Indian Journal of Biochemistry & Biophysics
Vol. 59, January 2022, pp. 103-110

DNA methylation is involved in the microRNA-886-3p gene expression and plays a potential role in hematopoietic stem and progenitor cell mobilization through affecting SDF-1

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Received 16 March 2021; *revised 06 November 2021*

The role of the sympathetic nervous system (SNS) in hematopoietic stem and progenitor cell (HSPC) mobilization has been largely investigated. However, there is a critical need for the identification of the underlying contributing factors to improve HSPC yield for transplantation. It has been demonstrated that miR-886-3p targets stromal-derived factor-1 (SDF-1), the central mediator of mobilization, and therefore may play a part in this process. Besides, miR-886-3p expression can be epigenetically regulated through DNA methylation modifications inits gene promoter. Here, to assess the contribution of miR-886-3p and other epigenetic factors in HSPC mobilization, human bone marrow-derived mesenchymal stem cells (MSCs) were treated with the β-adrenergic agonist of isoprenaline. The expression of miR-886-3p and SDF-1and the gene promoter methylation status of this miRNA were then, respectively, evaluated through the appropriate PCR techniques. As expected, despite a transient initial increase in SDF-1mRNA level, its expression reduced, and miR-886-3plevel remarkably increased 48 h following treatment. The gene promoter methylation pattern of miR-886-3p also changed from a full methylated state to a partially methylated one. Together, our findings suggest that miR-886-3p can be epigenetically regulated and through suppressing the expression of SDF-1 play an active role in the SNS-mediated HSPC mobilization.

Keywords: Hematopoietic Stem and Progenitor Cell, Methylation, miR-886-3p, Mobilization, β-adrenergic agonist

The induction of hematopoietic stem and progenitor cell (HSPC) mobilization from the bone marrow (BM) into the peripheral blood (PB) is now considered as an alternative approach for both autologous and allogeneic BM transplantation[1]. So far, different mobilizing agents have been tested to improve the efficacy of this process among them granulocyte colony-stimulating factor (G-CSF) has been accepted as the gold standard mobilizing regimen[2, 3]. However, it is reported that G-CSF is unsuccessful to achieve an adequate amount of mobilized HSPCs in about one-third of patients called poor mobilizers[4-6]. Therefore, a better understanding of the underlying mechanisms and finding new strategies for optimizing HSPC release into the blood circulation seems strongly beneficial.

The chemokine stromal cell-derived factor-1 (SDF-1), also termed as C-X-C motif chemokine ligand 12 (CXCL-12), is known to have a central role in HSPC mobilization. SDF-1 is expressed and secreted by some of the BM stromal cells like mesenchymal stem cells (MSCs)[7] and interacts with its cognate receptor, CXCR4, on the surface of HSPCs to retain these cells in the BM. This process is known as the SDF-1/CXCR4 axis and plays a pivotal role in both physiological and actively induced HSPC mobilization[8,9]. Studies have demonstrated that following the administration of a pharmacological dose of G-CSF to a patient, a proteolytic cascade begins that cleaves and inactivates molecules responsible for HSPC retention in the BM like SDF-1[10,11]. SDF-1 is also released to the PB and acts as a strong chemoattractant to recruit HSPCs from their niche in the BM to the PB where the concentration of this chemokine gradually increases[12]. Moreover, the sympathetic nervous system (SNS) has been demonstrated to mediate HSPC mobilization not only in the steady-state condition but also after G-CSF treatment[13,14]. It has been made clear that the terminal

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fibers of the SNS neurons directly innervate the BM microenvironment and regulate its actions through releasing adrenergic neurotransmitters[15-17]. The expression of b-adrenergic receptors responsive to these adrenergic signals by the BM stromal cells and the observed reduction in SDF-1 level after a β-adrenergic agonist administration confirm this claim[14].

It has been proved that SDF-1 expression can be adjusted through some other underlying factors including miRNAs. According to a previous study, miR-886-3p, also named nc886 or VTRNA2-1, targets SDF-1 in human bone marrow stromal cells and significantly reduces its level[18]. This result prompted us to investigate miR-886-3p exact contribution to SDF-1 related HSPC mobilization. To do so, we treated human BM-derived MSCs with a non-selective b-adrenergic agonist, isoprenaline, as the neural stimulus of mobilization and evaluated SDF-1 and miR-886-3p expression levels following this treatment. Additionally, due to the existence of a CpG island (CGI) in the miR-886-3p genomic region[19] and based on some previous studies which have reported that CpG DNA hypermethylation is responsible for the silencing of this miRNA in several malignancies[20,21], we examined whether the miR-886-3p promoter region undergoes DNA methylation changes during HSPC mobilization. For this purpose, after the treatment of the cells with the mentioned b-adrenergic agonist, we also analyzed the DNA methylation status of the miR-886-3p proposed gene promoter using a methylation-specific PCR (MSP) technique.

Materials and Methods

Cell isolation, culture, and treatment

Human bone marrow aspirate (20 mL) was obtained from the posterior iliac bone of a healthy donor after informed consent. MSC isolation was then carried out according to the common centrifugal density gradient method. Briefly, after diluting in an equal volume with phosphate-buffered saline (PBS; GIBCO-BRL, Grand Island, NY, USA), a 1:1 volume of Ficoll solution (Sigma Aldrich, St Louis, MO, USA) was added to the aspirate. The created biphasic sample was centrifuged (400 g, 20 min, 22°C) and the mononuclear cell fraction was carefully harvested. The separated sample was washed three times with PBS and finally transferred to a 75 cm^2 cell culture flask including 15 mL low glucose Dulbecco's modified eagle *medium* (DMEM; GIBCO-BRL) with 10% fetal bovine serum (FBS; GIBCO-BRL) and incubated at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was replaced every three days, and the cells were dissociated with 0. 25% *trypsin-ethylenediaminetetraacetic acid* (GIBCO-BRL) and sub-cultured in new flasks once they reached confluence.

The treatment procedure was done on approximately 80% confluent MSCs at passage three. The cells were divided into three groups, with group 1 as the control group and groups 2 and 3 as the treatment groups which were treated with isoprenaline hydrochloride solution (Sigma-Aldrich) to a final concentration of 100 μM for 12 and 48 h, respectively.

Cell characterization

MSC characterization was performed through the assessment of both adipogenic and osteogenic differentiation capacity of the isolated cells and flow cytometric analysis of the surface antigens. For the induction of adipogenic differentiation, the cells were incubated for three weeks with a complete culture medium containing 1 μM dexamethasone, 0.5 mM isobutyl methylxanthine, and 5 μg/mL insulin (all from Sigma-Aldrich). The adipogenic differentiation was finally confirmed through staining the accumulated lipid-rich vacuoles by oil red o (Sigma-Aldrich). The osteogenic differentiation was induced through supplementing the growth medium with 100 nM of dexamethasone (Sigma-Aldrich), 50 μg/mL ascorbic acid 2-phosphate (Sigma-Aldrich), and 10 mM b-glycerol phosphate (Merck, Rahway, NY, USA) for 21 days. The samples were then fixed, and the calcium deposits were stained using the alizarin red reagent (Sigma-Aldrich). For the analysis of the surface antigens, a total of 1×10^5 cells in 100 μL PBS (Sigma-Aldrich) were stained with anti-human CD45, CD90 (both fluorescein isothiocyanate (FITC) conjugated, Abcam, Cold Spring Harbor, NY, USA), CD73, and CD105 (both Phycoerythrin (PE) conjugated, Abcam) monoclonal antibodies for 30 min at 4°C. The corresponding isotype control antibody was also used for each test to ensure specificity. Analysis with FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA) was finally performed using Flowjo software, and the related histograms were generated.

RNA extraction and cDNA synthesis

All three groups were subjected to RNA extraction and cDNA synthesis. In brief, total RNA was manually extracted from MSCs using RNX^{TM}-Plus reagent (CinnaGen Inc, Tehran, Iran). Then, cDNA synthesis

was separately carried out from all expressed mRNAs (GenDEPOT, TX, USA) and miRNAs (Stratagene, CA, USA) according to the instructions provided in each kit. Based on the Stratagene instruction manual for miRNA cDNA synthesis, two reaction steps had to be taken successively to synthesize cDNA from the existing miRNA samples. In the first step, a polyadenylation reaction was done using an *E. coli* poly(A) polymerase, and cDNA synthesis was then completed by the application of a special adaptor primer and other reagents provided in the kit.

SDF-1 and miR-886-3p quantification

Possible alterations in miR-886-3p expression and its potential target gene, SDF-1, following treatment with isoprenaline were evaluated through real-time quantitative reverse transcription PCR (qRT-PCR). SDF-1expression levels before and after treatment with isoprenaline were quantified using a SYBR Green master mix (Ampliqon, Odense, Denmark). B2-microglobulin (B2M) was used as an internal control gene. The expression pattern of miR-886-3p was also quantitatively assessed using a miRNA QPCR Master Mix (Stratagene) which includes Eva green as a next-generation fluorescent DNA binding dye and a universal reverse primer attaching to polyadenylated-adaptor primer piece of the produced cDNA that was made in the cDNA synthesis step. Therefore, a forward primer was only designed to evaluate the miR-886-3p expression. MiRNA-886-3p Ct values were normalized using the U6 small nuclear RNA (snRNA) reference gene as one of the best housekeeping genes for miRNA quantification. The sequences of all of the designed forward and reverse primers and also Stratagene universal reverse primers have been provided in (Table 1).

Methylation-specific PCR and semiquantitative analysis

The genomic DNA was first extracted from all of the control and treatment groups using QIAamp DNA Mini-Kit (Qiagen, Valencia, CA, USA) according to the instruction manual. After that, the isolated DNA was subjected to sodium bisulfite treatment using the Bisul Flash DNA Modification Kit (Epigentek, NY, USA). This conversion reaction modifies the cytosine residues to uracil but leaves 5-methylcytosine residues unaffected. The gene promoter methylation pattern of miR-886-3p following treatment with isoprenaline was finally assessed through a bisulfite-PCR-based DNA methylation analysis called MSP. MSP was carried out using a 2X MSP Master Mix (Qiagen) and two pairs of primers, one for the probable methylated status and the other for the non-methylated DNA sequence. 1000 bp DNA upstream of the gene transcription start site was given as an input to the Methprimer online software to identify the CpG islands in the miR-886 gene promoter and design the corresponding MSP primers. Collagen type II alpha 1 (COL2A1) was also included as a validation reference for examining the qualification of bisulfite conversion in this MSP technique. The sequences of the designed forward and reverse primers for

Table 1 —Description of the designed forward and reverse primers used in qPCR and methylation-specific PCR reactions

Gene	Strand	Primer sequence (5'→3')	Amplicon size (bp)
SDF-1	Sense	AGAGCCAACGTCAAGCATCT	111
	Antisense	CTTTAGCTTCGGGTCAATGC	
B2M	Sense	ATGCCTGCCGTGTGAAC	91
	Antisense	ATCTTCAAACCTCCATGATG	
miR-886-3P	Sense	CTCAAGCGGTTACCTCCTCATG	75
	Antisense*	GACGAGCTGCCTCAGTCG	
U6	Sense	AAATTGGAACGATACAGAGAAG	96
	Antisense*	GACGAGCTGCCTCAGTCG	
miR-886-3P-M	Sense	GCGGGGAGTATATGTAGTTCGT	123
	Antisense	AAATCCGACATAAAAAAATAACCG	
miR-886-3P-U	Sense	GGGGTGGGGAGTATATGTAGTTT	125
	Antisense	AATCCAACATAAAAAAATAACCACT	
COL2A1	Sense	GTAATGTTAGGAGTATTTTGTGGGTA	86
	Antisense	CTACCCCAAAAAAACCCAATCCTA	

The sequence of the forward and reverse primers designed to assess the expression of SDF-1 and miR-8886-3P following isoprenaline treatment and their related internal controls (B2M and U6, respectively) and also the designed primers for both the methylated and unmethylated states of miR-886 gene promoter have been presented in the table. The sequence of the designed primers for the qualification of the COL2A1 gene bisulfite conversion has been also provided.

*Stratagene universal reverse primer was used as the antisense strand primer of miR-886-3P and U6

both methylated (miR-886-3p-M) and unmethylated (miR-886-3p-U) status of miR-886-3p gene promoter and the designed primers matched with bisulfite-treated COL2A1 gene have been given in (Table 1). The semiquantitative analysis of the PCR products using GelQuant. NET software was also performed to more precisely evaluate the anticipated DNA methylation changes at the miRNA gene promoter region.

Statistical analysis

The obtained data were analyzed statistically using the delta cycle threshold ($\Delta\Delta ct$) method to calculate the relative fold gene expression of SDF-1 and miR-886-3p to that of the housekeeping genes studied. P-value was determined using Student's t-test, Prism 7. 0 software (Graphpad), and the results were expressed as mean ± 1 SD.

Results

Cell culture and characterization testing

The BM-derived cells maintained their elongated, fibroblast-like appearance, which is the known morphological characteristic of MSCs, during the whole culture period. The cells showed their capability to differentiate toward osteogenic and adipogenic lineages and according to the performed flow cytometric analysis were positive for CD 73, 90, and 105 and negative for CD 45 surface markers, which all confirmed their identity as MSCs (Fig. 1).

SDF-1 and miR-886-3p expression pattern following treatment with isoprenaline

A transient significant increase of 6. 15±1.4 fold in SDF-1 mRNA level was first detected in MSCs 12 h post treatment with isoprenaline. Its expression then decreased to 0.49±0.05 fold at 48 h time point compared to the control group that was highly significant (Fig. 2A). The expression of miR-886-3p also gradually increased following the administration of isoprenaline and reached 3.5±0.43 and 75.23±4.79 fold 12 and 48 h post treatment, respectively, which was statistically significant in both cases (Fig. 2B).

The gene promoter methylation status of miR-886-3p following treatment with isoprenaline

Using the Methprimer online software, one CpG island located from −191 bp to −57 bp was found within the 1000 bp DNA upstream of the miR-886 gene, and the methylation pattern of the gene promoter at this area was accordingly evaluated performing the MSP reaction with the appropriate methylated- and unmethylated-specific primer sets (Fig. 3A). The MSP result of the mentioned CpG is land revealed a fully methylated DNA status for the

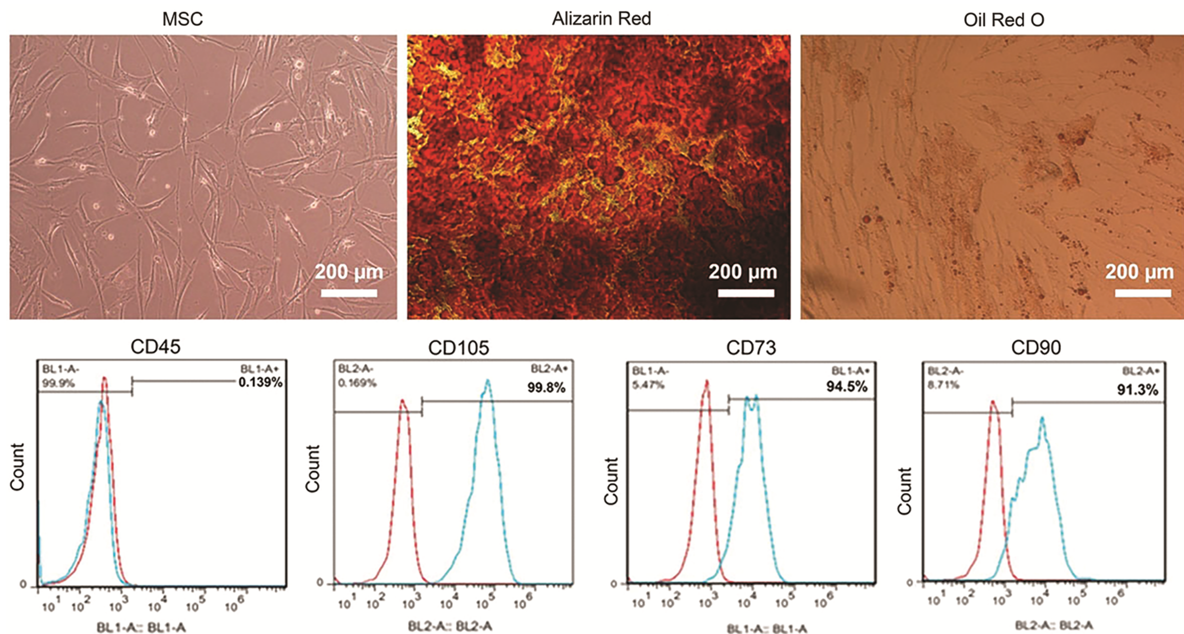

Fig. 1 — Characterization of the cultured human bone marrow-derived stem cells. The cells had a fibroblast-like, spindle-shaped morphology. The differentiation capacity of the bone marrow-derived cells into adipogenic and osteogenic lineages was verified through oil red o and alizarin red stating, respectively. Cell surface antigen analysis using flow cytometry also revealed that the cells were positive for CD73, CD90, and CD105 and also negative for CD45, which further confirmed their identity as MSCs

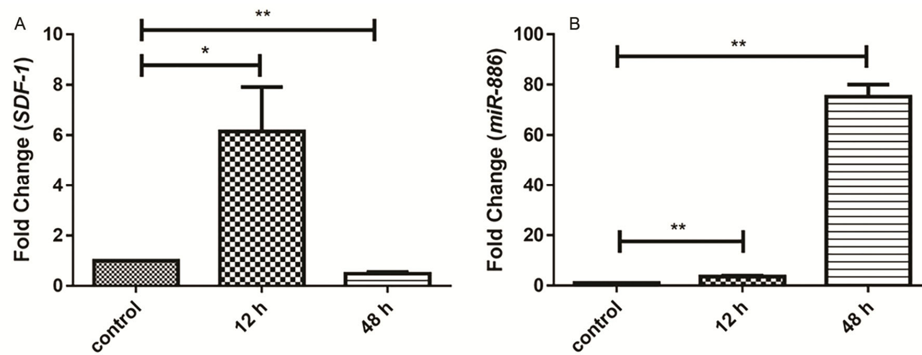

Fig. 2—SDF-1 and miR-886-3p expression levels before and after treatment with isoprenaline (A) SDF-1 mRNA expression showed a significant transient increase of 6.15-fold 12 h post treatment compared with the non-treated MSCs. Its expression level then decreased significantly to 0.49-fold at 48 h treatment time point * $P< 0.05$ and ** $P< 0.01$ were considered significant as compared to untreated control; and (B) miR-886-3p expression increased significantly to 3.5 and 75.23-fold 12 and 48 h post treatment, respectively. The increase observed at 48 h time point was highly remarkable ** represents $P< 0.01$

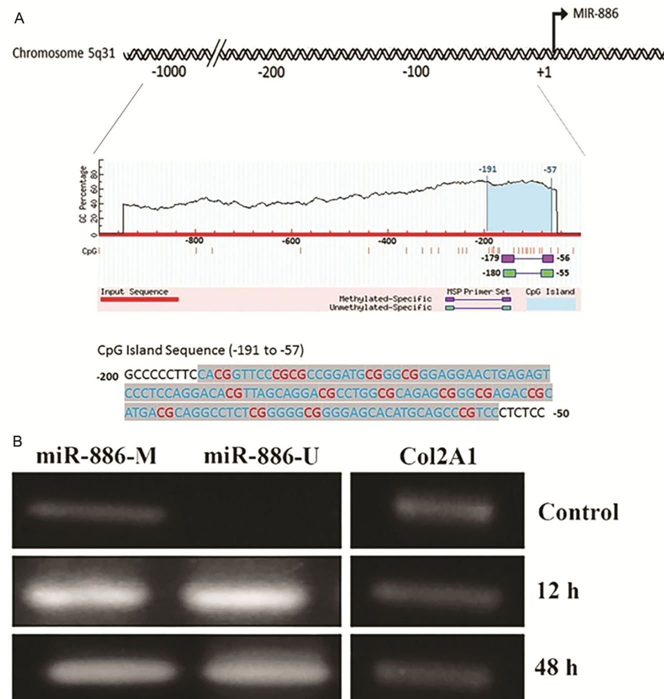

Fig. 3— Gene promoter methylation analysis of miR-886-3p (A) 1000 bp DNA upstream of the miR-886 gene was given as an input to the Methprimer online software. As shown in the picture, one CpG island was identified within the −191 to −57 bp promoter region of this gene whose methylation pattern before and after treatment with isoprenaline was assessed through designing methylated- and unmethylated specific primers. Positioning of the MSP primer sets and the sequence of the assessed CpG island have been also presented; and (B) A fully-methylated status was seen in the gene promoter of miR-886-3p before isoprenaline treatment; according to the picture, this pattern changed to a partial methylation state 12 and 48 h after the treatment. COL2A1 was used for the quality assessment of DNA bisulfite conversion reaction. miR-886-M: methylated miR-886-3p, miR-886-U: unmethylated miR-886-3p, COL2A1: collagen type II alpha1

miR-886-3p gene promoter before the treatment as a band was observed for miR-886-M but not miR-886-U of the control group. However, after treating the cells with isoprenaline, the gene promoter region of miR-886-3p showed a partially methylated state at both 12 and 48 h time points. The detection of specific bands in both methylated and unmethylated parts after the treatmentis the reason for this claim. In each of the control and treatment groups, a band containing the amplified product of the bisulfite-

Table 2 — Semiquantitative analysis of methylation-specific PCR products

Study Groups	Raw data (pixels volume)			Normalized ratio		Methylation ratio (%)
	miR-886-M	miR-886-U	Col2A1	miR-886-M/Col2A1	miR-886-U/Col2A1	
Control	7181	0	9448	0.76	0	100
12 h	16784	17796	8431	1.99	2.1	49
48 h	17269	15488	7557	2.3	2.05	53

The semiquantitative analysis of the MSP gel bands using Gel Quant. NET software showed the partial methylation of the miR-886-3p gene promoter 12 and 48 h post isoprenaline administration. A decrease in methylation from 100 to 49 and 53 percent (12 and 48 h post treatment, respectively) is observed in the table. The MSP results of methylated and unmethylated miR-886 (miR-886-M and miR-886-U, respectively) have been normalized relative to the Col2A1 gene

treated COL2A1 gene was also detected which confirmed that the bisulfite conversion of cytosine residues to uracil had been efficiently performed (Fig. 3B). The result of the semiquantitative analysis of MSP gel bands using Gel Quant. NET software has been also represented in (Table 2). Based on the information provided, treatment with isoprenaline resulted in the partial methylation of the miR-886-3p gene promoter. In another word, we observed a decrease of methylation over time from the pre-treatment state in which the promoter of this gene was methylated in all of the cells (fully methylated; 100% methylation ratio) to 12 and 48 h post-treatment that some of the cells had the methylated and others had the unmethylated status of miR-886-3p gene promoter (Partially methylated; 49% and 53% methylation ratio, respectively).

Discussion

Achieving a higher efficiency in HSPC mobilization has been long of considerable interest. It will therefore be advantageous to gain a better understanding of the factors involved in HSPC homing or migration and design more potent mobilization strategies.

By affecting the b-adrenergic receptors expressed on the BM cells, the SNS signals tightly regulate the SDF-1-related HSPC fluctuation between the BM and blood circulation[13,22]. It has been reported that treating the BM stromal cell line of MS-5 with either noradrenaline, as the natural neurotransmitter of the SNS, or isoprenaline, as a non-selective agonist of b-adrenergic receptors expressed on these cells, decreased both mRNA level and protein secretion of SDF-1 in a dose-dependent manner. Similar effects using the primary myeloid cultures of the BM were also detected, and a remarkable amount of progenitor cells mobilized to the supernatant[14]. Accordingly, in this study, we applied 100 µM isoprenaline to simulate HSPC mobilization. The expression of SDF-1 and its targeting miRNA, miR-886-3p[18], were then assessed 12 and 48 h after isoprenaline administration based on this hypothesis that miR-886-3p suppresses SDF-1 expression during the mobilization and is probably one of the key underlying factors of the SNS-mediated HSPC mobilization. As expected, SDF-1 mRNA level decreased and miR-886-3p level increased significantly 48 h post treatment. However, we observed a significant initial increase in SDF-1 mRNA expression at 12 h time point. This result is in accordance with the previous studies which reported a transient increase in SDF-1 expression following G-CSF administration and justified it with the initial induction of SDF-1 expression and secretion in the BM mesenchymal cells and the subsequent decrease of this molecule through proteolytic inactivation mechanisms[23]. Thus, isoprenaline can induce miR-886-3p expression in the BM MSCs and lead to SDF-1 mRNA down-regulation in these cells. The regulatory role of miR-886 on SDF-1-related cell mobilization is similar to that of some other miRNAs which have been shown to modulate SDF-1 chemotax is on related CXCR-4 expressing cells migrating to inflammatory and ischemic tissues or invading to a different tumor niche[24-26]. We come, accordingly, to regard miR-886-3p as a probable new biological regulator of the SNS-mediated HSPC egress from the BM into the PB and one of the main participants of mobilization.

Another aspect was to assess whether the gene promoter region of miR-886-3p undergoes DNA methylation modifications and works as a gene expression controlling mechanism during the mobilization. Our results revealed a full-methylated state in miR-886-3p gene promoter in untreated control, but after treatment with the mentioned b-adrenergic agonist, a partially methylated pattern was detected. Therefore, the elevated expression of miR-886-3p in MSCs following isoprenaline treatment can be due to the partial removal of the inhibitory methyl groups bound to its CGI-containing gene promoter.

MiRNAs are not only recognized as controlling epigenetic tools of gene expression, but they also can be a potential target for other regulatory epigenetic mechanisms, especially DNA methylation[27-30]. There is controversy, however, over the identity of miR-886-3p as a miRNA. Some studies have asserted that it is a vault RNA (VTRNA2-1), not a miRNA[31] while others believe that it is more appropriate to consider it as a non-coding RNA (nc886)[32]. In any case, the epigenetic repression of this RNA, which usually occurs through the hypermethylation of its CGI-containing genomic region, has been observed in several cancers. According to Fort *et al*., a suppressing increase of DNA methylation rate in nc886 gene promoter is seen in both tumor and metastatic prostate tissues[21]. Besides, the hypermethylation of the GC-enriched promoter region of miR-886-3p/VTRNA2-1/nc886 directly led to its silencing and subsequently induced the expression of target oncogenes involved in some other types of malignancies[20,33,34]. These findings are in line with our obtained results that miR-886-3p expression is under the regulation of epigenetic mechani ms and is probably dictated through alterations in the DNA methylation of its gene promoter. Investigations have suggested that as a tumor suppressor gene, miR-886-3p may also have various target genes participating in cell migration[20,35]. Therefore, there is this possibility that miR-886-3p is epigenetically regulated and mediates HSPC migration through targeting a special chemokine like SDF-1.

Many efforts have been made to achieve better HSPC mobilization outcomes. Collectively, miR-886-3p and the way its expression is managed can be considered as effective manipulation targets to clinically regulate and also improve HSPC mobilization for hematopoietic engraftment. Further *in vitro* and *in vivo* future studies, therefore, are required to prove the practicality of this hypothesis and translate it for human application.

Acknowledgement

This study was financially supported by the research grant from Tarbiat Modares University, Iran.

Conflicts of interest

All authors declare no conflict of interest.

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