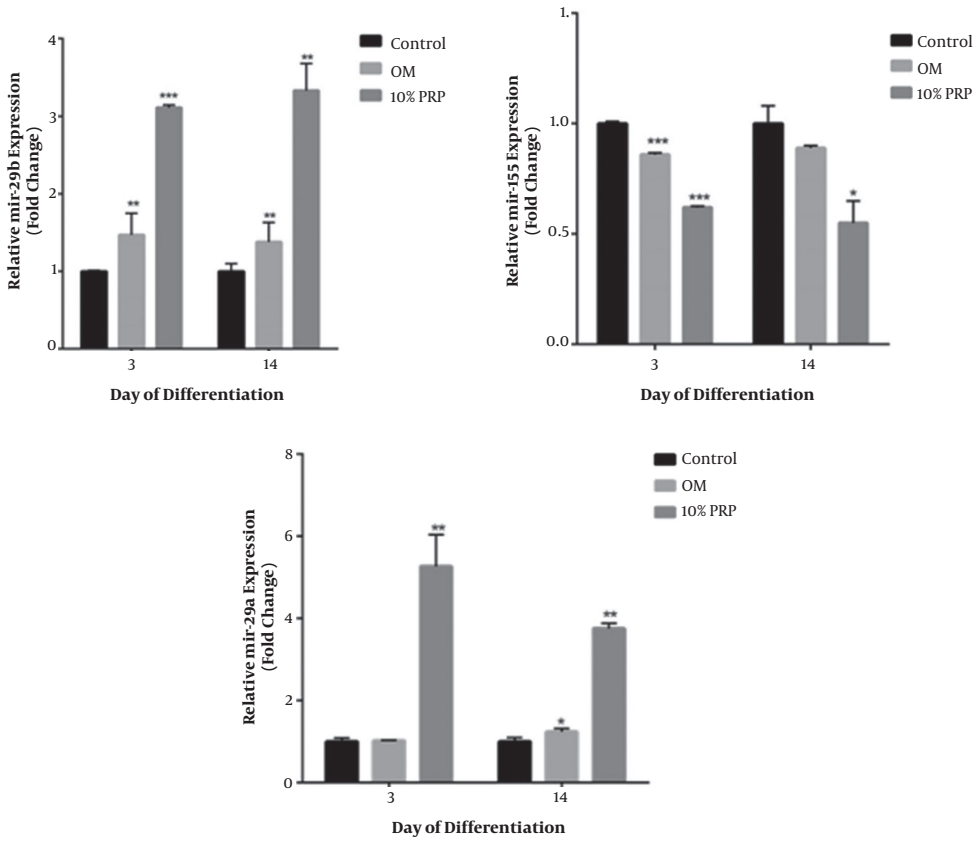

Figure 5. Relative expression of miR-155, miR-29a, and miR-29b on the 3rd and 14th days of osteoblast differentiation. ($P \leq 0.05$). Abbreviations: OM, osteogenic medium; PRP, platelet-rich plasma.

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