Molecular Biology Reports (2020) 47:8451–8463
https://doi.org/10.1007/s11033-020-05885-7

ORIGINAL ARTICLE

MicroRNA-218 competes with differentiation media in the induction of osteogenic differentiation of mesenchymal stem cell by regulating β-catenin inhibitors

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Received: 28 January 2020 / Accepted: 30 September 2020 / Published online: 13 October 2020

Abstract

Osteoporosis, a systemic skeletal disorder specified by low bone mass, is associated with bone fragility and the raised risk of fractures. Activation of the Wnt/β-catenin signaling pathway has been directly demonstrated as a prominent biological event in the prevention of osteoporosis. Recently, critical roles of microRNAs (miRNAs) were further revealed in Wnt/β-catenin signaling activation and thereby contributing to the development and maintenance of the human skeleton. In this study, we investigated whether miR-218 can significantly promote the osteogenic differentiation of mesenchymal stem cells in conditional media by regulating β-catenin signaling inhibitors. The pre-miRNA nucleotide sequence of miR-218 was cloned into the pEGP-miR vector. Next, human adipose tissue-derived mesenchymal stem cells (AD-MSCs) were isolated, characterized, and transfected using pEGP-miR-218.Subsequently, the osteogenic potential of AD-MSCs was investigated in different treated groups using alkaline phosphatase (ALP)activity, calcium mineral deposition, and the expression of osteogenesis-related genes. Finally, negative regulators of Wnt signaling targeted by miR-218 were bioinformatically predicted. Our results indicated a significant increase in the ALP activity, mineralization, and osteogenesis-related genes expression in the AD-MSCs transfected with pEGP-miR-218. Also, the bioinformatic surveys and gene expression results showed that adenomatosis polyposis coli (APC) and glycogen synthase kinase 3 (GSK3-β) were downregulated in the transfected AD-MSCs in both differential and conditional media. This study provided evidence that miR-218 can promote osteogenic differentiation of AD-MSCs even in conditional media. Therefore, our findings suggest miR-218 as a putative novel therapeutic candidate in the context of osteoporosis and other bone metabolism-related diseases.

Keywords Osteogenesis · miR-218 · Mesenchymal stem cells · Wnt/β-catenin signaling pathway

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-020-05885-7) contains supplementary material, which is available to authorized users.

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Abbreviations

miR, miRNA	microRNA
MSCs	Mesenchymal stem cells
AD-MSCs	Adipose tissue derived mesenchymal stem cells
ALP	Alkaline phosphatase

Introduction

Osteoporosis poses a significant public health issue in many developed countries. Two kinds of osteoporosis including senile osteoporosis and postmenopausal osteoporosis are described, which can cause a fracture in normal bones of elderly individuals [1]. Mesenchymal stem cells(MSCs) therapy is a new approach in bone regeneration. Several parts in our body is suitable for harvesting MSCs including visceral fat, subcutaneous fat, and organ fat [2]. The new bone formation promoted with secreting matrix components by stem cells for the osteogenic commitment [3].

Osteogenic medium applied in MSCs differentiation contains dexamethasone, ascorbic acid, and β-glycerol phosphate. Dexamethasone is the main regulator and key factor in differential media used to differentiate MSCs into osteogenic lineages. However, its exact role in osteogenic differentiation of MSCs remains unclear [1]. It has been demonstrated that corticosteroids like dexamethasone can raise the risk of osteoporosis. Corticosteroids are widely used to suppress immune system, and thereby to ameliorate allergy symptoms and rheumatism. However, the long-term use/exposure of corticosteroids can cause osteoporosis due to the prevention of the calcium sedimentation in bone structure [4]. Regulation of the molecular mechanisms in the differentiation of MSCs into osteoblasts has a great impact on the design of new anabolic therapies for osteoporosis [5].

The Wnt signaling pathway is an evolutionarily conserved pathway affecting crucial aspects of cell fate determination by influencing the proliferation, migration, and morphology of normal cells [6]. The canonical Wnt/β-catenin signaling pathway is mainly involved in cell proliferation and survival [7]. In the absence of Wnt signals, the destruction complex is formed and subsequently delivers β-catenin to the proteasome [8]. In fact, they phosphorylate and ubiquitinate β-catenin and make it ready for degradation. This destruction complex is composed of Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and serine kinase 1 (CK1). When Wnt binds to the cellular receptors Frizzled (Fzd), this destruction complex relocalizes to the membrane and therefore, the cytosolic concentration of β-catenin increases. The cytoplasmic β-catenins move to the nucleus and activate transcription of target genes that control cellular fates including differentiation, cell death, proliferation, and repairing of various tissues and organs [6].

MicroRNAs (miRNAs) are 21–25 nucleotides noncoding RNAs thatbind to the 3′ untranslated region of target mRNAs and negatively regulate gene expression at the post-transcriptional level [9]. Experimental evidence has been indicated that miRNAs have a significant role in bone tissue development, osteogenic differentiation, and also osteoporosis pathophysiology [5]. Although numerous miRNAs regulate osteogenesis during the transition of MSCs into osteoblasts, limited knowledge is present about the miRNAs that can stimulate osteogenesis in-vitro or in-vivo in the absence of differential media for instance, miR-637 suppress adipocyte differentiation and induce osteoblast differentiation, whereas miR-204 has the opposite effects [10, 11].

Bone-adjusting miRNAs (called 'osteomiRs') are most likely respond to a broad spectrum of physiologic signals and represent novel controlling candidates of bone formation and/or for regulating the completion of bone formation at the terminal mineralization stage [12].

High-throughput gene expression profiling analysis has demonstrated that the MSCs differentiation into different cell lineage needs certain miRNAs, therefore, it is unavoidable to determine the genes that can be targeted by miRNAs [13]. For example, miR-29a, miR-27, and Let-7 positively regulate osteogenesis by targeting Wnt pathway inhibitors such as secreted frizzled-related protein 1 (sFRP1), APC, and tissue inhibitor of metalloproteinases 1 (TIMP-1), respectively [14–16]. The positive biological role of miR-29a and miR-216 in control of glucocorticoid-induced bone loss has previously been described. In a study conducted by Ko et al. it was reported that the osteoblast lineage responds to excess glucocorticoid treatment by enhancing β-catenin deacetylation and degradation. Additionally, the authors emphasized that miR-29 precursors can stabilize the nuclear level of β-catenin in osteogenic activities [17].

miR-218 is a conserved miRNA in vertebrates with high expression in osteoblasts. No experimental data has been addressed on the role of miR-218 and the possibility of its application in the absence of glucocorticoid for osteoblast generation. Some previous studies have been reported that miR-218 induces Wnt signaling by downregulating its inhibitors, while the induction of Wnt signaling increases the expression of miR-218 in a positive feedback loop.

This study aims to ascertain whether miR-218 can replace osteogenic supplements in differential media of AD-MSCs, and to investigate whether miR-218 can affect APC and GSK3-β function on the inhibition of β-catenin in AD-MSCs cultures.

Therefore, we used the bioinformatic analysis to predict inhibitors of Wnt pathways that are the targets of miR-218 in osteogenesis. Furthermore, we overexpressed miR-218 in adipose tissue-derived mesenchymal stem cells (AD-MSCs) and compared its effect on osteogenic differentiation of stem

cells in differential medium (containing dexamethasone as a key component) and conditional medium. Besides, calcium accumulation, and molecular characteristics of AD-MSCs were investigated to evaluate the effects of miR-218 in osteogenesis with/ without osteogenic media supplements.

Methods and materials

Isolation and expansion of AD-MSCs

The AD-MSCs were isolated according to the previously published protocol [18]. Briefly, adipose tissues were obtained from healthy 25–30-year-old females undergoing abdominal plastic surgery at Modares hospital. Briefly, Informed consent was received from all donors and the procedure was under the supervision of the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics Committee No: 27-1395/8/23). AD-MSCs were isolated by enzymatic digestion; briefly fat tissues were Minced into small pieces and digested with 1% collagenase I (Gibco, Thermo Fisher Scientific, USA) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) and then, incubated at 37 °C for 20 min under shaking. The digested samples were neutralized with Dulbecco modified Eagle medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA). After centrifugation at 500 g for 10 min, pellets were suspended and cultured in MSCs complete medium, DMEM/F12 medium supplemented with 15% FBS (Gibco, USA) and 100 units/mL of penicillin/streptomycin and incubated at 37 °C with 5% CO_2 in a humidified atmosphere. Media was changed the next day and adherent cells were fed twice weekly with 1/2 media changes. The cells were subcultured when reached the confluency of approximately 80%. The passage three adherent cells were harvested were used for all experiments.

AD-MSC characterization and differentiation

To characterized AD-MSCs, the expression of the cell surface markers was analyzed using flow cytometry for CD45, CD34, CD14, CD90, CD73, and CD105. Briefly, the cells were harvested by trypsinization and suspended in PBS containing 0.05% Tween-20 to a final concentration of 10^6 cells/mL. Thereafter, 1 mL of the suspension was stained with antibodies against defined markers (all antibodies had been purchased from eBioscience, San Diego, CA, USA). After washing cells with PBS, the cells were fixed with 1% paraformaldehyde (Sigma-Aldrich, Saint Louis, Missouri United States) and analyzed using a FACSCalibur flow cytometer device (BD Biosciences, USA) and FlowJo® software (Ashland, OR, USA). The percentage of cells positive for each marker was measured to analyze the homogeneity of isolated AD-MSCs. Cells positive for CD73, CD90, and CD105 but negative for CD14, CD45, and CD34 are deemed to be AD-MSCs.

Osteogenic and adipogenic differentiation ability of AD-MSCs were also investigated. For adipogenic differentiation, 3×10^4 MSCs/well were cultured in 4-well tissue culture plates, and the next day, the medium was changed with adipogenic induction medium containing 5 mM insulin, 250 nM dexamethasone, 100 mM indomethacin, and 0.5 mM 3-isobutyl-1-methylxanthine. The medium was changed every 3 days. After 21 days, adipogenic differentiation was evaluated by the detection of intracellular lipid deposits by Oil Red O staining.

The osteogenic differentiation of MSCs was performed by seeding 3×104 cells/well in 4-well tissue culture plates in a complete medium. The following day, the medium was changed with the osteogenic differentiation medium containing 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid biphosphate, and 100 nM dexamethasone. The medium was replaced every 3 days. After 21 days, osteogenic differentiation was evaluated by Alizarin red S-staining.

For this purpose, the cell were fixed by 4% paraformaldehyde and stored at 4 °C overnigh and the specimen was washed repeatedly with PBS 3 times. It was stained with 2% ARS or Oil red solution for 10 min at room temperature. Next, the specimen was imaged by Olympus BX46 microscope. All the mentioned materials were purchased from Sigma-Aldrich, USA.

miR-218 containing vector construction

To generate the hsa-miR-218-expressing system, we cloned hsa-miR-218 precursor in pEGP-miR vector (Cell Biolabs Inc., USA) using BamHI/NheI Restriction enzymes. Accordingly, a 131-bp DNA fragment containing hsa-miR-218 precursor was amplified from human genomic DNA with the specific primers (Supplementary Table 1), which contained BamHI and NheI sites, respectively. After ligation, the DNA construct mixture was transformed into the *E. coli* TOP10 cells and cultured in the LB plate with 100 µg/mL ampicillin. Finally, the clones were verified using enzymatic digestion and sequencing.

AD-MSCs transfection

Human AD-MSCs were cultured at a density of 3×10^4 cells in DMEM supplemented with 10% FBS and incubated at 37 °C and 5% CO_2 overnight to reach 70% confluency. The AD-MSCs in a serum-free DMEM were transfected with pEGP-miR-218 using iN-fect™ reagent (iNtRON Biotechnology, Seongnam, South Korea).

Briefly, 2 µg plasmid and 0.6 µl iN-fect were separately incubated in 30 µl culture medium for 5–10 min with no serum and antibiotics. Afterward, both of them were mixed and incubated for 30 min. Then, this transfection complex was added to cells in serum and antibiotics free media treated for 8 h, replaced by DMEM (containing 10% FBS and antibiotics), and incubated for 24 h. Transfection was repeated once or twice. To determine the efficiency of transfection, cells were visualized using fluorescence microscopy (TE2000-S; Nikon).

After 24 h, the media was replaced with fresh DMEM containing 10% FBS (conditional media). All the experiments performed in four groups including AD-MSCs treated with differential media [19] comprised of dexamethasone, ascorbic acid and β-glycerol phosphate, AD-MSCs transfected with pEGP-miR-218 in conditional media (pEGP-miR-218-CM), AD-MSCs transfected with pEGP-miR-218 in differentiation medium (pEGP-miR-218-DM), and AD-MSCs transfected with pEGP-miR-Null in conditional media (control). Observation of GFP positive cells by fluorescence microscopy after transfection was an indicator of miR-218 expression in the cells.

Quantitative RT-PCR analyses for miR-218 and osteogenesis-associated gene expression

Total RNA was extracted from the cell groups using Hybrid-R™ kit (Gene All, Korea) according to the manufacturer's instruction. The quality and quantity of extracted RNA were evaluated using gel electrophoresis and Nanodrop (DeNovix Inc., USA). cDNA synthesis was carried out by reverse transcribing 1 µg of extracted RNA using 0.5 µL dNTPs (100 µM), 1 µL of random hexamer primer (10 pm), 2 µL reveres transcription buffer, and 0.3 µL RT enzyme (200 U/µL) (Thermo Fisher Scientific, USA). The reactions were incubated at 25 °C for 10 min, 42 °C for 60 min, and 70 °C for 10 min. In addition, miRNA cDNA synthesis was performed using 1 µg of extracted RNA, 1 µL of RT-Stem loop primers (1 pM), and 0.7 µL of nuclease-free distilled water. The reactions were incubated at 65 °C for 5 min, and then, 2 µL of 5X RT buffer, 0.3 µL of RT enzyme (200 unit/µL) (Thermo Fisher Scientific, USA), and 1 µL of dNTP mix (10 pM) were added to the mixture. Tubes were incubated in 25 °C for 10 min, 42 °C for 60 min, and 70 °C for 10 min. Quantitative RT-PCR (RT-qPCR) carried out in a final volume of 20 µL containing 0.5 µL of each forward and reverse primer (10 pm), 1 µL cDNA, and 10 µL RealQ Plus 2X Master Mix Green High ROX™ (Amplicon, Denmark) for the measurement of RUNX2, ALP, and Osteocalcin mRNAs expression. The relative expression of the miR-218 expression in groups was evaluated in a total volume of 20 µL containing 10 µL of RealQ plus 2X Master Mix Probe High ROX (Amplicon, Denmark), 0.8 µL forward primer (10 pM), 0.8 µL reverse primer (10 pM), 0.4 µL TaqMan probe (10 pM), and 2 µL of cDNA. Primer sequences are listed in Supplementary Table 1.

The cycling conditions were as follows: enzyme activation at 95 °C for 20 min followed by 40 cycles of 20 s at 95 °C and 15 s at 58 °C. The final acquisition of fluorescent was performed at the end of each annealing/extension cycle. For gene expression analysis based on SYBR Green I, at the end of the amplification cycles, melting temperature analysis was set by a slow increase in temperature (0.3 °C/s) from 60 to 95 °C using StepOne™ instrument (Applied Biosystems, USA). The relative expression of target genes was determined using the ΔΔCT method via REST® 2009 software. GAPDH and SNORD47 housekeeping genes were used as normalizers for the relative expression of the targeted mRNAs and miR-218, respectively.

Alkaline phosphatase activity

To measure alkaline phosphatase activity, total protein was extracted from cells transfected with pEGP-miR-218 or control in conditional media on days 7, 14, and 21 using 200 µL of radioimmunoprecipitation assay (RIPA) buffer. The lysate was centrifuged at 15,000×*g* at 4 °C for 15 min. Afterward, the supernatant was collected, and ALP activity assessment was performed using ALP assay kit according to the manufacturer's instructions (Pars Azmun, Tehran, Iran). The activity of alkaline phosphatase in cells (IU/L) was normalized against the total protein. ALP activity was obtained from the standard curve of absorbance in 405 nm versus a serial dilution of the enzyme [20].

Calcium content assay

The amount of calcium minerals deposited in different groups of AD-MSCs during osteogenic induction was measured using Cresolphthalein complex method as previously described [21]. Briefly, AD-MSCs were homogenized by 0.6 HCL (Merck, USA) followed by shaking for 4 h at 4 °C. Optical density [22] was measured at 570 nm after addition of the reagent to calcium solutions. Calcium content was measured from the standard curve of OD versus a serial dilution of calcium concentrations.

Bioinformatic prediction and in-silico study

In order to predict the miR-218 targets, different negative regulators of osteogenic differentiation were selected according to the literature review and https://www.genome.jp/kegg/. Correspondingly, the target genes of miR-218 were predicted using bioinformatic algorithms and programs including TargetScan (https://www.targe

tscan.org) [23], miRanda (https://www.microRNA.org) [24], PicTar (https://pictar.mdc-berlin.de) [25], miRmap (https://mirmap.ezlab.org/) [26], RNA hybrid (https://bibiserv.techfak.uni-bielefeld.de/rnahybrid) [27], and RNA22(https://cm.jefferson.edu/rna22/Interactive/).

Statistical analysis

All experiments were performed at least in triplicates for each sample. Ordinary one-way ANOVA of variance (ANOVA) was utilized followed by the correct multiple comparisons by Tukey tests. The statistical significance between treatment and control groups via SPSS software version 20 (IBM Corp., USA). A p-value of less than 0.05 was considered to be statistically significant.

Result

Characterization of AD-MSCs

Morphological analysis using light microscopy indicated that the isolated adherent cells possess a spindle fibroblast-like shape (Fig. 1a). Additionally, to investigate the differentiation activity of AD-MSCs, the cells were differentiated into osteogenic or adipogenic lineages, which is a key characteristic of MSCs. As indicated in Fig. 1b, osteocyte differentiation was confirmed by monitoring calcium phosphate accumulation using Alizarin Red S. Similarly, cell differentiation into adipocytes was evidenced by Oil Red O staining of cytoplasmic oil droplets (Fig. 1c).

Furthermore, flow cytometry analysis was performed to characterize the homogeneity of human AD-MSCs, using

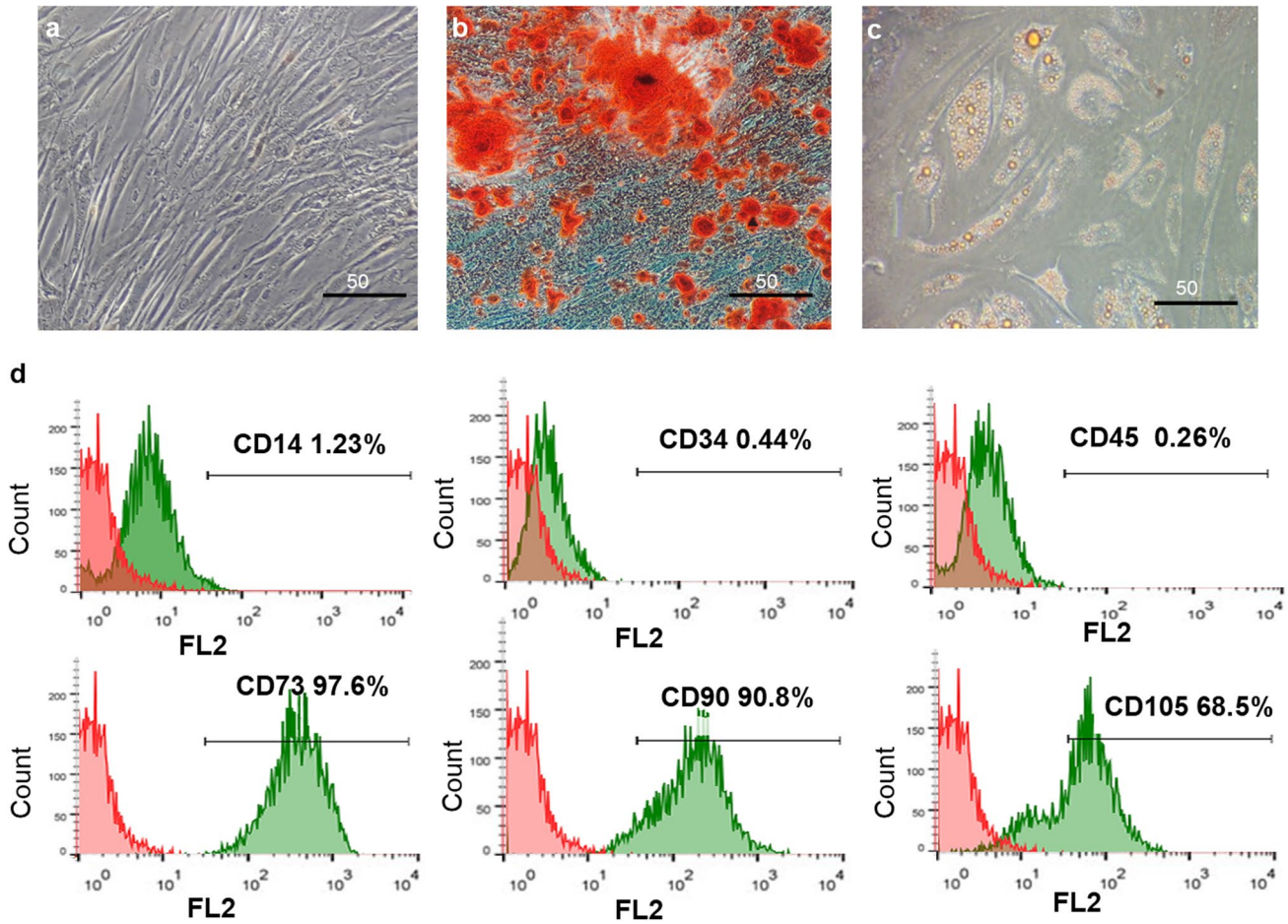


Fig. 1 **a** Morphology of hAD-MSCs by light microscopy at passage 3 showed fibroblast-like cells. **b** Osteocyte differentiation was evidenced by monitoring calcium phosphate accumulation using Alizarin red S. **c** Adipogenic differentiation was assessed by Oil red O staining. **d** Flow cytometric analysis of cell surface markers. This figure represents the flow cytometric analysis of cell surface markers on AD-MSCs. The X axis shows mean fluorescence intensity and the Y axis shows cell number. Isotype controls are presented as red histograms and analyzed markers as green histograms. AD-MSCs were positive for AD-MSC specific cell surface markers including CD73, CD90, and CD105, and negative for CD45 and CD34, and CD14. AD-MSC: adipose tissue-derived mesenchymal stem cell. (Color figure online)

specific monoclonal antibodies. Accordingly, at passage three, 97.6%, 90.8%, and 68.6% of AD-MSCs were positive for specific cell surface markers, including CD73, CD90, and CD105, respectively. They were also negative for CD45 (0.26%), CD14 (1.23%) and CD34 (0.44%). These results confirmed that the isolated cell types were AD-MSCs with high homogeneity (Fig. 1d).

AD-MSCs differentiation

The osteogenic differentiation of AD-MSCs was investigated compared with the control group using alizarin red [28] staining in the following three groups: (1) AD-MSCs cultured in differential media [19], (2) AD-MSCs transfected with miR-218 in conditional media (pEGP-miR-218-CM), and (3) AD-MSCs transfected with miR-218 in differential media (pEGP-miR-218-DM). The results of ARS on day 14 were almost similar in pEGP-miR-218-CM and the group that cells just cultured in differential media. Also, our results indicated that the calcium deposition was remarkably enhanced in the group transfected with pEGP-miR-218 in differential media. (Fig. 2a–d).

Characterization of the pEGP-miR-218-transfected AD-MSCs

To investigate the duration of miR-218-overexpressin in AD-MSCs during differentiation, the pEGP-miR-218 transfected cells in conditional media (pEGP-miR-218-CM), and the miR-218 transfected cells in differential media (pEGP-miR-218-DM) were examined for differentiation into osteogenic lineage in a period of 3 weeks. The qPCR results showed that the highest level of miR-218 expression was observed in day 7 in pEGP-miR-218-CM group (Fig. 2e). The expression of miR-218 in conditional and differential media was 3634 ± 0.15 and 772 ± 1.9-folds higher than the control group. miR-218 expression gradually decreased in 21 days of differentiation. However, decline in its expression was similar in miR-218-CM and miR-218-DM and it was siginificantly different from the cells which were cultured in DM. Figure 2f schematically illustrates the structure of pEGP-miR-218 vector.

RT-qPCR showed overexpression of osteogenic genes after miR-218 induction

The expression of osteogenesis-associated genes including RUNX2, ALP, and OCN were assesed in the different experimental groups using RT-qPCR assay (Fig. 3). The sequences of primers and probes are provided in Supplementary Table 1. In this study, a significantly higher RUNX2 expression was also observed in pEGP-miR-218-CM compared with control. Furthermore, RUNX2 expression levels in pEGP-miR-218-CM on the 7th and 14th days were 1.8 and 2.8-folds higher than those of pEGP-miR-218-DM, respectively (Fig. 3a).

In addtion, RT-qPCR data showed that ALP gene was remarkably overexpressed on the first week of differentiation in pEGP-miR-218 induced cells in CM and DM compared with the other two groups (Fig. 3b).

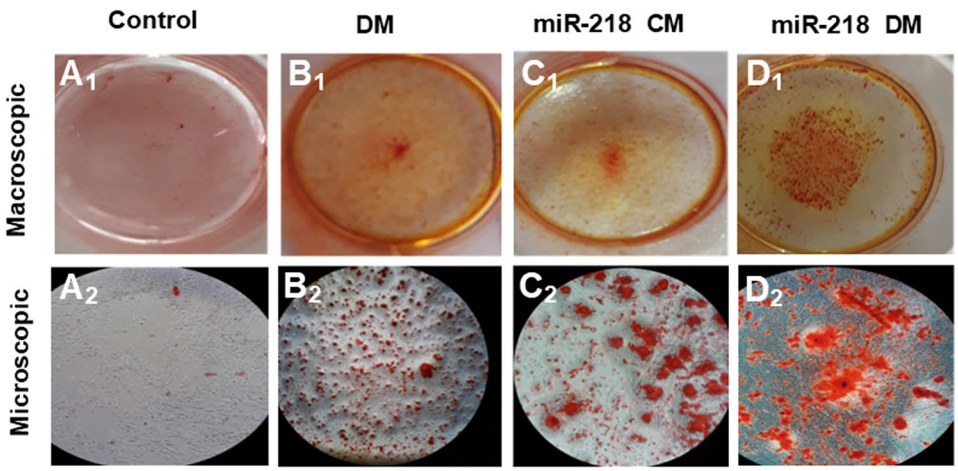
Moreover, OCN expressions in pEGP-miR-218-DM on days 7 and 21 were 2.68 and 5.73-folds higher than pEGP-miR-218-CM, respectively. pEGP-miR-218-CM were also presented 987.33, 109.5, and 33.068-folds OCN overexpression compared with the control groups on 7, 14, and 21 days, respectively (Fig. 3c).Therefore, the gene expressions analysis suggested that the presence of CM with miR-218 overexpression may efficiently increase ALP and RUNX2 expression. In contrast, OCN was revealed a higher expression in pEGP-miR-218-DM during 21 days of osteogenic differentiation.

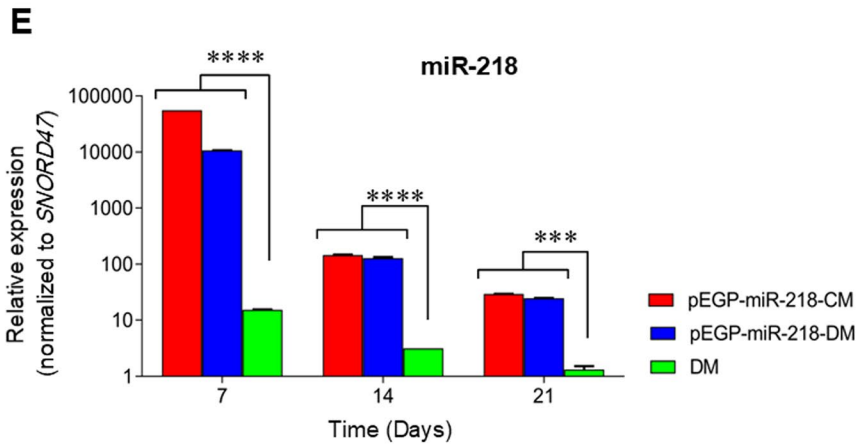
ALP activity and mineralization approved the effect of miR-218 in osteogenic commitment

Elevated activity of ALP is one of the most important early osteogenic commitment indicators. Our data showed that the ALP activity was increased during 21 days of differentiation in all groups except control. The ALP activity in three treated groups was detected almost the same on day 14. The highest amount of ALP activity detected on day 21 in pEGP-miR-218-DM (Fig. 4a, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$). The results demonstrated that ALP activity of DM and in pEGP-miR-218-CM were almost alike in the first two weeks of differentiation. Calcium deposition measurement revealed a significant increase in the amount of Ca^{2+} in all treated groups from day 7 to day 21 and the highest accumulation of Ca^{2+} detected at day 14 of treatment. Although calcium accumulation at 21 days after treatment with pEGP-miR-218 in differential media was at highest level, the differentiation of pEGP-miR-218-CM, control, and DM groups was also statistically significant (Fig. 4b). The calcium accumulation in Fig. 3 with Alizarin Red staining was in parallel with what we saw in calcium deposition quantification where cells transduced with pEGP-miR-218 in DM had the highest amount of calcium deposition.

Overexpression of miR-218-reduces the expression of endogenous APC and GSK3-β

Sequence analysis of miR-218 revealed that it is highly conserved in vertebrates and non-vertebrates, including Homo sapiens, Chimpanzee, Rhesus, Squirrel, Rat, Rabbit, Elephant, Opossum, Chicken, and Lizard. Considering the significant role of the Wnt pathway in bone formation, we examined predicted targets of miR-218 involved





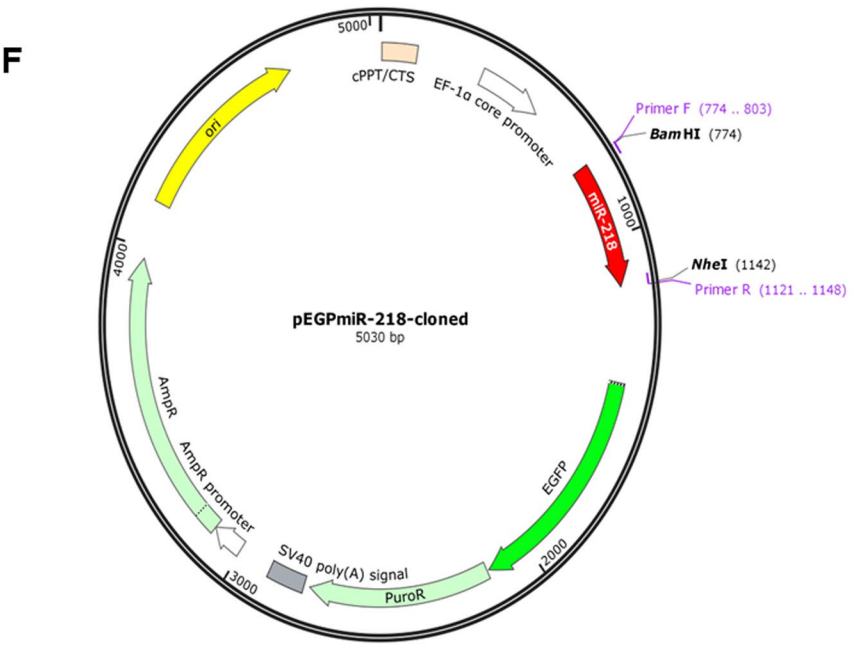


Fig. 2 Macroscopic (**A1**; **B1**; **C1** and **D1**) and microscopic (**A2**; **B2**; **C2** and **D2**) Alizarin red staining were performed to indicate mineral deposition on day 14. **A1** and **A2** control groups. **B1** and **B2** AD-MSC treated with differentiation medium (DM); **C1** and **C2** AD-MSC transfected with pEGP-miR-218 in conditional media (pEGP-miR-218-CM); **D1** and **D2** AD-MSC transfected with pEGP-miR-218 in differentiation medium (pEGP-miR-218-DM). **E**, miR-218 relative expression. pEGP-miR-218 was overexpressed in the two groups in conditional (pEGP-miR-218-CM) and differential (pEGP-miR-218-DM) media from day 7 to 21 (data are mean ± S.D. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). (Color figure online)

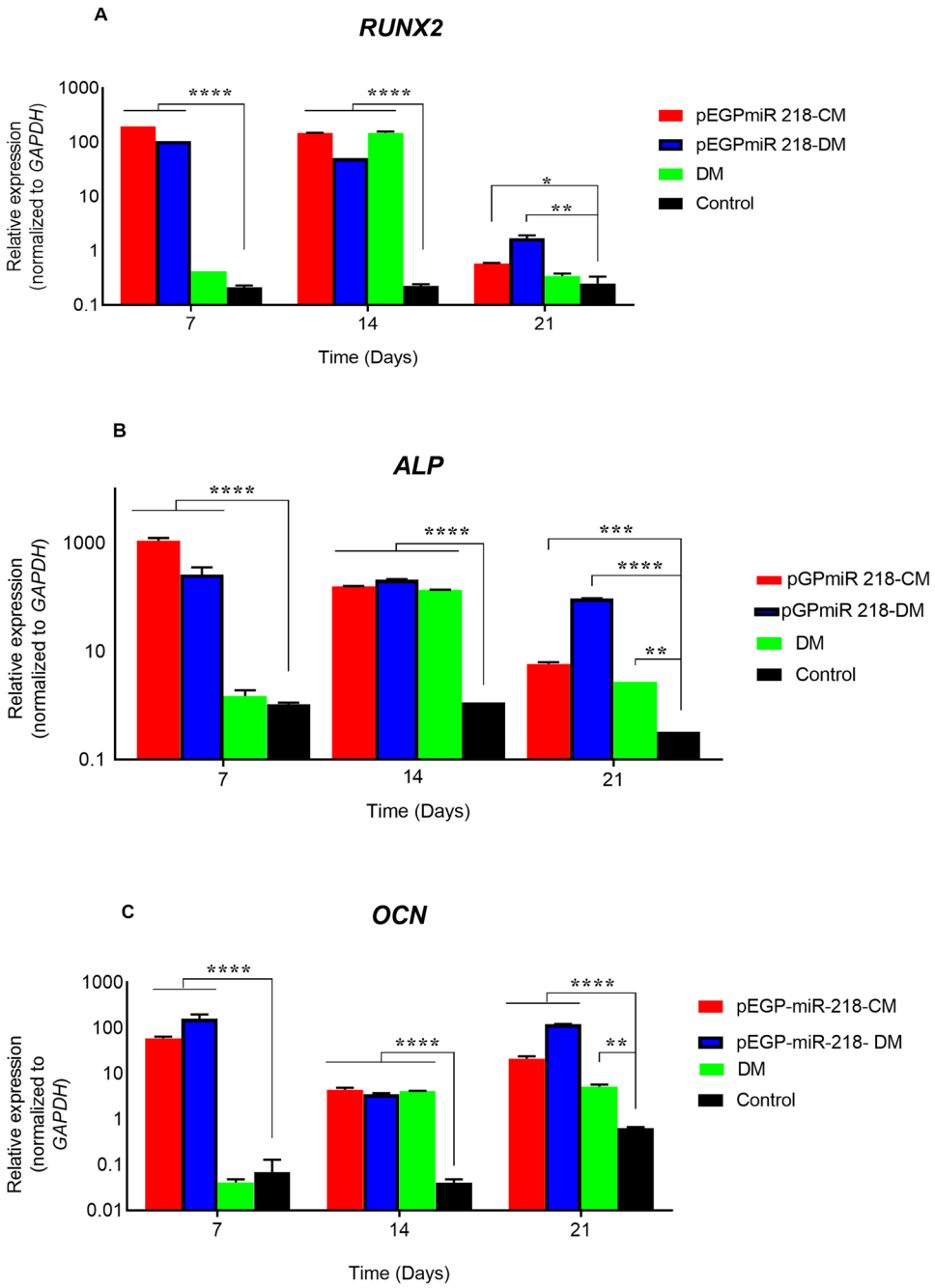


Fig. 3 Relative expression of Runx2 (**a**); ALP (**b**); and OCN (**c**) in AD-MSC after 7, 14, and 21 days of osteogenic differentiation (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). DM, AD-MSCs treated with differentiation medium; pEGP-miR-218-CM, AD-MSCs transfected with pEGP-miR-218 in conditional media; pGP-miR-218-DM, AD-MSCs transfected with pEGP-miR-218 in differentiation medium. (Color figure online)

in bone formation. Bioinformatic analysis was used to predict potential target genes of miR-218. To further analyze the role of miR-218 in osteogenesis, the expression of key inhibitor genes, including APC and GSK3-β was measured in AD-MSCs in different groups using RT-qPCR assay. Our results showed that the expression of APC was decreased significantly in pEGP-miR-218-CM and DM on days 7, 14 and 21. Additionally, the lowest amount of APC with statistically significant results was detected in pEGP-miR-218-DM on day 21. In addition, the expression level of APC in pEGP-miR-218-DM and pEGP-miR-218-CM were respectively 19.74 ± 0.013 and 12.91 ± 0.044-folds lower than that of the control group on day 14 (Fig. 5a).

The lowest amount of GSK3-β was also detected in pEGP-miR-218-DM on days 7 and 14. Compared to the cells cultured in differential media and control group, it was also observed a decrease in the GSK3-β expression in pEGP-miR-218-CM/DM at all time points. The GSK3-β expression level in pEGP-miR-218-DM and pEGP-miR-218-CM was lower than the controls, 888.46 ± 0.0014 and 385 ± 0.003 folds respectively (Fig. 5B). The differences between treatment groups and control in all tests

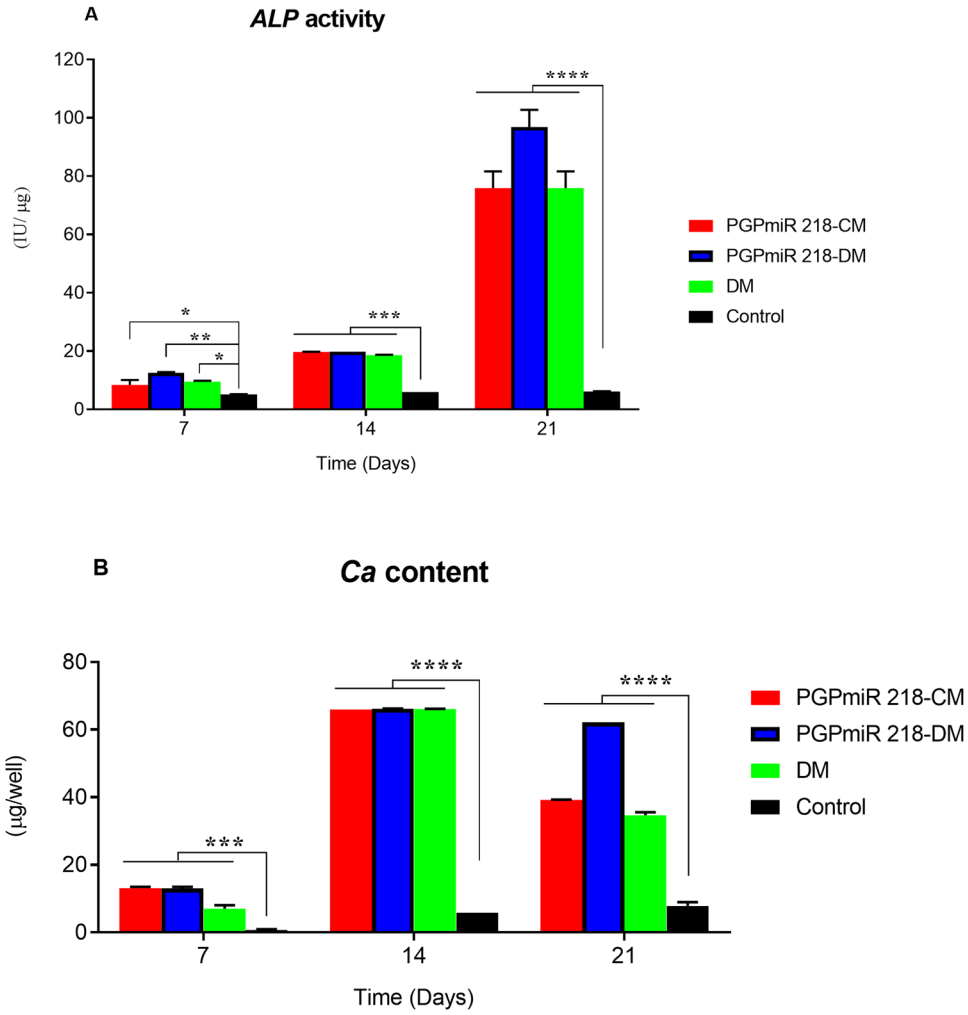


Fig. 4 ALP **a** activity and **b** Ca^{2+} content of AD-MSCs groups after 7, 14, and 21 days of osteogenic differentiation AD-MSCs (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). DM, AD-MSCs treated with differentiation medium; pEGP-miR-218-CM, AD-MSCs transfected with pEGP-miR-218 in conditional media; pGP-miR-218-DM, AD-MSCs transfected with pEGP-miR-218 in differentiation medium. (Color figure online)

were significant (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001).

Discussion

Mesenchymal stem cell differentiation can be affected by various intracellular and extracellular reprograming factors as well as different kinds of nanomaterials and scaffolds [29]. Recently, several miRNAs have been described to be involved in the process of osteoporosis and osteogenesis [12, 30]. Accordingly, some miRNAs have been identified as negative inducer factors in osteogenesis [31, 32] only few of them have been reported as the boosters of the osteogenesis [33].

In the present study, we performed bioinformatic analyses to investigate other inhibitors in the Wnt signaling pathway that are related to osteogenesis. The results indicated that APC and GSK3-β were among the highly probable targets of miR-218.

Moreover, we found that miR-218 in the absence of osteogenic media is able to induce differentiation and enhance the expressions of the RUNX2 and ALP. In this regard, in order to evaluate the effects of miR-218 overexpression with or without osteogenic media supplements, the morphology, calcium accumulation, and molecular characteristics of AD-MSCs were investigated. Literature mining indicated that miR-218 overexpression decreased the expression levels of DKK2 and SFRP2 at mRNA as well as protein levels in AD-MSCs and and protein levels in AD-MSCs and Bone Marrow-MSCs [34, 35]. In a study conducted by Hassan et al. the highest expression of miR-218 had been detected on the 7th day of osteogenic differentiation. The result of our study was in line with the mentioned study since a high level of miR-218 was observed on the first 7 days of differentiation. Zhang et al. also showed that miR-218 was up-regulated at early stages of differentiation (days 0–7) [34]. The result of our study was in line with the mentioned study since a high level of miR-218 was observed on the first 7 days of differentiation. Zhang et al. also showed that miR-218 was

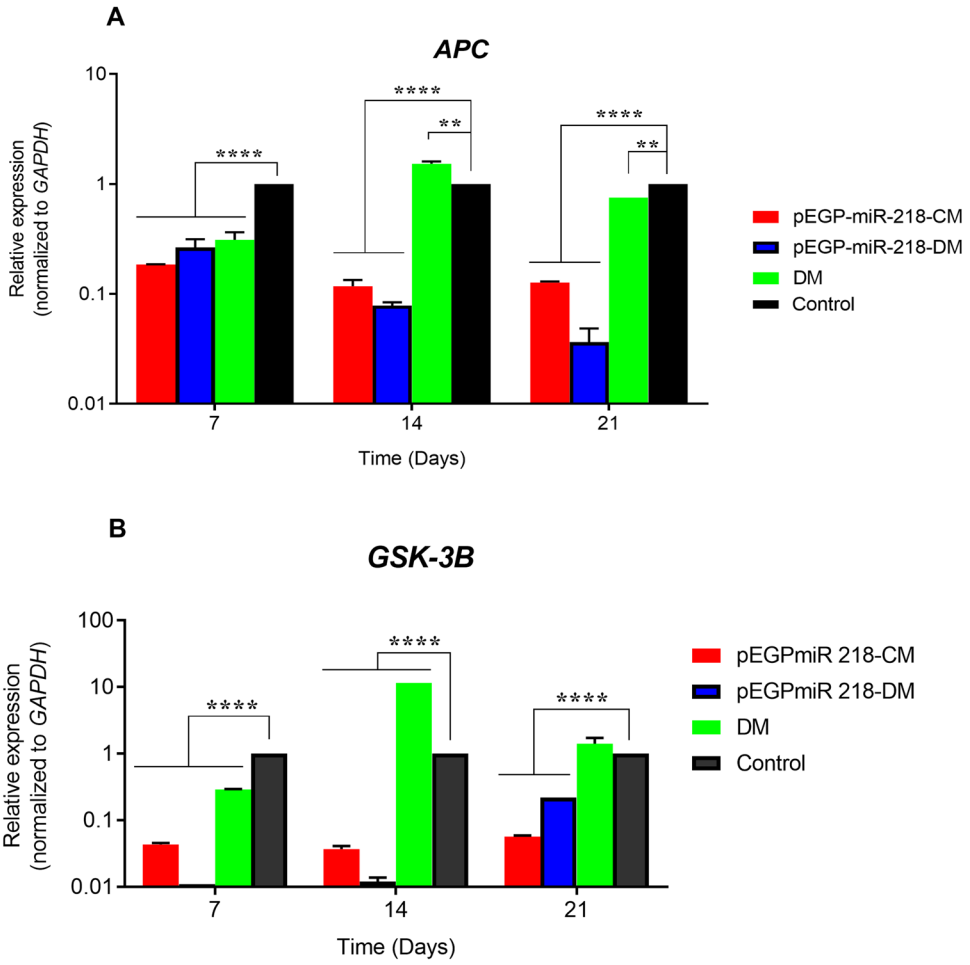


Fig. 5 Regulation on the endogenous expression of APC (**a**) and GSK3-B (**b**) by miR-218 in three groups of AD-MSC compared to control vector containing cells. DM, AD-MSCs treated with differentiation medium; pEGP-miR-218-CM, AD-MSCs transfected with pEGP-miR-218 in conditional media; pGP-miR-218-DM, AD-MSCs transfected with pEGP-miR-218 in differentiation medium. (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001). (Color figure online)

up-regulated at early stages of differentiation (days 0–7) [36].

Notably, the gene expression analysis is a powerful tool for investigating the cellular processes during osteogenesis; therefore, in the present study, the expressions of three osteogenic markers including RUNX2, ALP, and OCN were investigated using RT-qPCR assay. A key transcription factor playing an important role in the initiation of osteogenesis in stem cells is known as RUNX2, which was overexpressed at the onset of osteoblast differentiation lasted up to day14. Interestingly, our experimental results demonstrated that pEGP-miR-218-CM group could enhance RUNX2 expression more than pEGP-miR-218-DM group on days 7 and 14. In addition, the reduction in the expression level of RUNX2 on day 21 of osteogenesis was in line with the inhibition of RUNX2 during the last stages of osteoblast differentiation, which was also reported earlier by Zhang et al. Accordingly, they assumed that RUNX2 can be responsible for the reduced chondrogenesis and the increased osteogenesis [36].

ALPs are a group of enzymes that hydrolyze a variety of monophosphate esters at high pH level and are considered as an early and medium marker in osteogenesis. Our analysis indicated that the differences in ALP expression were significantly varied between three time points, 7, 14 and 21. No significant difference in expression was observed in DM group receiving differential medium compared to the control group on day 7 (P-value > 0.05). Interestingly, the groups that received miR-218 were shown higher expression of ALP on day 7 and among them the highest expression was noted in pEGP-miR-218-CM group. Although a sudden increase in the expression of ALP on day 7 was detected in the two groups receiving miR-218, ALP mRNA expreesion level was almost the same on day 14 in three groups, including DM, pEGP-miR-218-CM, and pEGP-miR-218-DM. ALP is one of the most prominent markers in the process of osteogenic differentiation of the primary cells, which is used as an indication of mineralization. The biochemical tests performed in this study showed the same trend as gene expression after 14 days of differentiation. The results of previous studies have been shown that the expression of this protein in transfected cells is high until the 10th day [35].

ALP expression decreased in AD-MSCs transduced by pEGP-miR-218 in both conditional and differential media until day 21. Our results are in agreement with other

researchers' studies indicating ALP is in peak during the first 10 days after miR-218 induction [35]. ALP activity in cells transfected with miR-218 and cultured in differential media on days 21 was the highest compared to the three other groups. Our results indicated that miR-218 in pEGP-miR-218-CM group can induce ALP activity similar to the cell cultured in differential media.

The most important protein playing a key role in bone matrix synthesis and collagen binding is OCN, which is considered as a late osteogenic marker. Furthermore, the results of our study reveal that there was a significant difference in its expression among three treated groups compared to the control in three time intervals. In two groups, as pEGP-miR-218-CM and pEGP-miR-218-DM, the amount of OCN mRNAs was higher than the untransfected group on day 7. According to Ca^{2+} content test, the amount of Ca^{2+} accumulation on AD-MSCs was significantly higher in three groups on day 14 compared to that of controls; however, this difference was not significant. The miR-218 transfected groups showed the highest amount of OCN on day 21 and among all groups, and the pEGP-miR 218-DM group revealed the highest OCN expression. Moreover, it was indicated that miR-218 in differential media ingredient (pEGP-miR-218-DM) could more enhance the expression of OCN compared to CM, DM, and control groups. On the 21st day, AD-MSCs transfected with pEGPmiR-218 in differentiation media had still a higher amount of calcium deposition compared to the other groups. According to these results, it can be concluded that miR-218 fasten the speed of mineralization in the osteogenic commitment of AD-MSCs. In this study, the effect of miR-218 expression on the osteogenesis induction in AD-MSCs was investigated, considering its possible enhancing effect on the differentiation process. In the present study, the effects of miR-218 on the osteogenic inhibitors of AD-MSCs were systematically analyzed through transfection with a non-viral vector expression system. We also investigated the mechanism underlying miR-218's enhancement on AD-MSCs osteogenesis. The results of our study demonstrate that the expression of miR-218 could inhibit the expression of key inhibitors of β-Catenin signaling, which is considered as crucial in bone formation. Moreover, from the post-modification translation of β-Catenin, acetylation resulted in an increased effective form [37].

In vitro experiments showed that transfection of AD-MSCs with miR-218 could lead to the downregulation of APC and GSK3-β in cells cultured in conditional and differential media. In a study by Mao et al., the result of luciferase assay confirmed that wild type of APC is targeted by miR-218 in cervical cancer cells [38]. In another study by Wang et al., the levels of β-catenin and the inactivated form of GSk3-β protein in the Wnt signaling pathway were significantly increased by the overexpression of miR-218 mimic during cardiac stem cells differentiation [39]. Altogether, these data support our result, in which miR-218 can reduce the expressions of APC and GSK3-β in AD-MSCs, and consequently, it can induce osteogenesis.

How does miR-218 enhance AD-MSCs osteogenesis? In this regard, previous studies strongly pointed that Wnt antagonists DKK2 and SFRP repress osteogenesis, and miR-218 targets these mRNAs. The possibility of targeting other mRNAs applying miR-218 was also investigated in this research and the obtained data showed that accumulation of β-catenin in the cytoplasm is resulted from the inhibition of destruction complex. The inhibition of various targets in the osteogenesis pathway by miR-218 has probably provided evidence on that it can promote osteogenesis alone. Furthermore, miR-218 can be translocated to the nucleus and form a complex with transcription factors like TCF1, which consequently increase the expression of RUNX2. Dexamethasone has been generally used to upregulate osteogenesis of MSCs [40, 41]. In high-dose dexamethasone (10^{-6}–10^{-7} M), C/EBP-α, as an adipogenic master transcription factor, remains unmethylated and activated. Moreover, in this concentration, dexamethasone prevents osteoblast conversion potential by improving β-catenin degradation and GSK3-β activation. On the other hand, upregulation of the secretion of Wnt inhibitors such as DKK2 and SFRP enhances adipogenesis [42]. In some other studies, the obtained results indicated that the dexamethasone (10^{-8}–10^{-9}) could induce expression and localization processes of β-catenin. This process needs to ensure cells undergo normal proliferation and differentiation [43]. Our results indicate the enhanced level of miR-218 decreased the expressions of GSK3-β and APC. Therefore, overexpression of miR-218 can compete for the need to dexamethasone on the osteoblast differentiation of AD-MSCs. However, more investigations are required to reveal the exact role of miR as well as the possibility of its application for stem cell differentiation, rather than differential media. Finally, we suggest that the therapeutic up-regulation of miR-218 in AD-MSCs may promote bone formation and even reverse osteoporosis.

Acknowledgements We express special thanks to members of Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran and the Department of Biotechnology, University of Tehran, Iran for providing technical support.

Funding This project was funded by Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant Number: 6612).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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