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Promoter methylation and expression pattern of *DLX3*, *ATF4*, and *FRA1* genes during osteoblastic differentiation of adipose-derived mesenchymal stem cells

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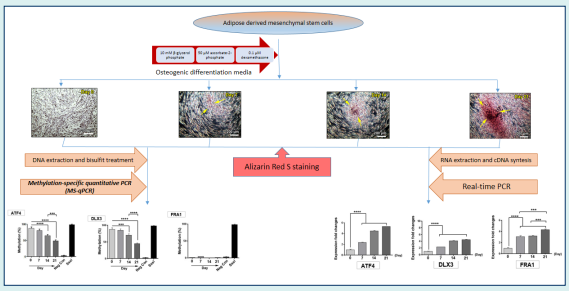
Abstract



Introduction: Nowadays, mesenchymal stem cells are touted as suitable cell supply for the restoration of injured bone tissue. The existence of osteogenic differentiation makes these cells capable of replenishing damaged cells in the least possible time. It has been shown that epigenetic modifications, especially DNA methylation, contribute to the regulation of various transcription factors during phenotype acquisition. Hence, we concentrated on the correlation between the promoter methylation and the expression of genes *DLX3*, *ATF4,* and *FRA1* during osteoblastic differentiation of adipose-derived mesenchymal stem cells *in vitro* after 21 days.

Methods: Adipose-derived mesenchymal stem cells were cultured in osteogenesis differentiation medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerol phosphate, and 50 μM ascorbate-2-phosphate for 21 days. RNA and DNA extraction was done on days 0, 7, 14, and 21. Promoter methylation and expression levels of genes *DLX3*, *ATF4,* and *FRA1* were analyzed by methylation-specific quantitative PCR and real-time PCR assays, respectively.

Results: We found an upward expression trend with the increasing time for genes *DLX3*, *ATF4,* and *FRA1* in stem cells committed to osteoblast-like lineage compared to the control group ($P<0.05$). On the contrary, methylation-specific quantitative PCR displayed decreased methylation rates of *DLX3* and *ATF4* genes, but not *FRA1*, over time compared to the non-treated control cells ($P<0.05$). Bright-field images exhibited red-colored calcified deposits around Alizarin Red S-stained cells after 21 days compared to the control group. Statistical analysis showed a strong correlation between the transcription of genes *DLX3* and *ATF4* and methylation rate ($P<0.05$).

Conclusion: In particular, osteoblastic differentiation of adipose-derived mesenchymal stem cells enhances *DLX3* and *ATF4* transcriptions by reducing methylation rate for 21 days.

Introduction

Bone tissue is composed of a discrete calcified matrix with collagen fibers and different cell types such as osteoblasts, osteocytes, osteogenic cells, and osteoclasts.[1] Although bone tissue has the potency to restore and repair small-sized defects, critical large-sized bone defects require interventional approaches.[2] Grafting and metal implants are the gold standard methods for bone defects, but these modalities have numerous disadvantages such as implant rejection, tissue dysfunction, and microbial infections.[3]

From the past to present, regenerative medicine and stem cell-based therapies have shown promising preclinical and clinical outcomes to accelerate and reconstitute injured tissues and organs.[3,4] Recently, mesenchymal stem cells

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(MSCs) account for a small fraction of mononuclear cells in different tissues and have been touted as a promising supply for cell-based therapies.[5-8] Due to the existence of multipotentiality and self-renewal bioactivity, it potentiates MSCs to give rise to various lineages such as osteoblasts, adipocytes, and chondrocytes.[9] It has been shown that lineage-specific differentiation potential of stem cells correlates with the expression of special transcription factors which are *per se* regulated by diverse extracellular stimuli such as hormones, cytokines and etc.[10,11]

To induce osteogenic differentiation of MSCs, the critical role of several transcription factors has been previously determined.[12-14] For instance, Runx2 and Osterix are two osteoblastic specific transcription factors that regulate the differentiation of MSCs to osteoblasts.[14] Later on, other bone markers such distal-less homeobox 3, 5, and 6 (*DLX3, 5*, and *6*), activating transcription factor 4 (*ATF4)*, *FRA1*, *MSX1*, *MSX2,* and Twist participate in osteoblastic differentiation of MSCs.[15] Accumulating data support a notion that *ATF4* is activated in the late stages of osteoblastic differentiation leads to the expression of osteogenic specific genes.[16] An orchestrated interactions of *ATF4* and *Runx2* with *OSE2s* (Runx2-binding sites) and *OSE1* (ATF4-binding site) promotes the transcription of gene osteocalcin (*OCN*).[15,16] The phosphorylation of DLX3 at serine 10 via protein kinase A (PKA) activity enhances BMP2-induced osteoblastic differentiation.[17] A highly orchestrated osteogenic differentiation follows by the activation of Fos-related antigen 1 (Fra-1) belongs to the activator protein-1 (AP-1) family.[18] RUNX2 can induce osteoblastic differentiation of C2C12 myogenic progenitor cells by positively targeting FRA-1.[19]

Based on these findings, it seems that *ATF4, DLX3,* and *FRA-1* genes are essential transcription factors during bone tissue development and regeneration. To our knowledge, few experiments have explored the dynamics of *ATF4, DLX3,* and *FRA-1* genes in different stem cells committed to multiple lineages except osteoblast-like lineage. The activity of *ATF4, DLX3,* and *FRA-1* genes during osteoblastic differentiation of MSCs is not fully addressed before. An intriguing question in the current experiment correlates with the determination of *ATF4, DLX3,* and *FRA-1* genes promoter methylation and expression levels during osteoblastic differentiation of adipose-derived MSCs (ADSCs) after 21 days.

Materials and Methods

Cell expansion

Human ADSCs were obtained from Royan Institute (Tehran, Iran). ADSCs were re-suspended in low glucose content Dulbecco's Modified Eagle Medium (DMEM/LG; Gibco; Ireland) containing 10% fetal bovine serum (FBS; Gibco; Ireland) and 1% Pen-Strep (Gibco). Cells were maintained at 37°C with a humidified atmosphere and 5% CO_2. To replenish the exhausted medium, cell supernatants were changed every 3-4 days. Cells at a confluence of 80% were detached by using 0.25% Trypsin- (1mM) EDTA solution (Gibco; Ireland). ADSCs at passages 3-6 were used in different analyses.

Osteoblast-like differentiation of ADSCs

For this propose, ADSCs were transferred into 6-well culture plates (SPL) and allowed to adhere and expand for 48 h. Thereafter, ADSCs were incubated with osteogenic induction medium containing 10 mM β-glycerol phosphate (Cat no: G9891; Sigma-Aldrich, Germany), 50 µM ascorbate-2-phosphate (Sigma-Aldrich, Germany), and 0.1 µM dexamethasone (Sigma-Aldrich, Germany) for 21 days. The exhausted medium was exchanged every 3-4 days until the experimental procedure finished.

Alizarin Red S staining

To confirm the osteoblast differentiation of ADSCs, we performed Alizarin Red S (Sigma-Aldrich, Germany) staining to detect calcium deposition. For this propose, ADSCs were seeded at an initial density of 1×10^6 per well of 6-well plates and exposed to the osteogenic culture medium supplemented with 1-2% FBS. After the completion of the differentiation protocol, we stained the cells on days 0, 7, 14, and 21 with Alizarin Red S (2 g Alizarin Red S in 100 mL of distilled water; pH = 4.1-4.3). To stain the cells, the osteogenic medium was discarded and cells were washed with 1.5 ml DPBS and fixed with pre-cooled formalin solution (10%) for 20 minutes at room temperature. After twice washing with PBS, Alizarin Red S solution was poured to each well and kept for 30 minutes at room temperature. Finally, Alizarin Red S solution was discarded and the wells washed with PBS (2 times; each for 5 minutes).

Real-time PCR analysis

The expression of osteopontin (*OPN*) and *ATF4*, *DLX3,* and *FRA1* was studied at time points 0, 7, 14, and 21 days to confirm the osteoblastic differentiation of ADSCs. For this propose, the RNA was extracted from differentiating ADSCs using the Trizol® Reagent (Cat no: T9424; Sigma-Aldrich) according to the manufacturer's instruction. Isolated RNAs were qualified by 1% agarose electrophoresis and concentrations measured by using Nanodrop® (Model: ND-1000; Thermo Scientific). Then, RNAs were reverse-transcribed to cDNAs by using cDNA synthesis kit (Cat no: YT4500; Yekta Tajhiz Azma, Tehran, Iran). Real-time PCR was performed using the SYBR Green solution (batch no: 17D2701; Amplicon) and Mic qPCR Real-Time Cycle. The $2^{-\Delta\Delta CT}$ method was used to calculated relative gene expression. Primer was designated by the Primer Quest tool (version 2.2.3) and the sequence outlined in Table 1.

DNA extraction and sodium bisulfite treatment

On days 0, 7, 14, and 21, nuclear DNA was extracted from ADSCs. Cells were collected and incubated with white

Table 1. Primer list used for real-time PCR analysis

Gene		Primer sequences	TM (°C)	Product size
ATF4	F	TTCTCCAGCGACAAGGCTAAGG	61.5	122
	R	CTCCAACATCCAATCTGTCCCG	60.5	122
DLX3	F	AAGCCCAAGAAGGTCCGAAA	60.80	107
	R	TTTCACCTGTGTCTGCGTGA	60.90	107
FRA1	F	GGAGGAAGGAACTGACCGACTT	59	127
	R	CTCTAGGCGGTCCTTCTGCTTC	60	127
OPN	F	CGAGGTGATAGTGTGGTTTATGG	60	120
	R	CACCATTCAACTCCTCGCTTTC	60	120

blood cells lysis buffer (0.5 M EDTA, 1 M Tris-base, and 75 mM NaCl) at 56˚C for 1 hour followed by treatment with proteinase K at 56˚C for 30 minutes. After the addition of chloroform, the samples were centrifuged at 12000 rpm for 20 minutes and supernatants transferred into tubes containing EtOH (Merck). Then, the samples were mixed gently and stored at -20˚C for 15 minutes. Finally, samples were air-dried and double distilled water added to each tube. The integrity of isolated DNA was evaluated by a NanoDrop® system (Model: ND-1000; Thermo Scientific). To measure methylation rate, unmethylated cytosine residues of DNA were converted to uracil residues with sodium bisulfite by using EZ DNA Methylation-Gold Kit–Zymo Research (Cat no: D5006). To provide positive control, we used peripheral blood DNA samples and methylated by CpG-specific methylase from Spiroplasma namely *SSS1* methylase (Biolabs; USA) as previously described. For negative control, we used EpiTect Control DNA (Cat no: 59655; Qiagen).

Methylation-specific quantitative PCR (MS-qPCR)

DNA methylation of *ATF4*, *DLX3,* and *FRA1* was analyzed using MS-qPCR on days 0, 7, 14, and 21. Two sets of primers for the detection of methylated and unmethylated genes *ATF4*, *DLX3,* and *FRA1* are designated using Gene Runner software and MethPrimer website (Table 2). For MS-qPCR analysis, 7.5 µl SYBR Green reagent (Amplicon Master Mix) was mixed with 2 µL of each primer, 4.5 µL of DDW MS-qPCR and 1 µL DNA samples template. The percentage of *ATF4*, *DLX3* and *FRA1* methylation was estimated using the formula as follows: 100/1 + (efficiency $Met^{Ct\ (Met)}$)/(efficiency $Unmet^{Ct\ (unmet)}$). We considered index 2 for efficiency Met and Unmet.

Statistical analysis

Data are presented as mean ± SD. One-way ANOVA analysis with Tukey post hoc test was used to find statistical differences between groups by using Graph Pad Prism software version 6.07. P value < 0.05 was considered statistically significant in this study.

Results

Confirmation of osteoblastic differentiation

ADSCs were incubated with the osteogenic differentiation medium for 0, 7, 14, and 21 days. We performed Alizarin Red S staining to detect calcium mineral deposition in extracellular space (Fig. 1A). According to our results, red-color calcium deposits were observed in differentiating ADSCs 7 days after incubation with osteogenic medium and these features reached the maximum levels after 21 days, confirming successful osteoblast-like differentiation of ADSCs while no calcium mineral deposition was found in the control group (Fig. 1A). We also monitored the transcription of the osteoblast-specific marker, *OPN*

Table 2. Primer list used for MS-qPCR assay

Gene			Primer sequence	TM (°C)	Product size
ATF4	Methylated	F	5′-ATCGGGAAAGCGTAGTCG-3′	60.6	203
		R	5′-CAAATACGACCAAAACGACCG-3′	61.6	203
	Unmethylated	F	5′-GATTGGGAAAGTGTAGTTGGG-3′	60.1	206
		R	5′-TCCAAATACAACCAAAACAACCA-3′	60.8	206
DLX3	Methylated	F	5′-GTAATGGTGTAAGCGTTTTTCG-3′	60	177
		R	5′-ACCACTCATCCTAACGAACG-3′	60.9	177
	Unmethylated	F	5′-GGTAATGGTGTAAGTGTTTTTTGG-3′	60.2	179
		R	5′-AACCACTCATCCTAACAAACACT-3′	61.4	179
FRA1	Methylated	F	5′-TTGGTAGGTGCGTTAGTTCG-3′	61.5	111
		R	5′-CCCGAAAACTACGAACCCG-3′	62.2	111
	Unmethylated	F	5′-TTTTGGTAGGTGTGTTAGTTTGTAG-3′	60.7	114
		R	5′-CCCCAAAAACTACAAACCCAC-3′	61.1	114

to assess the osteogenic potential of ADSCs (Fig. 1B). Based on the data from real-time PCR analysis, *OPN* was up-regulated in ADSCs committed to osteoblast-like cells. According to our data, the expression of *OPN* was increased by time and reached at day 21. Compared to cells from various time points, the control ADSCs did not express *OPN* expanded in normal culture medium (Fig. 1B). These data demonstrated that ADSCs had the potential to give rise to osteoblast-like cells after being exposed to osteogenic medium over 21 days.

Methylation of ATF4, DLX3, but not FRA1, genes was decreased during osteogenesis

Considering the role of epigenetics in osteogenic differentiation of ADSCs, we examined whether promoter methylation of *ATF4*, *DLX3,* and *FRA1* could be varied during differentiation. Based on our results, the rate of methylation in the promoter region of *ATF4* and *DLX3* was increased by time in ADSCs cultured in osteogenic medium (Fig. 2). However, we found an irregular pattern related to methylation in the promoter region of *FRA1* in ADSCs exposed to osteogenic differentiation medium (Fig. 2). These data added a notion that during osteogenic differentiation of ADSCs, the methylation in the promoter region of some genes such as *ATF4* and *DLX3* was modulated in ADSCs after being-exposed to osteogenic medium over 21 days.

Expression of ATF4, DLX3, and FRA1 was induced in ADSCs during osteogenesis

Levels of *ATF4*, *DLX3,* and *FRA1* mRNA were examined on days 0, 7, 14, and 21. During osteoblasts differentiation of MSCs, the transcription levels of *ATF4*, *DLX3,* and *FRA1* were semi-quantified by real-time PCR assay. Based on data from this panel, a significant increase in mRNA expression of *ATF4*, *DLX3,* and *FRA1* was shown by time and these values reached a maximum level at the end of the experiment. We found a significant difference in the transcription level of three genes on days 14 and 21 compared to the initial time point (day 0; $P < 0.05$). These results showed a possible critical role of *ATF4*, *DLX3,* and *FRA1* during osteoblast differentiation of ADSCs. Based on the data from this panel, we found a strong correlation between transcription level and methylation in the promoter region of genes *ATF4* ($P < 0.05$; $R^2 = 0.9379$) and *DLX3* ($R^2 = 0.7375$, $P > 0.05$). We found a non-significant relationship between methylation in the promoter region and the expression of *FRA1* ($P > 0.05$; $R^2 \simeq 0$).

Discussion

Technologies for the restoration of bone diseases in the clinical setting would be a basic requirement in human medicine. The osteogenic potential of various stem cell types, especially ADSCs, offer hope for healing of injured

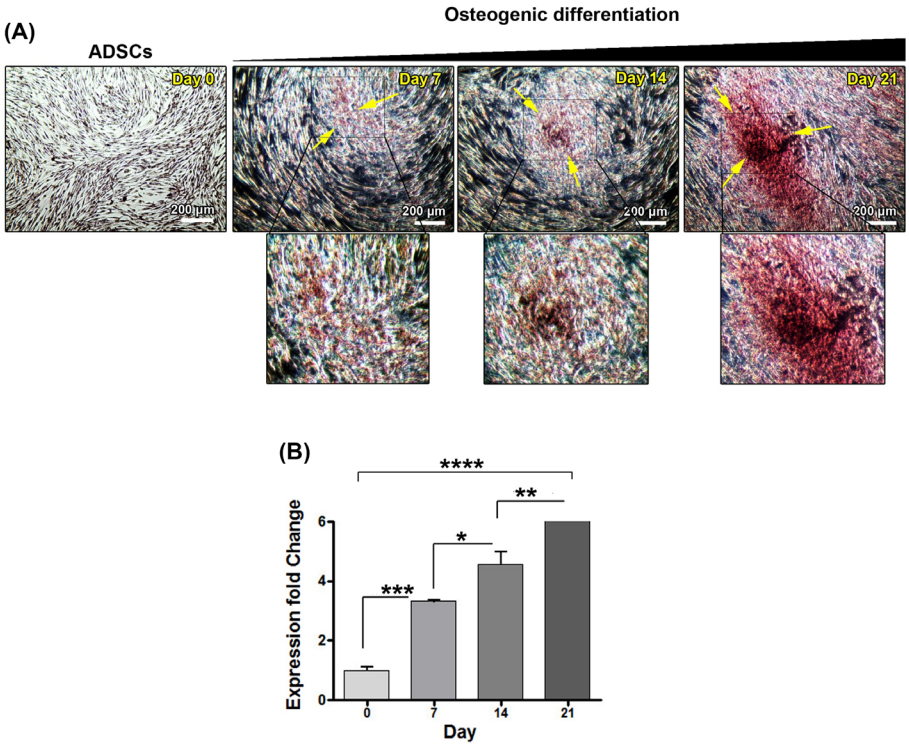

Fig. 1. Confirmation of osteoblastic differentiation evaluating Alizarin Red S staining (**A**) and *osteopontin* gene (**B**) expressions Adipose-derived mesenchymal stem cells were imaged during osteoblastic differentiation on days 0, 7, 14 and 21 by an inverted microscope. The cells were stained at respective time points by Alizarin Red S solution. By the time the level of red-colored deposition increased, indicating the calcium deposits at extracellular space. These changes were evident on day 21 (**A**). Osteopontin (*OPN*) gene expression levels have been shown on days 0, 7, 14, and 21 during osteoblastic differentiation (**B**). The expression of this gene was increased by time and reached a maximum level on day 21. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

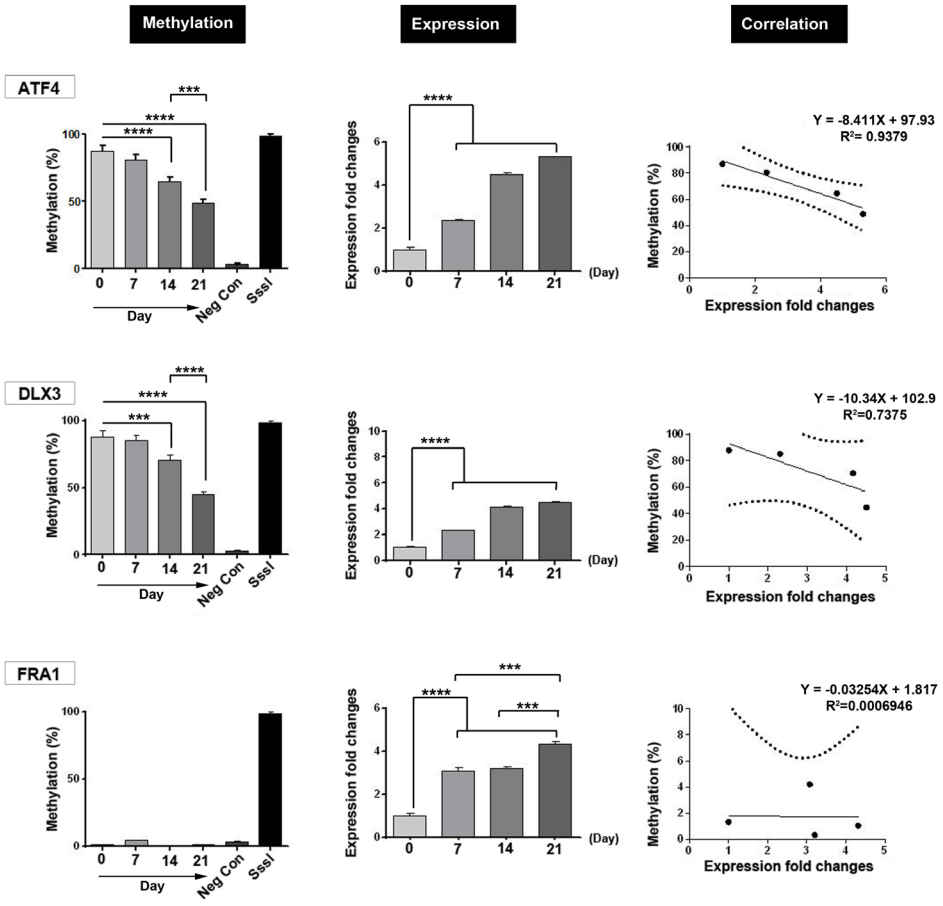

Fig. 2. Representative image of DNA methylation, mRNA level and the correlation between methylation and expression for *ATF4*, *DLX3* and *FRA1* genes during osteoblastic differentiation of ADSCs on 0, 7, 14 and 21 days. A significant increase in the mRNA level of all three genes was observed during MSCs differentiation. The amount of DNA methylation of both genes *ATF4* and *DLX3* was significantly reduced, but no significant changes were observed in the DNA methylation level of *FRA1*. Also, correlation analysis revealed a strong relationship between *ATF4* and *DLX3* gene expression and DNA methylation. SssI: CpG-specific methylase from Spiroplasma ***P<0.001; ****P<0.0001.

bone tissue.[15] Here, we aimed to monitor the expression and promoter methylation rate of *ATF4*, *DLX3,* and *FRA1* during ADSCs differentiation into osteoblast-like cells. Alizarin Red S *in vitro* staining confirmed the efficiency of the current protocol to induce of ADSCs differentiation into osteoblast-like cells after 21 days. By applying the osteogenic differentiation medium, we noted the novel phenotype acquisition coincided with the formation of red-colored calcium mineral deposits required for bone matrix construction. Along with these changes, the expression of *OPN* was simultaneously induced and reached a maximum on day 21. Although as yet not well understood, the osteogenic potential of stem cells coincides with the changes in the expression of genes such as *OCN*, *OPN*, *OSN,* and formation of calcium hydroxyapatite.[20,21]

Our data confirmed that ADSCs' exposure to osteogenic differentiation medium led to the up-regulation of genes *ATF4*, *DLX3,* and *FRA1*.[22-24] It has been shown that *ATF4* participates in osteoblast survival and proliferation at both early and late phases of osteogenesis by modulating specific gene transcription via RSK2-dependent activity and promotion of DDR2-mediated p38 MAPK signaling pathway.[16,25] In support of *ATF4* role on bone function, Yang and colleagues found that the suppression of *ATF4* reduced bone formation capacity by the inhibition of *OCN* in mice model. All these changes contributed to decreased bone mineral density and deposition of the extracellular matrix.[16] Interestingly, it is well-established that the expression of *ATF4* could promote osteogenic capacity in non-osteoblastic lineages.[26] These data show the unique role of *ATF4* in ADSCs undergone a phenotypic trans-differentiation to osteoblast-like phenotype. However, it is yet vague that what extent *ATF4* participates in the direct orientation of ADSCs toward osteoblast lineage.

DLX3 plays a crucial role in the osteogenic orientation of progenitor cells during the development of the embryo.[27] The activity of DLX3 has been shown in the placenta, periosteum, osteoblasts, and chondrocytes and cells differentiating toward osteoblasts.[27,28] Remarkably, DLX3 transcription factor controls the co-expression of *OCN* and *RUNX2* with a particular activity on the promoter regions.[29,30] Further molecular assessments revealed that the suppression of *DLX3* contributed to mouse pup death even prior to bone development.[31] Similar to finding

described by Choi and co-workers, they found that *DLX3* ablation accelerated differentiation of multipotent mesenchymal C2C12 mouse myoblasts to osteoblast lineage at late stages.[32] However, this group did not describe an underlying mechanism related to the activity of *DLX3* on osteoblastic differentiation. This hypocrisy may be explained by the fact that *DLX3* activity is solely associated with the stem cell type undergo differentiation into osteoblast-like cells at different time points.

Fos-related protein (Fra-1) is a member of the AP-1 transcription factor family. Previous experiments revealed that *Fra-1* expressing transgenic mice developed osteosclerosis characterized by high bone mass coincided with enhanced osteoblastic differentiation and bone formation.[33] In support of this claim, *Fra-1* knockout ($fra1^{-/-}$) mice developed osteopenia, a disorder with low bone mass. Also, it was observed that the expression of bone matrix components including osteocalcin and matrix Gla protein decreased in these mice.[34] Over-expression of *Fra-1* in adipose-derived stromal cells inhibited osteoarthritis in mice.[35] Similar to the expression of the above-mentioned genes, we found an increased expression of *FRA1*. These data demonstrated that the expression of all genes including *ATF4*, *DLX3,* and *FRA1* was increased during the osteogenic differentiation of ADSCs. As above-mentioned, changes in the expression of these factors are integral to the trans-differentiation of MSCs. The methylation of DNA, changes in nucleosome histone tails and the remodeling of a chromosome are epigenetic changes, which are crucial in cellular genomic profile expression during differentiation to different lineages. Recent data in the field of embryonic stem cell differentiation revealed an inevitable role for dynamic epigenetic regulation.[36] In this regard, Aranda et al stated that the differentiation potential of SCs correlated with epigenetic status changes.[37] Among these modulations, the changes related to DNA methylation is the most dominant procedure. DNA methylation includes the addition of a methyl group to the 5 cytosines in a CpG dinucleotide, altering genomic integrity, and modulating the various genes. These changes may relate to gene silencing.[38] A recently published data demonstrated that embryonic stem cells differentiating to neuronal lineage showed the CpG methylation at a distinct locus.[39,40] In MSCs, DNA methylation of Trip10 promoter had the potential to accelerate the neuronal and osteoblast differentiation of MSCs.[41] The analysis of promoter methylation revealed different patterns for *FRA1*, *DLX3,* and *ATF4* genes. FRA1 promoter remained in the hypomethylated form pre- and post-osteoblastic differentiation procedure. On the other hand, *ATF4* and *DLX3* promoter region showed the decrease of DNA methylation by time which reached a minimum level on day 21. Correlation of DNA methylation and gene expression changes of *ATF4* and *DLX3* genes demonstrated a strong relationship. In a better word, osteogenic differentiation promoted the expression of these genes while decreased the methylation rate. Previous works showed that hypermethylation could decrease the transcription and bioactivity of a distinct gene.

Research Highlights

What is the current knowledge?

√ Mesenchymal stem cells have great potential in the restoration of bone-related injuries.

√ Promoter methylation and expression of specific genes promote lineage-specific differentiation of stem cells.

What is new here?

√ Promoter methylation of special transcription factors (*DLX3, ATF4,* and *FRA1* genes) induces osteoblastic differentiation of adipose-derived MSCs.

Conclusion

Our data added a notion that continuous incubation of ADSCs with osteogenesis medium decreased the net promoter methylation rate of *ATF4* and *DLX3* genes which could possibly control the expression of these genes. These data showed that monitoring the methylation and expression of specific genes during stem cell differentiation into target cell types could enable us to efficiently control differentiation and orientation rates.

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Ethical statement

Not applicable.

Competing interests

There is no conflict of interests.

Authors' contribution

Conceptualization: AB, MFH; Experiments design: RR, MN; Data analysis: SR, ZL; Provision of study materials and equipment: AB, AN, HNC; Study validation: RR; Supervision: AB, MFH; Data presentation: SR, SA; Draft preparation: HR; Manuscript writing and edition: SR, RR.

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