Mir-4699 Promotes the Osteogenic Differentiation of Mesenchymal Stem Cells

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Article

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Abstract

Mesenchymal stem cells (MSCs) are drawing considerable attention in the field of regenerative medicine due to their differentiation capabilities. The miRNAs are among the most important epigenetic regulators of MSC differentiation. Our previous study identified miR-4699 as a direct suppressor of the DKK1 and TNSF11 genes expression. However, the precise osteogenic-related phenotype or mechanism caused by miR-4699 change has yet to be dealt with in depth. In the present study, miR-4699 mimics were transfected into Adipose tissue-derived mesenchymal stem cells (AdMSCs) and osteoblast marker genes expression were analyzed to investigate whether that miR-4699 promotes osteoblast differentiation of hAd-MSCs through targeting the DKK-1 and TNFSF11. We further examined and compared the effects of recombinant human BMP2 with miR-4699 on cell differentiation. In addition to quantitative PCR, analyses of alkaline phosphatase activity and Alizarin red staining were used to explore osteogenic differentiation. The overexpression of miR-4699 in hA-MSCs resulted in the stimulation of alkaline phosphatase activity, osteoblast mineralization, and the expression of ALP and OCN osteoblast marker genes. Our findings indicated that miR-4699 supported and synergized the BMP2-induced osteoblast differentiation of mesenchymal stem cells. We suggest, thereof, the utilization of hsa-miR-4699 for further in vivo experimental investigation to reveal the potential therapeutic impact upon regenerative medicine for different types of bone defects.

Introduction

Impaired healing of severe bone defects in cases of osteoporotic fractures, osteolytic metastases, and severe trauma can cause disability of patients as well as burdensome financial costs on the society [1–3]. The delayed process of bone healing calls for the need to develop new regenerative medicine approaches [4]. Protein-based growth factors on collagen scaffold are the most comprehensive approaches currently approved to be applied in the field of bone regeneration [5]. Recombinant human bone morphogenetic protein 2 (rhBMP2) and rhBMP7 are among the most widely used growth factors, which can promote osteogenic differentiation of Mesenchymal stem cells (MSCs) [5, 6]. Potential adverse situations related to recombinant proteins such as an immune response to BMPs, ectopic bone formation and fetal development complications restrict the use of BMPs [7–10]. Another regenerative strategy is the use of cell-based therapies such as transplantation of MSCs for fracture healing [11]. MSCs can be found and isolated from different types of tissue resources, including bone marrow, adipose tissue, and Warton jelly. The human adipose tissue-derived mesenchymal stem cells (AdMSCs) are drawing considerable attention thanks to their abundance while the procedure needed to obtain them is also less invasive than that of bone marrow-derived MSCs [11, 12].

Different signaling pathways such as WNT and TGF-β/BMP regulate osteoblast differentiation of MSCs and bone formation [13]. DKK1 is one of the main inhibitors of Wnt beta catenin pathway, acting through a blockage of LRP5 and LRP6 (WNT signaling receptors) [13, 14]. In non-union patients the process of bone healing will permanently fail unless either surgical treatment or another form of intervention is

introduced. MSCs in non-union patients have boosted level of dickkopf-related protein 1 (DKK-1) secretion [15]. Osteoblasts acnd bone marrow stromal cells (BMSCs) can secrete another protein, TNFSF11, which is an osteoclastogenic factor [16]. Antibodies targeting TNFSF11 (denosumab) and DKK1 (BHQ880) are FDA-approved and in phase II of clinical trials for osteoporosis therapy, respectively [17]. Production and purification of recombinant human growth factors and monoclonal antibodies come at an exorbitant price [18]. To overcome side effects and problems associated with recombinant proteins, pharmaceutical companies attempt to discover innovative drugs that are more cost effective. Since a single miRNA can regulate multiple target genes and pathways, the next decade is likely to witness a tremendous development of drugs based on gene silencing by miRNAs and siRNAs [19].

Given our previous findings that have demonstrated miR-4699 to be a direct suppressor of DKK1 and TNSF11 genes expression [20], we evaluated the role of miR-4699 in the osteogenic differentiation of human adipose mesenchymal stem cells (AdMSCs). We also examined and compared the effects of recombinant human BMP2 with miR-4699 on cell differentiation. The overexpression of miR-4699 in hA-MSCs resulted in the stimulation of alkaline phosphatase activity, osteoblast mineralization, and the expression of ALP and OCN osteoblast marker genes. Our findings indicated that miR-4699 supported and synergized the BMP2-induced osteoblast differentiation of mesenchymal stem cells.

Materials And Methods

hAD-MSCs isolation and characterization

In order to harvest and isolate hAD-MSCs, liposuction operation samples from three donors (aged 28 ± 5, Modaress Hospital in Tehran, Iran) after informed consent were collected. Specimens were obtained in accordance with guidelines of the Medical Ethics Committee, Ministry of Health I. R. Iran (IR.SBMU.REC.1401.002). 10 ml of lipoaspirates was partially digested with 0.2% collagenase II (Gibco, Grand Island, NY) at 37°C, vigorously shaken every 5 minutes in a period of 30 minutes. The digestion was stopped after mixture with DMEM supplemented by 10% FBS (up to a total volume of 50 ml) then centrifuged at 200 g for 5 minutes (Sorvall RC2-B, GSA rotor) with the supernatant discarded. Cell pellet was seeded in cell culture T25 flasks in DMEM supplemented with a 20% fetal bovine serum (FBS) and 100U/ml penicillin/streptomycin (P/S) (Sigma, Munich, Germany). The seeded cells were transferred to a humidified incubator at 37 °C, 95% air, 5% CO2. 48 hours later, the debris and floating cells were removed. MSCs were maintained and grown to reach 80-90% confluency. Confluent cells were treated with trypsin and seeded into two new flasks. The cells were expanded in DMEM containing 10% FBS. In order to characterize cellular and molecular phenotype of primary hAD-MSCs, differentiation induction and stem cell-specific surface antigens evaluation were carried out. Adipogenic induction was carried out using DMEM low glucose containing 10% FBS, 0.5 mM isobutyl-methyl xanthine, 1 µM dexamethasone, 10 µM insulin and 200 µM indomethacin. Osteo-lineage differentiation was induced by basal medium (DMEM + FBS 10%), which is supplemented with 0.1 µM dexamethasone, 50 µM ascorbate-2-phosphate, 10 mM β-glycerolphosphate (all of the ingredient were from Sigma, Germany). Adipogenic and osteogenic media

were replaced every 3 days. Alizarin red and oil red staining were performed to assess lipid accumulation (adipogenesis) and mineralization (osteogenesis), respectively. In order to investigate the expression of stem cell-specific surface antigens, cells were immunostained with different anti-human antibodies (BD Biosciences) including: CD73-PE, CD44-FITC, CD105-PE, CD90-PE, CD271-FITC, CD15-FITC, CD34-FITC, and CD45-PE. The instrument employed was FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software v8.0.

miRNA transfection and BMP signaling induction

The passage 4 hAD-MSC cells of primary culture (7000 cell/cm^2 into 24 well plates), seeded and grown to 80% confluence in 10% FBS supplemented DMEM, were transfected with 25nM synthetic miRCURY LNA™ Premium miRNA Mimic, FAM-hsa-miR-4699-3p (Exiqon, Cat NO#479997611) or miRNA mimic from C. elegans (Cel-miR-39) (Exiqon, Cat NO#40479995), that served as a control tool, with Lipofectamine® 2000 transfection reagent at use (Invitrogen, Carlsbad, CA). Six hours into the treatment, the initial medium was replaced with standard medium (DMEM supplemented with 10% FBS). Throughout this paper the acronym MIMIC stands for cells which was transfected with hsa-miR-4699-3p, SCR cells which was transfected with scramble control, for MIMIC+BMP2 for cells which was transfected with hsa-miR-4699-3p and treated with BMP2, BMP2 for cells which was treated with BMP2, DM for differentiation media (osteogenic media) and CM for control media (basal media or DMEM supplemented with 10% FBS). On the second day post-transfection, the medium was again replaced with osteogenic media in (MIMIC, MIMIC+BMP2, BMP2, SCR, DM) groups. The (CM+MIMIC, CM) groups were grown with DMEM supplemented with 10% FBS. The CM group whose cells are not transfected with miRNA mimic or scramble and are cultured in DMEM supplemented with 10% FBS media, served as the control and reference group.

We briefly modified the BMP2 induction methods as explained by Grunhagen [21]. At confluence, cells were serum starved for four hours then they were stimulated with 50 ng/ml human recombinant BMP2 (Sino Biological) for three days. In the next three days, 100 ng/ml of BMP2 was added. During the time span between days seven and ten, 200 ng/ml of BMP2 was applied. Mineralization of hASCs was tested on the second, seventh, 14th and 21st days after osteogenic differentiation induction.

Quantitative real-time PCR (RT-qPCR)

The total RNA was extracted using Hybrid-R™ (GeneAll, Korea) in accordance with the manufacturer's instructions. The quantity and the quality of the extracted RNA samples were determined using gel electrophoresis and spectrophotometry (Eppendorf, Hamburg, Germany). In brief, reverse transcription was performed from one microgram of the total RNA, using RevertAid® RT enzyme (Thermo Scientific™) along with random hexamer primers (Metabion, Germany) for genes and specific RT Stem-loop primers for miR-4699-3p. The expression of osteogenic marker genes such as RUNX2, ALPL, OCN and the target genes of miR-4699-3P, DKK1 and TNFSF11 were analyzed. All RT-qPCR tests were performed in triplicate, using ABI StepOne™ (Vernon, CA) thermal cycler. The RT-qPCR reactions contained 2μL of cDNA (<100ng),

10µL of 2× RealQ Plus Master Mix for Probe-high ROX (Amplicon, Denmark) for the miRNA and 10µL of 2× RealQ Plus Master Mix Green-high ROX (Amplicon) for the genes, 0.4µM of each primer (10pm), and 7.2µL of double-distilled water. The thermal cycle of the reactions was one cycle of 95°C for 10min and 40 cycles of 15s at 95°C, 40s at 60°C, and data acquisition at the end of each cycle. The melting curve analysis was carried out for target genes. GAPDH and SNORD47 (U47) reference genes were used for normalization of the gene and miRNA expression data, respectively. Data analysis was performed using REST® 2009 and LinReg® (Heart Failure Research Center, Netherlands) software. Sequences of primers used in the current study can be found in Table S1.

Quantitative alkaline phosphatase and calcium content analysis

To determine the quantification of alkaline phosphatase activity of each group, the quantitative alkaline phosphatase ES Characterization Kit (Millipore, USA) was used based on the manufacturer's recommendations. In brief, hAD-MSC cells were detached and aliquoted to 20,000 cells per reaction in the p-nitro phenyl phosphate (p-NPP) buffer. Alkaline phosphatase can hydrolase p-NPP into phosphate and p-nitro phenol, a yellow-colored by-product. The amount of alkaline phosphatase is directly proportional to the quantity of p-nitro phenol, which can be assessed through reading the absorption at 405 nm on a spectrophotometer. Extracellular matrix calcium deposits measurement was taken according to cresolphthalein complexone colorimetric analysis (Pars Azmoon, Iran). Calcium deposits were dissolved in 0.6 N HCL (Merck, Germany). Calcium and cresolphthalein complexone under alkaline conditions (pH>10) form a purple complex, the amount of which is proportional to the amount of calcium content, and can be reliably measured based on reading the absorbance at 570 nm on a spectrophotometer.

Western Blot Analysis

Cultured hAD-MSC cells transfected with miR-4699-3p and un-transfected hAD-MSC cells that were treated with osteogenic media were lysed in radio immunoprecipitation (RIPA) buffer (Sigma) supplemented with phenyl methane sulfonyl fluoride (PMSF) (Roche, USA). Total protein concentration was quantified using the BCA kit (Pars Azmoon, Iran) per the manufacturer's protocols. Equal amounts of proteins were resolved by SDS-PAGE, 10% polyacrylamide gel and blotted to nitrocellulose membranes, then incubated overnight at 4°C with primary antibodies against DKK1(1:10000, ab109416, Abcam, USA), and OCN (ab13418, Abcam, USA). Horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibody (Santa Cruz Biotechnology, USA) were used to visualize protein bands by Amersham ECL chemiluminescence detection system. Mouse anti- GAPDH (1:1000, T-5168, Sigma, USA) was applied as a control tool.

Statistical analysis

Genes and miR-4699-3p expression levels were determined using the Linreg® software and REST® 2009. All statistical tests were conducted with the GraphPad Prism v 7.01 software (GraphPad, USA), expressed as mean ± SD (standard deviation) / standard error of mean (SEM). One-way ANOVA (with Tukey post hoc test) and Kruskal-Wallis were used to calculate the differences among the means of parametric and

non-parametric data of the groups, respectively. The changes of osteogenic parameters over time (day 2, 7, 14, 21) were analyzed through the use of factor repeated measure ANOVA. P-values below 0.05 were considered statistically significant.

Results

Characterization of isolated hAD-MSCs

Flow-cytometry analyses of the isolated spindle fibroblast-like shape demonstrated the expression of typical MSC surface marker, including CD73 (95.4%), CD90 (98%), and CD105 (77.1%) whereas hematopoietic cell markers such as CD14 (3.87%), CD34 (1.09%), and CD45 (0.9%) had low expression (figure 1). Another crucial characteristic of MSC is the potential to differentiate into osteogenic and adipogenic lineages. Calcium deposits in mineralized MSC and intracellular lipid droplets in adipocytes were determined by Alizarin red S staining and Oil red O staining, respectively (figure 2a, 2b, 2c).

The expression of miR-4699 and its target genes

To investigate the effect of hsa-miR-4699 on osteogenic differentiation, MSCs were transfected with either 25nM synthetic scramble or miR-4699 mimic, and grown under osteogenic and regular growth medium. We evaluated the expression level of miR-4699 and its targets at different time points during differentiation. Gene expression analysis revealed increased levels of miR-4699 in transfected groups (MIMIC+DM, MIMIC+DM+BMP2, MIMIC+CM) and it was detectable at 21 days after transfection. The expression level of miR-4699 in non-transfected-groups, which were grown under osteogenic media (DM+BMP2, DM+SCR, DM) proved higher than the control group (CM), which was grown under the DMEM supplemented with 10% FBS media. However, such upregulations were not significant (figure 3A). The expression of the DKK1 gene, one of the miR-4699 target genes, in groups in which the cells were grown under osteogenic media stood higher than groups grown under the control medium, but the overexpression of DKK1 was higher in non-transfected groups in comparison with the transfected groups. At the last time point (21th day), the downregulated expression of DKK1 in transfected groups and groups that were treated with BMP2 appeared more obvious and significant. The combination of BMP2 treatment and miR-4699 transfection reduce the expression of DKK1 more than either treatment individually. The comparison between the two groups (MIMIC+CM, CM), which were grown under the control medium indicated that there was a drop in DKK1 expression in the transfected group (MIMIC+CM) when compared to the group that received only the control media (CM). Such decrease was statistically significant on the first and fourteenth days (time point 1 and 3) (figure 3B).

TNFSF11 gene is another miR-4699 target gene that has been investigated in the present study. In the groups where cells were cultured in the differentiating medium, the expression of this gene stood above the groups where cells were cultured in the control medium. Non-transfected cells had a higher expression of the TNFSF11 gene in comparison with transfected cells. The lowest expression of TNFSF11 among the groups grown in the differentiation medium was observed in a group, in which cells were transfected with miR-4699 on the seventh day of the study (second time point). In the group whose cells were

transfected and received the basal medium, there was a decrease compared to the control group. Nevertheless, such decrease was also statistically significant with the exception of the second time point (day seven) (Figure 3C).

The expression of osteoblast-related marker genes

Given the roles played by miR-4699 in regulating the DKK1 and TNFSF11 expression, we investigated afterwards whether the osteoblast-related marker genes expression mounted in transfected cells. The expression levels of the Runx2, OCN and ALP went up significantly in cells grown in the differentiation medium, with the level of expression proving higher in transfected cells or in the cells, which were treated with BMP2. More details about the results obtained from the Runx2, OCN, and ALP genes expression are presented in Figures 1S, 2S and 3S.

Alkaline phosphatase levels

The alkaline phosphatase level among groups which had been treated at differentiation mediums, is significantly higher than the ones kept only at a basal medium(Figure 4S). At the first time point, the day upon treatment, the alkaline phosphatase level of the group maintained at the basal medium and treated by the mimic miR-4699, equals the ones exposed to the differentiation medium. When it comes to the other time points, however, the level is significantly higher than that of the control group, though the level still remains below that of the ones kept at the differentiation medium. On day 7, the second time point, the alkaline phosphatase level at groups which were all treated either by Mimic or BMP2 are remarkably higher than the control groups that had been treated at the differentiation medium and transfected with scramble, as well as cells that had been maintained at the differentiation medium. On day 21, alkaline phosphatase level of the group which had been kept at the differentiation medium and the BMP2, was significantly higher than other groups (Figure 4S).

Calcium content

During the differentiation process, which involves the deposition of calcium out of the stem cells, the bone is mineralized. To analyze the calcium content, a photometric method is applied, as a result of which the calcium content mixes with cresolphthalein complexone to produce a purple complex. The density of the color is an indication of the calcium level in the mixture. As shown in figure 5S, across all the time points involving groups which had been treated with Mimic and BMP2 and kept at the differentiation medium, the calcium content levels proved to be way higher than the groups maintained at either only a differentiation, or differentiation and scrambled mediums. Meanwhile, cells which had been simultaneously treated with mimic and BMP2, demonstrated the greatest jump in calcium levels. The result indicates the mineralization boosting role jointly played by the two as a result of their simultaneous use.

In cells where the scramble had been transfected and the treatment occurred at the differentiation medium, experienced a slight and not a significant reduction of calcium levels when compared to cells

which was only exposed merely to the differentiation medium, an indication that the scramble has served as a proper balancing tool for transfection and has not interfered with the expression of the genes involved in the differentiation process.

The calcium level in Mimic-transfected cells, which were grown with basal medium, accelerated with more rapidness than cells which grown with only basal medium. As shown in figure 5S, the highest calcium level was witnessed in the group where Mimic miR-4699 was transfected in the cells and the treatment was conducted in the differentiation medium and the BMP2 (figure 5S).

Alizarin staining

The Alizarin red staining was conducted on day 21 of the differentiation for the purpose of analyzing the calcium deposits. The results, which corresponded with the rest of the observations in the present study, indicated a higher rate of calcium deposits in groups where miR-4699 had been transfected into the cells and BMP2 was induced.

As for cells, which was not exposed to the differentiation medium but miR-4699 was transfected into its cells, tiny particles of calcium were spotted in the outer cell areas. Those particles were non-existent in the control group, an indication of the positive role played by miRNA in the bone differentiation process (figure 4).

Analysis of DKKI protein level and osteocalcin using Western blot

Employing the Western blot method, an analysis was carried out on the impact by miR-4699-3p upon the gene expression at the protein level and its osteogenic role. To do so, the lysate of mesenchymal stem cells which had been transfected with scramble and mimic miR-4699-3p and had been exposed to the differentiation medium for 14 days, were examined by Western blot. The expression level of the DKK1 protein as the target gene for miR-4699-3p, osteocalcin as the marker for bone differentiation and GAPDH as the internal control in Mimic-transfected cells and scrambled-transfected, were examined (Figure 5A). The Western blot results were analyzed using Image J software, which converted the density of bands into numbers.

Western blot results indicated that miR-4699-3p can significantly (p-value=0.012) reduce the DKK1 expression in the protein level. The drop in the negative regulator of differentiation can lead to a jump in the expression of bone markers, including osteocalcin. As demonstrated by the present study, when it came to groups where miR-4699 had been transfected, a significant increase (p-value=0.0007) in the expression level of osteocalcin was also detectable (Figure 5B).

Discussion

In our previous study, we first applied several prediction tools to find a miRNA that can target negative regulator of osteogenic differentiation, and we selected miR-4699-3p for furtherer investigation. Then we overexpressed miR-4699 in HEK-293 cells, and using RT-qPCR we observed significant decline in

expression of DKK1 and TNFSF11. For validation the interaction between miR-4699 and 3'UTR of DKK1 and TNSF11, we used luciferase reporter assay and its results indicated that miR-4699 could directly target DKK1, TNSF11 3'-UTRs [20]. Yet, our previous study did not cover the impacts of miR-4699 on stem cells' bone differentiation. Mesenchymal stem cells are of remarkable significance when it comes to treatment and regenerative medicine in general and so is the optimization of the efficiency of differentiation [11]. In this vein, and to verify the positive role played by miR-4699 in the differentiation process, the selected MSC cells were the ones derived from adipose tissues. The findings on the impact by miR-4699-3p indicated that the mentioned miRNA has managed to effectively and significantly induce bone differentiation in mesenchymal stem cells and increase the expression of bone marker genes.

MiR-4699 is a novel miRNA whose role and functions remain largely unexplored. It was introduced in 2020 as one that is overexpressed in early relapse in pediatric B-cell acute lymphoblastic leukemia [22]. The target genes that have been proposed for miR-4699 are E2F3 and SOS1 [22]. In one recent study the expression level of miR-4699 in ovarian cancer cell lines was found to be lower than normal cells, while the increased miR-4699 expression reduces the proliferation and migration of OVCAR-3 cells as it targets MRPS23 genes [23].

DKK1 is a secreted antagonist in the Wnt signaling pathway, which blocks linkage between Wnt ligands and their receptors through binding to LRP5/6 receptors. DKK1 increased expression has been witnessed in such diseases as osteoporosis, metastasis-caused bone degeneration, multiple myeloma and rheumatoid arthritis [24, 25]. According to a study by Kapinas et al. in 2010, the miR-29a expression mounts in primary human osteoblast cells as well as in cell line hFOB 1.19. The same study points out that miR-29a can induce bone differentiation following interaction with DKK1, FRP2s and Kremen2 genes [26]. Our results have a number of similarities with Kapinas et al.'s findings that a drop in DKK1 expression through miRNA overexpression can lead to a jump in bone differentiation. An advantage in the present research is that in addition to investigation of negative regulator of Wnt signaling, the TNFSF11 gene, which is a target for osteoporosis treatment, is also covered. Yet another study conducted by Zhang et al. in 2011 focused on miRNA's expression profile in MC3T3-E1 and MLO-A5 stem cell lines. The results indicated that miR-335-5p specifically reduces the DKK1 expression [27].

Meanwhile, in a 2018 research on the synovial pseudo-fibroblast, Lawamoto et al. found that miR-218 does increase osteogenesis through reducing DKK1 [28]. They investigated the miRNA expression profile in the FLS cells differentiation process using the microarray technique, the study revealed that some of the miRNAs, including miR-218, have been over-expressed. Using miRecords database, the miR-218 target genes were predicted, based on which the ROBO1 is set as the target. To examine the miR-218 function, it was transfected into the cells, and to verify the role played by ROBO1, the siRNA against ROBO1 was used to reduce its expression. The results indicated that an increase in miR-218 or a drop in ROBO1 expression through siRNA lead to reduced DKK1 and further bone differentiation. As a result, it is likely that ROBO1 is the transcription factor in DKK1 expression. miR-218 can cause osteoblast differentiation of RA-FLS cells (rheumatoid arthritis- fibroblast-like synovial cells) even without a differentiation medium

[28]. Similarly, the present research demonstrates that in a group, where the cells were transfected with miR-4699 and received the basal medium, some level of differentiation was witnessed when compared to control cells, which were merely received the basal medium. Yet such differentiation is not that considerable to support the argument that complete differentiation is only attributed to miR-4699 transfection. An advantage of the present study, when put alongside the research conducted by Lawamoto et al., is that the former has used a BMP2 recombinant protein as the basis and criterion for optimized differentiation and a simultaneous examination of the synergy power of both miRNA and BMP2.

Another study conducted by Zheng et al. in 2018, revealed that during osteoblast differentiation the expression of DICER gene, which has been responsible for the miRNAs trimming, and Runx2 gene are increased [29]. Using luciferase assay, they demonstrated that Runx2 through interaction with the promoter of DICER gene, increase its expression. Furthermore, the DICER expression in Runx2 (Runx2 −/−) knocked-out mice is lower than Runx2 +/− knocked-out and Runx2 +/+ mice. The same group proved that in C3HH10T and MC3T3 cell lines, DICER decrease DKK1 expression through maturation of miR-335-5p [29]. Another research carried out by the same group has already pointed out that inhibition of DKK1 using miR-335 with the help of lipid nanoparticle carriers can help better repair the damage left on the skulls of C57BL/6J mice [30], thus reaffirming the key role played by miRNA in targeting DKK1 genes for the purpose of repairing the bone damage at the *in vivo* level.

In a 2016 research, Liu et al. found that the miR-9 induces osteogenic differentiation through targeting DKK1. The interaction between miR-9 and DKK1 was examined based on the luciferase assay and western blot [31]. The purpose behind the selection of miR-9 for the present study had to do with past literature, in which mirR-9's repairing role in the spine cord [32] had been examined. The choice of DKK1 as the target gene was based on bioinformatic prediction using the Miranda software. That study was also the only one so far to have explored the part played by miRNA as well as bone differentiation, which like our research, compared the differentiation induction by miRNA with that of the BMP2 recombinant protein, while also reviewing the pair's synergy impact [32]. As it was the case with our research, the mentioned study demonstrated that miR-9, when working alongside BMP2, proves more effective than when BMP2 or miRNA are there alone [32]. And similarly again, the synergy of the two agents was exhibited after the Alizarin red staining, the measurement of the alkaline phosphatase activity as well as the examination of the bone markers' expression such as OCN, type 1 collagen, and bone sialoproteins [32].

Based on the results yielded by the present study, the expression of target genes of miR-4699-3p in transfected cells was significantly lower than the non-transfected ones. Interestingly enough, the reduction pace in the stem cells, which were both transfected and exposed to BMP2-induced differentiation, hit the lowest rate on day 14, a clear indication of the synergy of BMP2 and miR-4699-3p in lowering the expression of target genes.

Another crucial differentiation inhibitor gene investigated in the present research was TNFSF11, one of the major cytokines in the osteoclast differentiation, whose binds to its receptor RANK on the osteoclast precursor surfaces, causing differentiation of osteoclast from those precursor [33]. The over-expression of TNFSF11 gene resembles that of DKK1, leading to such corrosive diseases as osteoporosis, metastatic bone degeneration and multiple myeloma [34]. As far as the review of the previous literature demonstrated, only a limited number of papers have been written on the miRNA's direct targeting of TNFSF11, while there is considerable evidence alluding to the fact that the expression of OstemiRs (miRNAs that induce bone differentiation), including miR-29b, miR-146a and miR-155, reduced when cells are being treated by TNFSF11 [35].

In 2015, Pitari et al. examined, miR-21's role in bone diseases linked to multiple myeloma, they did realize that it increases the TNFSF11/OPG proportion by targeting 3'UTR of OPG mRNA, thus leading to a jump in the osteoblast differentiation [36]. The TNFSF11 gene, on the other hand, is expressed by the STAT3 transcription factor. The protein inhibitor of activated STAT 3 (PIAS3) gene is one of the direct and verified targets of miR-21. Therefore, as miR-21 expression mounts, the PIAS3 level plunges, eventually triggering a jump in the TNFSF11 expression [36]. The bulk of studies conducted on the link between miRNA and the TNFSF11 gene have not been able to establish a direct link between miRNA and the TNFSF11 gene [37, 38]. They have rather only shown that miRNA directly target the STAT3 gene (the main transcription factor of TNFSF11), which as a result reduces the TNFSF11 expression through reducing its expression. Nonetheless, the study carried out by Zhang et al. in 2016 employed the luciferase assay to conclude that miR-338-3p can target TNFSF11 and they observed that miR-338-3p brings down the differentiation of osteoclast precursors into osteoclast [39].

For the first time ever, the present study demonstrated that the expression of miR-4699-3p mounts during the osteogenic differentiation of MSC cells. The examination of the calcium content and the cells' alkaline phosphatase levels as well as the Alizarin red staining all indicated that miR-4699-3p induce an osteogenic property, both at the gene expression level and when it comes to function. The present study further analyzed the positive role played by the simultaneous treatment of cells with both the BMP2 recombinant protein and miR-4699-3p. The results showed that the use of the two molecules, when applied together, accelerate the differentiation process and improve the quality of such differentiation. The findings yielded after the western blot test was applied to DKK1 genes and osteocalcin highlighted the part played by miR-4699-3p in the targeting of DKK1 genes and the consequent higher expression of osteocalcin bone markers.

Overall, the research has demonstrated how positively the miR-4699-3p can affect the differentiation, paving the way for future studies that are worth being carried out at the *in vivo* level for further light to be shed on the osteogenic role played by the same miRNA, following which miR-4699-3p could be applied to the treatment of bone deficiencies and fractures along with proper scaffolds for tissue engineering purposes.

Declarations

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CONFLICT OF INTEREST

The authors declare no competing conflict of interests concerning the work described.

CONTRIBUTION

Vahedeh Hosseini: Conceptualization, investigation, formal analysis, methodology, resources, writing– original draft, writing–review, and editing.Ameneh koochaki: Data curation, investigation, formal analysis, resources, writing–original draft, writing–review, and editing. Henry Manuel Cesaire: Methodology and writing– review and editing. Samira Mohammadi-Yeganeh and Mahdi Paryan: Conceptualization, formal analysis, funding acquisition, methodology, writing– original draft and writing– review and editing.

ETHICS APPROVAL

This project was approved and supervised by the ethical committee of Shahid Beheshti University of Medical Sciences, (ethics number Iran (IR.SBMU.REC.1401.002)).

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

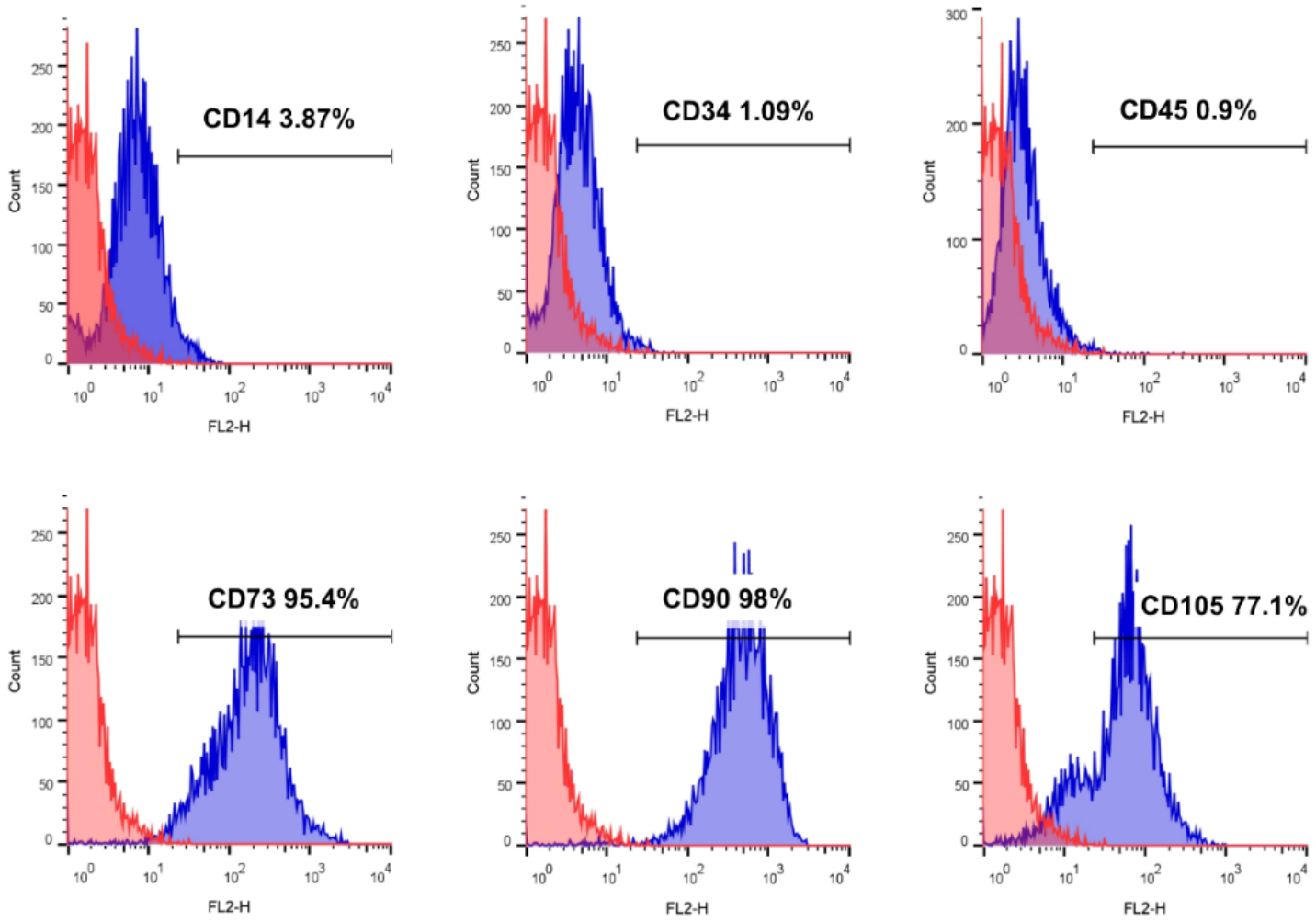

Figure 1

Flow cytometric analysis of hAD-MSCs cells. Isotypic controls are shown in red and analyzed markers in blue. Cells are positive for stem cell markers (CD73, CD90 and CD105) and negative for hematopoietic markers (CD14, CD34 and CD45).

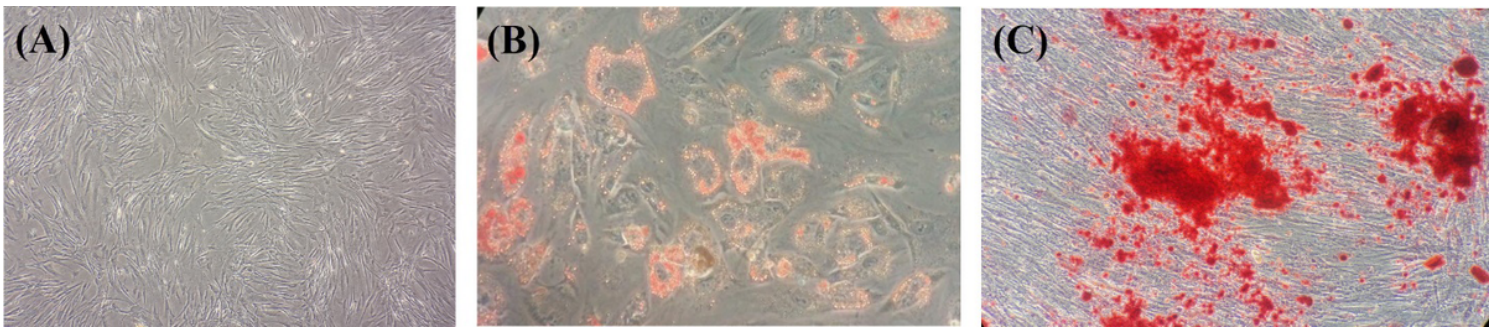

Figure 2

Confirmation of MSC cells by differentiation. A)Confirmation of spindle-shaped and fibroblast-like morphology of mesenchymal stem cells (100X magnification). B) Differentiation of cells into adipose tissue and red color of intracellular lipid droplets inside the cell (200X magnification). C) Differentiation of cells into osteocytes and red color of extracellular calcium deposits (200X magnification).

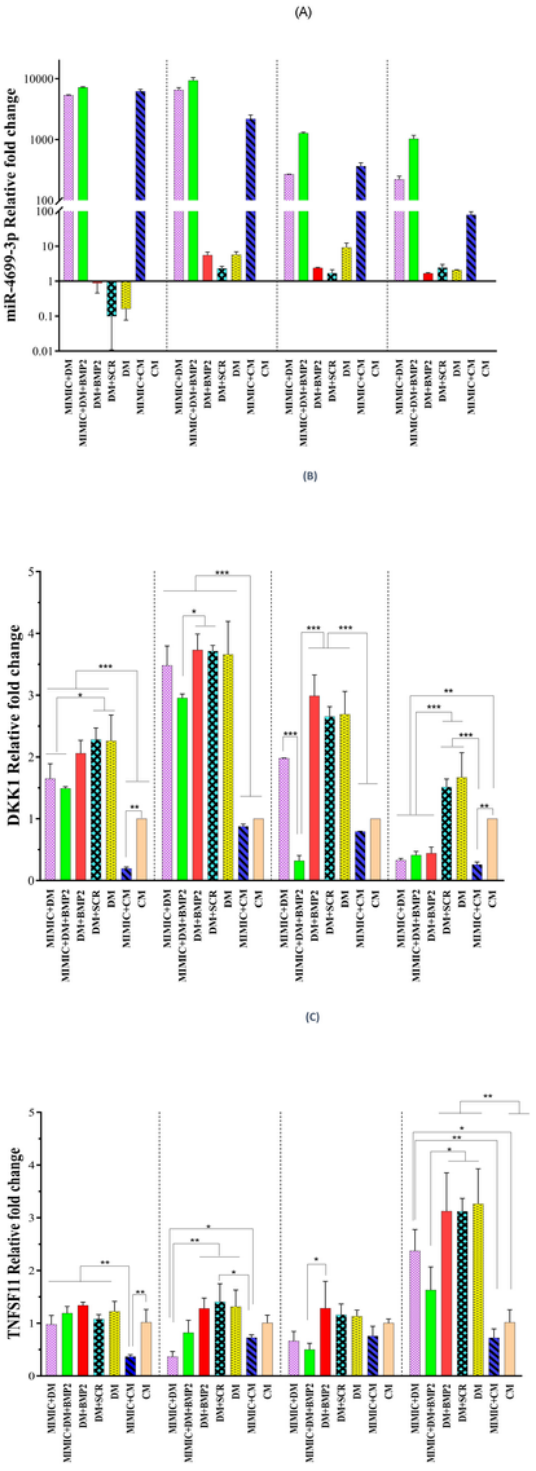

Figure 3

Figure 3: (A) Expression of miR-4699 in different groups and at different time points. Expression in transfected groups (MIMIC+DM, MIMIC+DM+BMP2, MIMIC+CM) is significantly increased. (B) Expression of DKK1 in different groups and in different time points. (C) Expression of TNFSF11 in different groups and in different time points. * indicates significance below 5%, ** implies significance below 1% and *** remains below 0.01%. The data represent the mean obtained from three replications ± SD.

MIMIC (cells which was transfected with hsa-miR-4699-3p), SCR (cells which was transfected with scramble contro, for MIMIC+BMP2 (cells which was transfected with hsa-miR-4699-3p and treated with BMP2), BMP2 (cells which was treated with BMP2), DM (cells which was grown with differentiation osteogenic media) and CM (cells which was grown with control media, basal media or DMEM supplemented with 10% FBS).

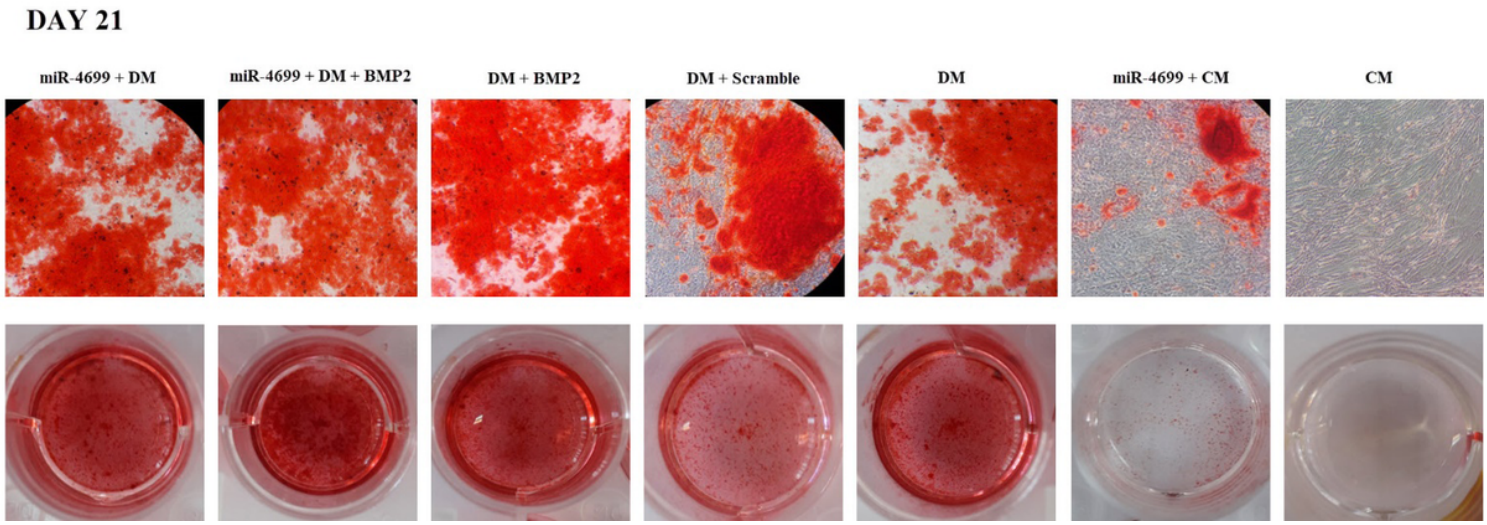

Figure 4

Alizarin staining on day 21 upon differentiation. Picture magnified by 200X.

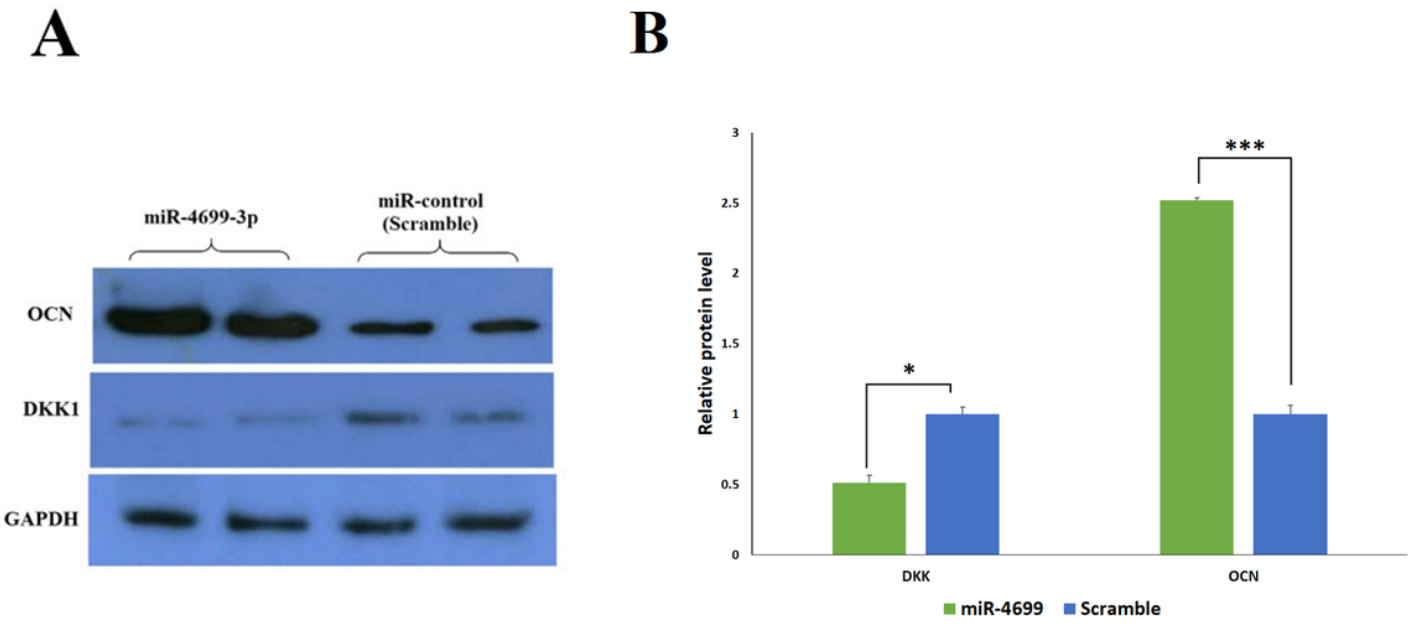

Figure 5

A) Western blot of OCN (osteogenic marker), DKK1 (target gene of miR-4699), GAPDH (internal control) B) Results of Western blot data analysis was performed using Image J software. DKK1 and OCN protein levels are normalized to the level of GAPDH protein. DKK1 protein levels in miR-4699 transfected cells were lower than its level in control cells. OCN protein levels are higher in miR-4699 transfected cells than its level in control cells. * indicates significance below 5%, and *** significance below 0.01%. The data represent the mean obtained from three replications ± SD.

Supplementary Files

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