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In vitro characterization of human bone marrow mesenchymal stem cell-derived motor neurons induced by epigenetic modifiers

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Abstract

Background: Motor neurons (MNs) are distinct types of cells in the dorso-ventral axis of the spinal cord. These cells are developed in the presence of two main morphogens, including Sonic hedgehog (Shh) and retinoic acid (RA). On the other hand, human bone marrow mesenchymal stem cells (hBM-MSCs) are known as a multipotent type of cells with neural differentiation capacity. In this regard, the aim of this study was to quantitatively evaluate the expression of MN-related genes and the potent epigenetic regulatory genes involved in neurogenesis, including *Enhancer of zeste homolog 2* (*EZH-2*) and *P300*, during hBM-MSC differentiation into MN-like cells, using RA and Shh. After isolating and inducing the cells with Shh and RA, the results were evaluated using immunocytochemistry and qRT-PCR.

Results: Our findings showed that the treated cells could express choline acetyltransferase (ChAT) and insulin gene enhancer binding protein-1 (Islet-1) antigens at the protein level, 2 weeks after induction. Moreover, at the second week after induction, the induced cells expressed MN-related genes (*ChAT* and *ISLET-1*) and epigenetic regulatory genes (*EZH-2* and *P300)* at significant levels compared to the control (non-treated BM-MSCs) and to the induced cells at the first week (day 7). In addition, the expression of *EZH-2*, as a histone-modifying gene, was also significantly upregulated at the first week compared to the control. No significant upregulation was detected in the expression of *motor neuron and pancreas homeobox 1 (MNX-1)* in the treated groups compared to the control group.

Conclusion: We concluded that epigenetic modifiers, P300 and EZH-2, are important mediators for regulating the process of motor neuron differentiation induced by RA and Shh.

Keywords: Motor neuron, Bone marrow, Mesenchymal stem cell, Epigenetic, *P300*, *EZH-2*

Background

Motor neurons (MNs) are known as a distinct type of cells with defined functions along the dorso-ventral axis of the spinal cord. Two main morphogens, including retinoic acid (RA) and Sonic hedgehog (Shh), make role in the developmental specification of these cells [1]. Motor neuron-related diseases threaten the lives of many patients throughout the world. Since there is no efficient clinical treatment for these patients, medical interventions such as cell therapy still bring hope to these people. Human bone marrow mesenchymal stem cells (hBM-MSCs) are multipotent cells characterized by self-renewal and immunomodulatory properties [2, 3]. Cell fate of MSCs would be changed depending on the source of origin and by the ingrained epigenetic memory signatures of the cells [4]. Synergistic administration of RA and Shh leads to the expression of motor neuron-related markers in cultured stem cells [5]. Retinoic acid

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plays a major role in the development of stem cells; however, little is known about the mechanisms for the repression of RA-regulated genes [6]. Moreover, the evolutionary conserved developmental morphogens, the Hedgehog family, have critical roles in stem cell development and functions [7]. During the development of human stem cells, differential gene expression is retained through mitosis. Such stable alterations in the gene expression occur during progression from stem cells into differentiated progenies. The commitment of stem cells to differentiate into a particular cell type requires upregulating the expression of genes responsible for a specific phenotype and suppressing the expression of other genes that maintain the stemness properties [8, 9]. By imposing functionally relevant modifications to the genome, histone-modifying enzymes regulate the accessibility of transcription factors and other modulators to the genes [9, 10]. For example, a histone-lysine *N*-methyltransferase enzyme called enhancer of zeste homolog 2 (EZH-2) regulates the transition from proliferation to differentiation and accelerates the onset of neurogenesis [11, 12]. Moreover, it regulates the balance between self-renewal and differentiation in the cerebral cortex cells [11], controls neural stem cell state [13], and prevents premature differentiation [11, 14]. Through an epigenetic mechanism, Shh signaling regulates the expression of *EZH-2* and manages the expression of genes involved in numerous physiological functions such as neural cell survival and differentiation, in vivo [15, 16].

On the other hand, a histone acetyltransferase, P300, makes a role in the regulation of neurogenesis in the spinal cord motor neurons [5, 17]. Tight control on the function of P300 is critical to ensure precise histone acetylation and gene activation. Numerous studies have examined the functional requirement of P300 to act as a co-activator or an acetyltransferase for other transcription regulators [18]. In embryonic spinal motor neurons, P300 and CREB-binding protein (CBP) contribute to the maintenance of Islet-1 expression as the main motor neuron-related transcription factor [19]. The decrease in the number of MNs in CBP/P300 compound mutant embryos confirms that these co-activators act redundantly to promote MN specification, in vivo.

The gene expression and epigenetic regulation underlying the process of mesenchymal stem cell differentiation into motor neurons are poorly understood. Therefore, the purpose of this study was to quantitatively investigate the expression of motor neuron-related genes and the potent epigenetic regulatory genes involved in neurogenesis, including *EZH2* and *P300*, during differentiation of human BM-MSCs into motor neuron-like cells, using RA and Shh.

Methods

Isolation and culture of human bone marrow-derived mesenchymal stem cells

The protocol for the aspiration of human bone marrow was approved by the university research ethics committee. After diluting 5 ml of the aspirated samples with an equal volume of phosphate-buffered saline (PBS; Gibco, USA), to isolate bone marrow mesenchymal stem cells (BM-MSCs), the solution was loaded onto 20 ml of Ficoll (Inno-Train, Germany) and centrifuged (400×*g*, 20 min). The mononuclear cells were then collected, washed twice with PBS, and seeded in tissue culture flasks 75 cm^2 (TPP, Switzerland) at a density of 10^5 cells/ml using Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents from Gibco, USA). The cells were cultured at 37 °C in an incubator containing 5% CO_2. The culture medium was refreshed every 3 days. The cells were passaged at the ratio of 1: 3, upon reaching 80% confluence [20]. The third passage cells were used for subsequent experiments.

Characterization of mesenchymal stem cells

Mesenchymal stem cells were characterized using mono-color cytofluorimetric analysis, according to our previous protocol [21]. To do that, 1×10^5 of these cells with 10% goat serum were incubated at 4 °C for an hour. After removal of the serum, the cells were labeled with monoclonal antibodies conjugated with red phycoerythrin (PE) or green fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human antigens including CD34, CD44, CD45, CD73, and CD90 (BD bioscience) at 4 °C for 40 min. In each case, an isotype-matched control was used. Antigen expression data obtained by FACSCalibur Flow Cytometer (Becton Dickinson, UK) were analyzed by the FlowJo software.

Induction of mesenchymal stem cells into motor neuron-like cells

Human bone marrow mesenchymal stem cells were induced into motor neuron-like cells based on our previous protocol [20, 21]. In summary, the cells were cultured overnight at a density of 10^5 cells/well in a 24-well plate containing a complete expansion medium at 37 °C and 5% CO_2 humid incubator. Afterward, the existing medium was replaced with a pre-inductive medium consisting of DMEM-F12, 20% FBS, 10 ng/ml bFGF (Sigma, USA), 250 mM isobutylmethylxanthine (IBMX; Gibco, USA), 100 mM β-mercaptoethanol (β-ME; Gibco, USA), and 2% B27 (Invitrogen, USA) and stored overnight in an incubator. On the following day, after removing the inductive medium, the induced cells were treated with the first differentiation medium

including DMEM-F12 supplemented with 0.01 mM RA (Sigma, USA), 100 ng/ml Shh (R&D, USA), and 0.2% B27 for a week. The first differentiation medium was then substituted with the second differentiation medium containing DMEM-F12 with brain-derived neurotrophic factor (BDNF; Invitrogen, USA) and 0.2% B27 as surviving factors, and differentiating cells were cultured in this medium for 1 week later.

Immunocytochemical staining

To evaluate the expression of relevant antigens at the protein level, the treated cells were fixed using 4% paraformaldehyde. Then, the cells were permeabilized by treatment with 0.2% Triton X-100 (Gibco, USA) in PBS. After washing, they were incubated with diluted 10% goat serum in PBS as a blocking buffer at room temperature for an hour to prevent nonspecific interactions. Thereafter, primary antibodies against human choline acetyltransferase (ChAT; Abcam, USA), Islet-1 (Santa Cruz, USA), and Mnx-1 (Abcam, USA) were incubated overnight with samples at 4 °C. After washing with PBS, the cells were labeled by incubation with the corresponding secondary antibodies (Sigma, USA) conjugated to FITC or PE at 37 °C for 45 min. Eventually, DAPI (Sigma, USA) was used to stain the nuclei, and cell observation was accomplished by a DP70 fluorescence microscope (Olympus, Japan).

Table 1 List of primers used in qRT-PCR

Target gene	Primer sequence
GAPDH	F: CTCATTTCCTGGTATGACAAC R: CTTCCTCTTGTGCTCTTGCT
CHAT	F: GCA GGAGAAGACAGCCAACT R: TGCAAACCTCAGCTGGTCAT
MNX-1	F: AGCACCAGTTCAAGCTCAACA R: ACCAAATCTTCACCTGGGTCTC
ISLET-1	F: ATATCAGGTTGTACGGGATCAAATG R: CACGCATCACGAAGTCGTTC
P300	F: GTTCTCCCTTACAGCAGCAACA R: GCAGAGGATTCATGTTCTGCAAG
EZH-2	F: CCGGGCTAGGTTAATTGGGACCAAA R: CTCGAGTTTGGTCCCAATTAACCT

Q-RT PCR

The expressions of motor neuron-associated antigens and the regulating histone acetyltransferase and histone methyltransferase genes were confirmed by real-time PCR. After cell harvesting at days 7 and 14 post-induction, total RNA was extracted by TRIzol Reagent (Sigma, Germany). Then,

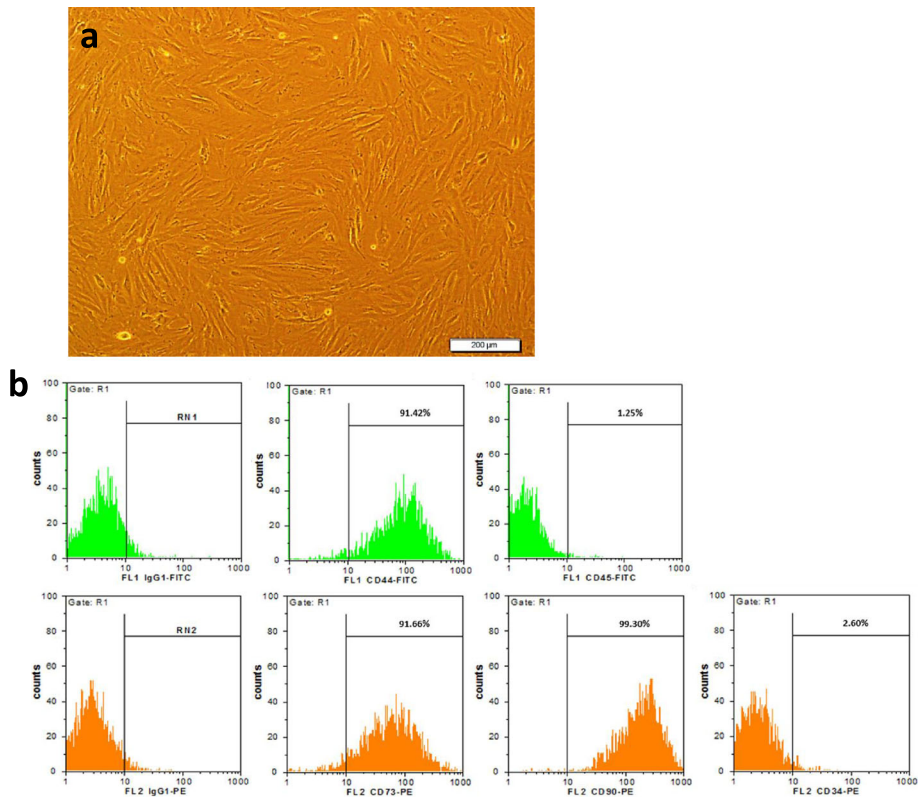

Fig. 1 Isolation and characterization of human bone marrow-derived mesenchymal stem cells. **a** BM-MSCs had fibroblast-like morphology after three passages (magnification ×4). **b** Cytofluorimetric analysis of the isolated cells at passage 3 revealed that they expressed CD44, CD73, and CD90 antigens, but they could not express hematopoietic and leukocyte markers, including CD34 and CD45

the purity of RNA was quantitatively evaluated by spectrophotometer, and cDNA was synthesized from the extracted RNA using a cDNA Synthesis Kit (Fermentase, Canada). Eventually, RT-PCR reactions were performed in a 7500 real-time PCR system (Applied Biosystems, USA) in such a way that in each reaction, 2 μl of 12.5 ng cDNA was mixed with 5 μl of SYBR Green Master Mix (Applied Biosystems, USA) and 1 μl of corresponding forward and reverse primers (Table 1), and the total volume was reached to 20 μl by adding double-distilled water. The expression level of the GAPDH gene was considered as an internal control to normalize the expression levels of selected genes. Each target gene expression was evaluated in duplicate for three different mRNA samples.

Results

Isolation and characterization of human bone marrow mesenchymal stem cells

The isolated MSCs taken from human bone marrow had spindle-like morphology at passage 3 (Fig. 1a). These cells were able to express CD44, CD73, and CD90 markers on their surfaces, but they could not express hematopoietic antigens, such as CD45 and CD34 (Fig. 1b).

Differentiation of bone marrow mesenchymal stem cells into motor neuron-like cells

The expression of motor neuron-related markers, including ChAT, Islet-1, and Mnx-1, were evaluated using immunocytochemical staining. The results showed that the cells could express ChAT (45 ± 13.4%) and Islet-1 (53 ± 10.1%). No expression of Mnx-1 was detected on day 14 (Fig. 2).

Evaluation of expression by real-time PCR

Treatment of hBM-MSCs with the induction medium led to the upregulation of motor neuron-related genes, including *ISLET-1* and *ChAT* as well as histone-modifying enzyme genes, *P300* and *EZH-2*, at the second week of induction when RA and Shh were removed ($p \leq 0.05$). Moreover, significant upregulation of *EZH-2* was also detected at the first week when the result was compared with non-treated BM-MSCs as the control ($p \leq 0.05$). We could not detect any upregulation in the expression of *MNX-1* compared to the control (Fig. 3).

Discussion

Mesenchymal stem cells derived from bone marrow are known as multipotent cells with immunomodulatory properties which have the potential to differentiate into

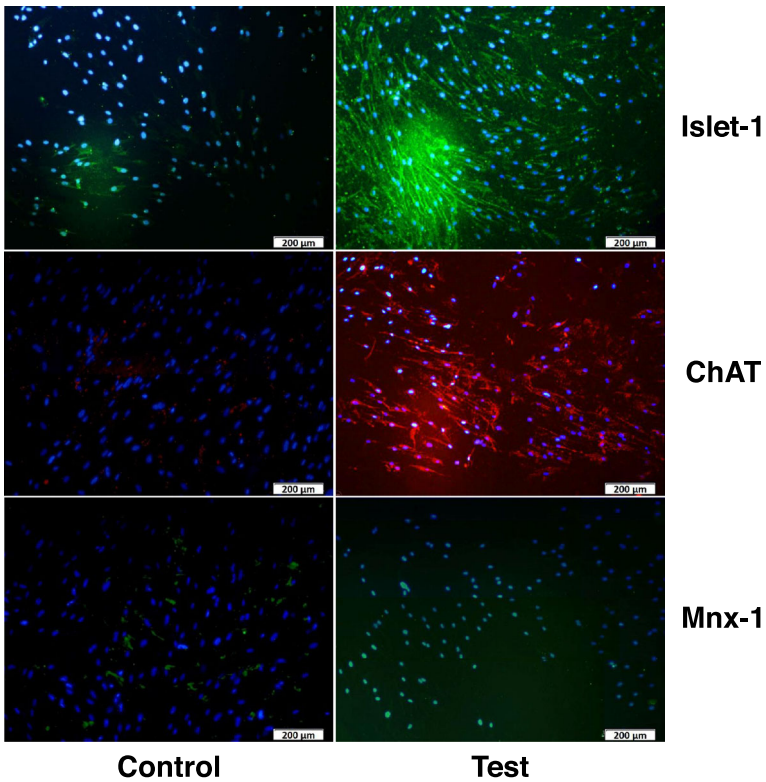

Fig. 2 Immunostaining of motor neuron-like cells derived from hBM-MSCs. The expression of Islet-1 (53 ± 10.1%), ChAT (45 ± 13.4%), and Mnx-1 antigens were investigated at the second week post-induction in the test group (treated hBM-MSCs) compared with the control group (non-treated hBM-MSCs)

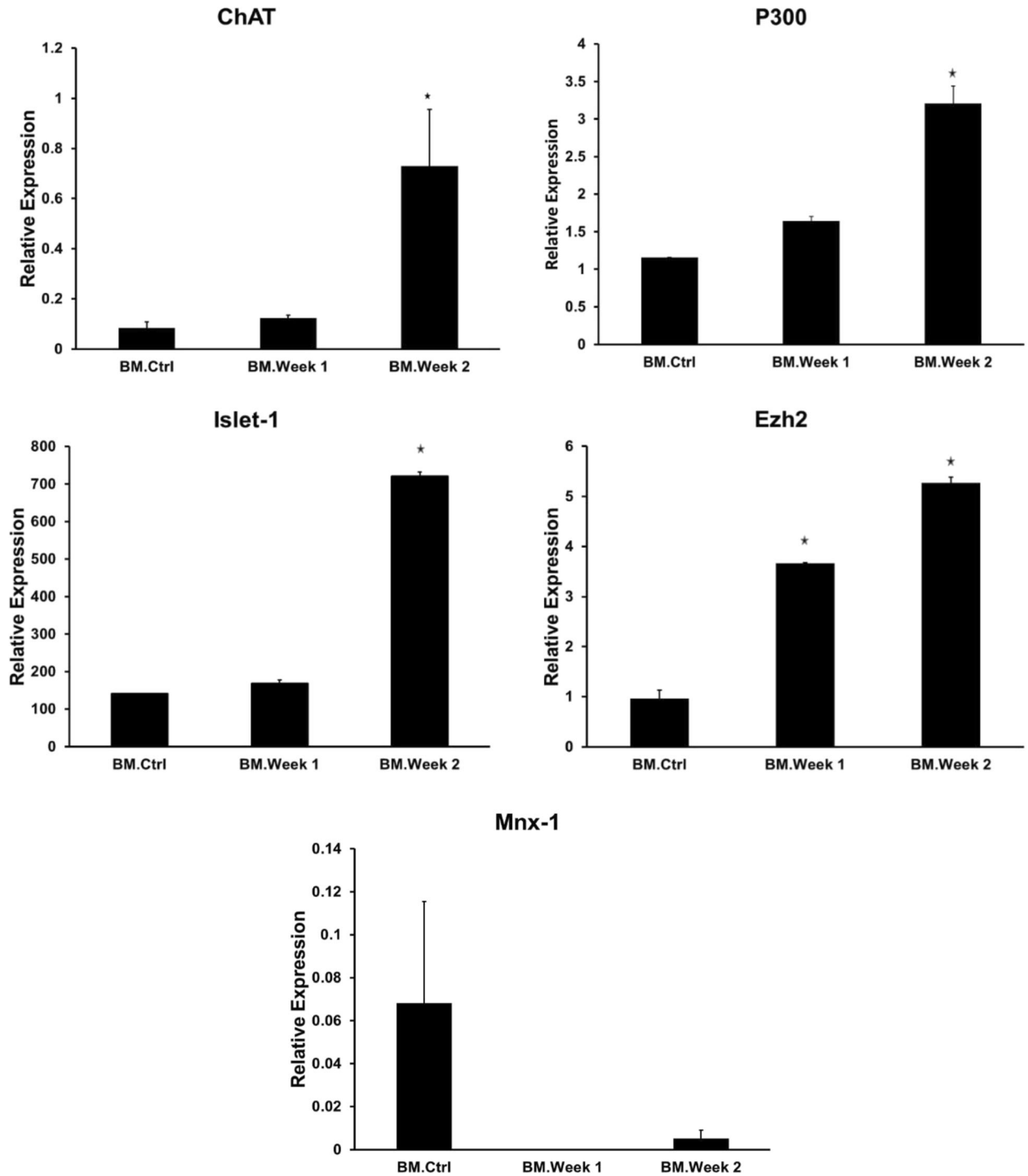

Fig. 3 Quantitative gene expression. The expression of *ISLET-1*, *ChAT*, and *MNX-1* was evaluated in human BM-MSCs at 7 and 14 days after induction in comparison with the control group (non-treated BM-MSCs) (n=3; mean ± SE; $p \leq 0.05$). The expression of motor neuron-related genes (*ISLET-1* and *CHAT*) and histone-modifying enzyme genes (*P300* and *EZH-2*) was significantly upregulated at the second week of induction when RA and Shh were removed. Significant upregulation in the expression of *EZH-2* was also detected at week 1 when the result was compared to the control. No upregulation was detected in the expression of *MNX-1* when the results were compared with the control

neural cell lineages. During stem cell differentiation, some specific genes associated with the development of a particular phenotype are upregulated, and the genes accountable for stemness characteristics are inhibited [8, 22]. For example, in histone-mediated epigenetic regulation of cell differentiation, some modifications alter the access of transcription factors and other modulators to gene promoters [9, 10].

In the process of motor neuron differentiation from mesenchymal stem cells, the relationship between gene expression and epigenetic regulation is poorly understood. So, the aim of this study was to quantitatively investigate the expression of the candid genes associated with motor neuron differentiation along with two potent epigenetic regulatory genes, called *EZH-2* and *P300*, in motor neuron-like cells derived from hBM-MSCs. To induce the differentiation, we used two morphogens called RA and Shh which are responsible for the dorso-ventral specification of the spinal cord and the development of motor neurons, in vivo. To our knowledge, this is one of the first studies to evaluate the expression of *EZH-2* and *P300* during motor neuron differentiation of hBM-MSCs.

According to our results, the treated cells could express ChAT and Islet-1 as motor neuron-specific markers at both mRNA and protein levels at the second week of induction. Data were confirmed by qRT-PCR and immunocytochemistry. It has already been approved that the synergistic administration of RA and Shh upregulates the expression of Islet-1 and ChAT [23]. Islet-1 as a LIM-homeodomain transcription factor is involved in the development of cholinergic amacrine cells and differentiation of motor neurons [24]. We could not detect any upregulation in the expression of *MNX-1* upon differentiation. Suppression of the expression of *MNX-1* in mice can reduce the number of developing motor neurons; however, the target genes downstream of *ISLET-1* are independent of *MNX-1* [25]. We could detect significant upregulation in the expression of *ISLET-1*and *ChAT* at the second week of the induction after removal of RA and Shh. Accordingly, it seems that the morphogens tend to keep the cells in an immature state.

We could also detect the expression of *EZH-2* and *P300* genes for histone-lysine *N*-methyltransferase and histone acetyltransferase enzymes, respectively. EZH-2 is crucial for regulating the transition from proliferation to differentiation as well as suppressing forebrain traits [26]. It also controls the condition of neurogenesis by coordination between neuro-regeneration and differentiation in the cerebral cortex [11, 13]. In this regard, it seems that the expression of *EZH-2* sets on the process of neurogenesis initiation by the transition from proliferation to differentiation and prevents premature differentiation in our experiment. It has been shown that *EZH-2* has a serious time-dependent role during neurogenesis [27]. In our study, the expression of *EZH-2* upregulated in the presence of RA and Shh and continued to be expressed even after the removal of these two morphogens.

Based on our results, the expression of the *P300* gene increased at the second week of differentiation after the removal of RA and Shh. The expression of P300 is required for astrocyte development and axon regeneration [14, 28]. The development of motor neurons in the spinal cord is dependent on CBP [29]. Through synergistic interaction with RA receptor (RAR) and Neurogenin 2, CBP and its paralog, P300, as co-activator are involved in regulating neurogenesis and following stages of neural differentiation in spinal motor neurons [28, 29]. This co-activator participates in maintaining the expression of *ISLET-1* in embryonic spinal MNs. As we could see, the expression of *ISLET-1* was also upregulated at the second week post-induction. According to Toch et al., the mutation in the CBP/P300 compound reduces the number of developing MNs in the embryos. This indicates that P300 acts redundantly in neural progenitors to promote MN specification [19]. Interestingly, other researchers suggest that CBP and P300 also act in post-mitotic MNs to regulate later aspects of development [29]. This phenotype is due to the downregulation of *ISLET-1* in MNs, suggesting that the activity of CBP and P300 in post-mitotic MNs may depend on the downregulation of *ISLET-1* [19].

Conclusion

It can be concluded that neuronal inducing morphogens, including RA and Shh, have regulatory effects on the expression of epigenetic regulatory genes, *EZH-2* and *P300*, during differentiation of mesenchymal stem cells into motor neurons. These regulatory effects eventually lead to the expression of motor neuron-related markers such as *ChAT* and *ISLET-1*, which indicate the completion of the differentiation process.

Abbreviations

hBM-MSCs: Human bone marrow mesenchymal stem cells; MNs: Motor neurons; Shh: Sonic hedgehog; RA: Retinoic acid; EZH-2: Enhancer of zeste homolog 2; ChAT: Choline acetyltransferase; ISLET-1: Insulin gene enhancer binding protein-1; MNX-1: Motor neuron and pancreas homeobox 1; CBP: CREB-binding protein; IBMX: Isobutylmethylxanthine; β-ME: β-Mercaptoethanol; BDNF: Brain-derived neurotrophic factor

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Not applicable

Authors' contributions

DS and PV contributed to the investigation, data curation, and formal analysis. ZB contributed to the methodology. FF contributed to the supervision, conceptualization, and writing—review and editing. AL contributed to the conceptualization and writing—review and editing. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.
The protocol for aspiration of human bone marrow was approved by the Research Ethics Committee of Iran University of Medical Sciences (IR.IUMS.REC 1395.95-03-117-29420).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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