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Inhibition of DNA methylation enhances HLA-G expression in human mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are immunosuppressive multipotent cells under investigation for potential therapeutic applications in regenerative medicine and prevention of graft-versus-host disease. Human leukocyte antigen (HLA)-G contributes to the immunomodulatory properties of MSCs. HLA-G expression in MSCs is very low and diminishes during *in vitro* expansion. Epigenetic regulation activates HLA-G expression in some cancer cell lines but not in MSCs. In the present study, adipose- and bone marrow-derived MSCs were exposed to the DNA demethylating agent 5-aza-2-deoxycytidine (5-aza-dC) and histone deacetylase inhibitor valproic acid (VPA) and HLA-G mRNA levels assessed using semi-quantitative reverse-transcription PCR. Exposure to 5-aza-dC resulted in HLA-G1 and -G3 upregulation in both early and late passage MSCs. VPA treatment did not induce HLA-G expression in both bone marrow and adipose derived MSCs. Our results provide the first evidence that HLA-G3 could be expressed in MSCs and that methylation-mediated repression is partly responsible for the observed low levels of HLA-G expression in MSCs. Our findings provide insight that treatment of MSCs with specific epigenetic regulatory modulators may improve their immunoregulatory capability for therapeutic applications.

1. Introduction

Mesenchymal stem cells (MSCs) are a population of multipotent adult stem cells with a high capacity for proliferation, self renewal, and differentiation into osteogenic, chondrogenic, and adipogenic cells [1,2]. MSCs can be isolated from different sources such as bone marrow [1], adipose tissue [3], and umbilical cord blood [4], and are characterized by their shared expression of CD29, CD73, CD90, and CD105, and absence of hematopoietic markers such as CD14, CD34, and CD45 [1,2].

MSCs exhibit an immunomodulatory potential and have been shown to inhibit T and NK cell proliferation *in vitro* [5,6]. Because of their immunosuppressive, self-renewal, and multi-lineage differentiation properties, MSCs have been used in human clinical trials for treatment of graft-versus-host disease [7], multiple sclerosis [8], and spinal cord injury [9]. The mechanisms underlying MSC's immunosuppressive properties are not yet fully elucidated; however, several mediators such as HLA-G [10], indoleamine 2,3-dioxygenase (IDO) [5] and prostaglandin E2 (PGE_2) [11] have been proposed.

HLA-G is a non-classical human leukocyte antigen (HLA) class I molecule that is widely known for its limited polymorphism and restricted tissue expression, mainly in immune-privileged sites such as the placental trophoblast [12] and cornea [13]. However, HLA-G expression can also be induced in various pathological conditions such as cancer [14] and inflammation [15]. HLA-G was shown to inhibit peripheral blood T cells and natural killer (NK) cell cytolysis, and to suppress $CD4^+$ T cell alloproliferation through interaction with inhibitory receptors expressed on immune effector cells [16,17]. Thus, constitutive expression of HLA-G in placental trophoblasts serves to maintain fetal-maternal immune tolerance during pregnancy [18]. In addition, HLA-G expression has been associated with a reduced incidence of rejection during transplantation [19]. HLA-G transcript is expressed as multiple isoforms that encode membrane-bound (HLA-G1, -G2, -G3, -G4) and soluble proteins (HLA-G5, -G6, -G7) [20]. HLA-G1 isoform contains extracellular (α1, α2, α3), as well as cytoplasmic and transmembrane domains [21]. The remaining membrane-bound isoforms are devoid of one or more of the extracellular domains: α2 for HLA-G2, α2 and α3 for HLA-G3, and α3 for HLA-G4 [21].

Abbreviations: AMSCs, adipose derived MSCs; BMSCs, bone marrow MSCs; 5-aza-dC, 5-aza-2-deoxycytidine; HDAC, histone deacetylase; HLA, human leukocyte antigen; MHC, major histocompatibility complex; MSC, mesenchymal stem cell; VPA, valproic acid.

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DNA methylation and histone modification (acetylation/deacetylation) are the major epigenetic mechanisms known to play a key role in transcriptional regulation of gene expression [22]. Studies demonstrated that DNA demethylating agents like 5-aza-2′-deoxycytidine (5-aza-dC) induced HLA-G expression in some tumor cell lines [23,24] but not in MSCs to date. Since the yields of MSCs isolated from human tissue compartments are very low (0.001–0.01% of recovered nucleated cells) [1,25], MSCs must be expanded *in vitro* for therapeutic application and this results in gradual loss of their immunosuppressive potential due decreased HLA-G expression [26].

Strategies to enhance and maintain MSC immunosuppressive effects involve either stable HLA-G expression [27] or chemical induction of endogenous HLA-G expression. In this study, we investigated the effects of epigenetic modulators on endogenous HLA-G expression in adult adipose- (AMSCs) and bone marrow-derived MSCs (BMSCs) during *in vitro* expansion.

2. Materials and methods

2.1. Isolation and culturing of adipose MSCs (AMSCs)

The study was approved by the Stanford University Institutional Review Board and Ethics Committee and all patients were consented prior to treatment. AMSCs were isolated from adult human lipoaspirate as previous described [28]. Cells were seeded at 1–2 $\times$ 10^4 cells/cm^2 and cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator and media changed every 2–3 d. After reaching 70–80% confluence, cells were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin–EDTA (Invitrogen), then centrifuged and expanded at 1:3 split ratios.

2.2. Culturing bone marrow MSCs (BMSCs)

BMSCs were obtained from Cell Applications, Inc. (San Diego, CA) and cultured in alpha-minimum essential medium (α-MEM) (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. At 70–80% confluence, cells were harvested with 0.25% trypsin–EDTA (Invitrogen) and expanded at 1:3 split ratios.

2.3. Cell lines

JEG-3 (ATCC, Manassas, VA), a choriocarcinoma cell line, was maintained in Eagle's minimum essential medium with Earl's balanced salt solution (ATCC) + 10% FBS at 37 °C in a humidified 5% CO_2 incubator. K562 cells (ATCC) were maintained in Iscove's modified Dulbecco's medium (ATCC) supplemented with 10% FBS. JEG-3 and K562 cells were used as positive and negative controls for HLA-G expression, respectively.

2.4. Immunophenotyping

MSCs were harvested and expression of surface markers was characterized with flow cytometry. Briefly, cells were collected, washed with PBS, and blocked for 30 min with 10% goat serum/3% BSA diluted in PBS. Cells were washed with cold PBS and incubated with 1:100 dilution of mouse anti-human CD105, CD73, CD34, and CD45 (all from BioLegend, San Diego, CA) and CD90 (Becton–Dickinson, San Jose, CA) for 60 min on ice. Isotype-matched control antibodies were used to evaluate non-specific binding. After 3 washes, cells were incubated with allophycocyanin (APC) labeled goat anti-mouse IgG secondary antibody (abcam, Cambridge, MA) for 30 min, followed by 3 washes with PBS. Cells were resuspended in PBS and 10,000 events were acquired with a FACScanto II flow cytometer (BD) and results were analyzed using cyflogic software (CyFlo Ltd., Finland).

2.5. Adipogenic and osteogenic differentiation

Adipogenic and osteogenic differentiation was induced, as described previously [29] with slight modification. Briefly, to induce adipogenic differentiation, MSCs were seeded at 1 $\times$ 10^4 cells/cm^2 in growth medium and allowed to reach 70–80% confluence. Medium was supplemented with 0.5 mM 3-isobutyl-methylxanthine (IBMX) (Sigma), 1 μM dexamethasone (Sigma), 10 μM insulin (Santa Cruz Biotechnology, Santa Cruz, CA), and 100 μM indomethacin (Santa Cruz Biotechnology). Medium was replaced every 3–4 d for 14 d and fat vacuoles stained with Oil Red-O solution. To induce osteogenic differentiation, MSCs were seeded at 2.5 $\times$ 10^3 cells/cm^2 in growth medium and allowed to reach 50% confluence. Growth medium was supplemented with 10 mM β-glycerophosphate, 0.1 μM dexamethasone, and 0.2 mM ascorbic acid-2-phosphate (all from Sigma) and induction medium replaced every 3–4 d. After 21 d, mineral deposition was visualized by staining with Alizarin Red (Sigma).

2.6. Cell treatments

Demethylating treatment was carried out as previously described [24]. MSCs were cultured for 72 h with 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma) at a final concentration of 10 μM or with the solvent DMSO as a control. Histone deacetylase (HDAC) inhibitory treatment was carried out for 72 h with valproic acid (VPA) (Sigma) at final concentration of 10 mM.

2.7. RNA isolation and RT-PCR

After 72 h treatment, cells were trypsinized and total RNA extracted using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Semi-quantitative RT-PCR reactions were performed on a DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, CA) as previously described [30]. Briefly, first

Table 1
Primers used for HLA-G gene expression analysis in MSCs.

Gene	Primer sequence (5′-3′)		Reference ID	Tm (°C)	Amplicon size (bp)
HLA-G1	F	CCAATGTGGCTGAACAAAGG	NM_002127.4	60	426
	R	AGG GAA GAC TGC TTC CAT CTC			
HLA-G3	F	AGCGAGGCCAAGCAGTCTTC	NM_002127.4	60	167
	R	GCGAGATCTTCACATTGCAGCCTGAGA			
HLA-G5	F	ATA CCT GGA GAA CGG GAA GG	NM_002127.4	60	363
	R	AGG CTC CTG CTT TCC CTA AC			
β_2M	F	GCTCGCGCTACTCTCTCTTT	NM_004048.2	60	85
	R	ATTCTCTGCTGGATGACGTG			

strand cDNA was synthesized in 50 µl reactions from 2 µg total RNA using oligo dT primers and TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Reverse transcription (RT) reactions consisted of 24 °C for 10 min, 48 °C for 1 h, and 95 °C for 5 min. RT samples were diluted at 1:4 in DNase, RNase-free water and PCR performed in 20 µl reaction using Taq PCR master mix (Qiagen), 1 µl of diluted template, and HLA-G primers (Table 1). Beta-2-microglobulin (β2 M) was used as an internal control. PCR program consisted of 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were separated using 1.5% agarose gel in the presence of 0.5 µg/ml ethidium bromide and imaged using the FluorChem® HD2 Imaging System (Alpha Innotech Corporation, San Leandro, CA). Band intensity was analyzed using AlphaEase FC software (Alpha Innotech Corporation) and relative HLA-G expression levels were calculated by dividing band intensity values of HLA-G to that of β2M.

2.8. Statistical analysis

All data are expressed as means ± SEM (standard error of the mean) of 3 independent experiments. Statistically significant differences were evaluated with two-tailed student *T*-test with $p < 0.05$ considered significant.

3. Results

3.1. Phenotypic characterization

Flow cytometry analysis of MSCs revealed that both AMSCs and BMSCs were strongly positive for CD105, CD73 and CD90, but negative for hematopoietic markers lineages CD34 and CD45 (Fig. 1A). MSCs treated with adipogenic and osteogenic media differentiated into adipocytes and osteocytes, respectively (Fig. 1B). Hence, both AMSCs and BMSCs fulfilled the 3 proposed criteria to define MSCs [31].

3.2. Inhibition of DNA methylation upregulates MSC HLA-G1 and -G3 transcription

To quantitate variations in the amount of HLA-G transcripts between 5-aza-dC treated and untreated MSCs, RT-PCR products were separated on agarose gel and band intensities (also called integrated density values) were analyzed using AlphaEase FC software as previously described [32]. Relative HLA-G expression

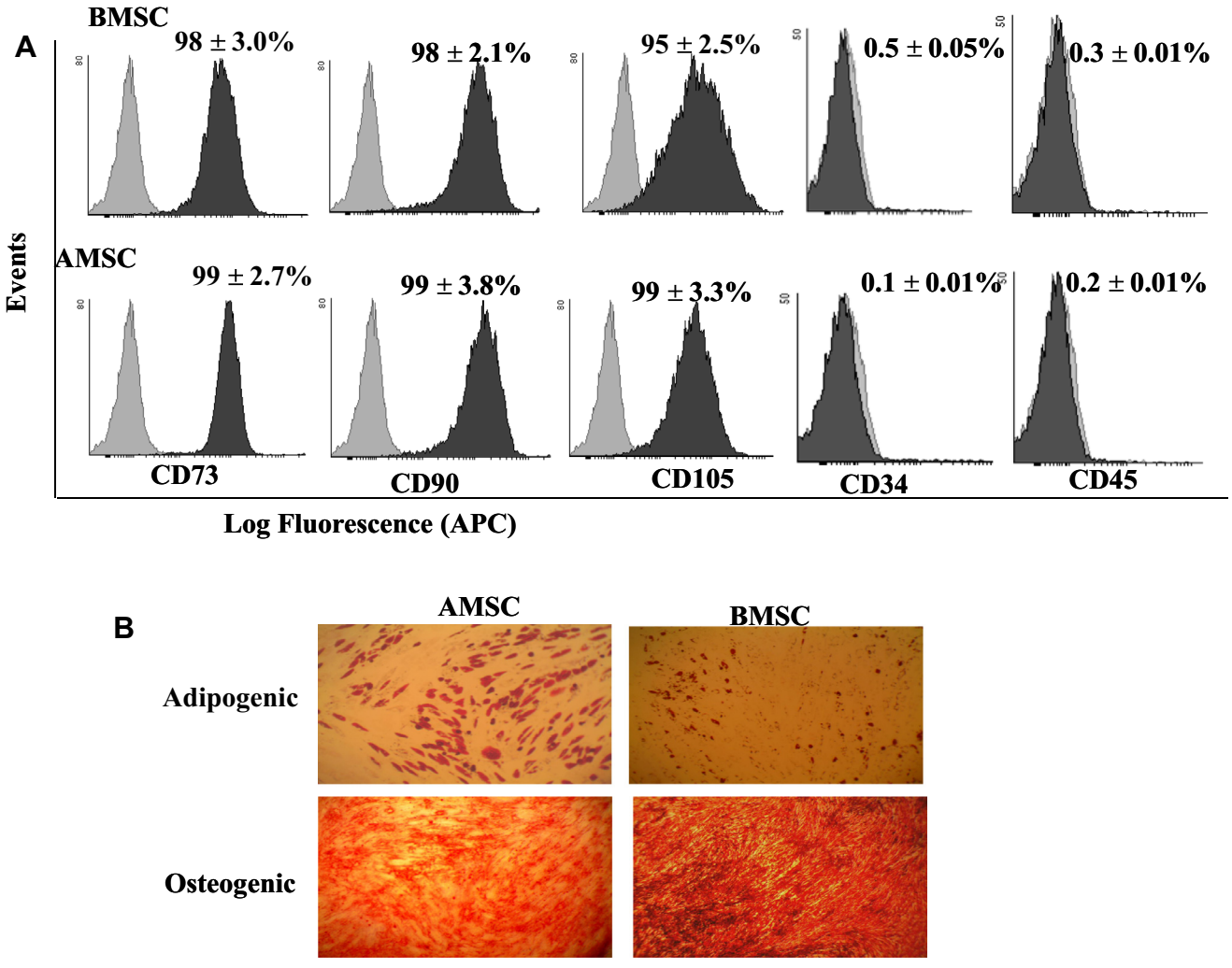

Fig. 1. Characterization of MSC. (A) MSCs were characterized for cell surface marker expression using flow cytometry. Specific markers are presented as dark histograms and isotype-matched control antibodies as gray histograms. Representative histograms from 3 independent runs are shown. (B) MSCs were cultured in adipogenic and osteogenic media and visualized by Oil-Red-O (adipogenic) and alizarin red (osteogenic) staining.

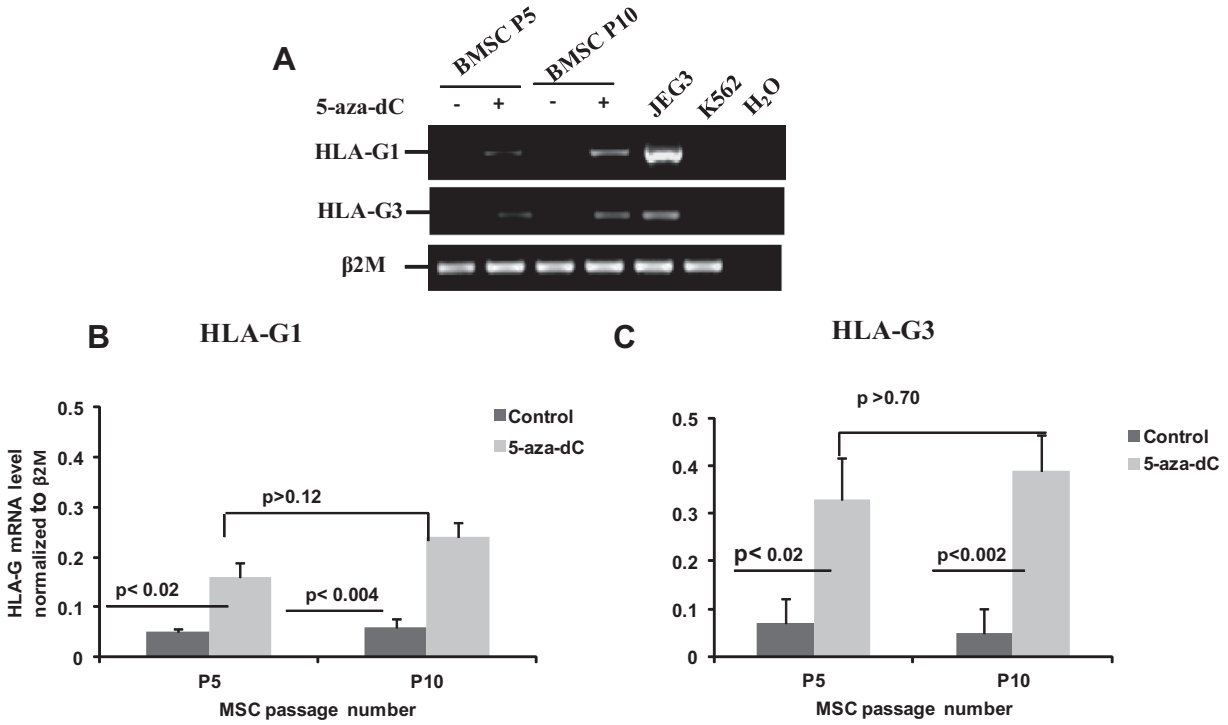

Fig. 2. RT-PCR evaluation of HLA-G expression in BMSCs treated with 5-aza-dC. (A) RT-PCR products were analyzed on 1.5% agarose gel. Representative image from 3 independent runs is shown. Bar graphs showing the relative expression level of HLA-G1 (B) and HLA-G3 (C) transcripts in treated and untreated BMSCs. Relative expression levels were obtained by dividing band intensity values of HLA-G to that of endogenous reference β2M. Means ± SEM from 3 independent runs are illustrated.

levels presented herein are normalized values obtained by dividing band intensity values of HLA-G to that of endogenous reference β2 M. Relative HLA-G1 expression in 5-aza-dC treated P5 (0.16 ± 0.01) and P10 BMSCs (0.24 ± 0.01) was statistically significantly higher than untreated P5 (0.04 ± 0.007, $p < 0.02$) and P10 (0.06 ± 0.01, $p < 0.004$) cells, respectively (Fig. 2A and B). No

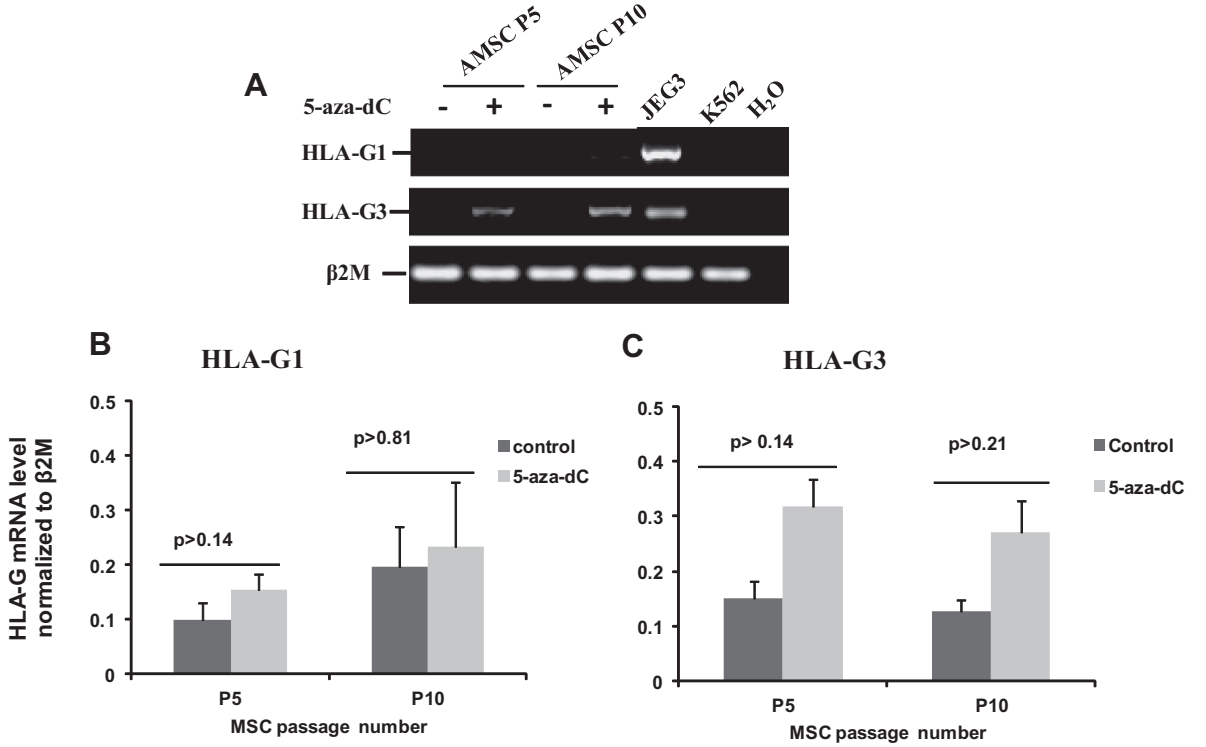

Fig. 3. RT-PCR evaluation of HLA-G expression in AMSCs treated with 5-aza-dC. (A) RT-PCR products were analyzed on 1.5% agarose gel. Representative image from 3 independent runs is shown. Bar graphs showing the relative expression level of HLA-G1 (B) and HLA-G3 (C) transcripts in treated and untreated BMSCs. Relative expression levels were obtained by dividing band intensity values of HLA-G transcript to that of endogenous reference β2M gene. Means ± SEM from 3 independent runs are illustrated.

significant difference ($p > 0.12$) between P5 and P10 was observed (Fig. 2B). Relative HLA-G3 expression in treated P5 (0.33 ± 0.08) and P10 (0.38 ± 0.07) BMSCs was statistically significantly higher than untreated P5 (0.07 ± 0.01, $p < 0.02$) and P10 (0.05 ± 0.01, $p < 0.002$) cells, respectively (Fig. 2A and C). No significant difference ($p > 0.70$) between P5 and P10 was observed (Fig. 2C).

Although the difference was not significant, relative HLA-G1 expression in treated P5 (0.15 ± 0.02, $p > 0.14$) and P10 (0.23 ± 0.19, $p > 0.81$) AMSCs was higher than untreated P5 (0.09 ± 0.03) and P10 (0.19 ± 0.07) AMSCs, respectively (Fig. 3A and B). Relative HLA-G3 expression in treated P5 (0.31 ± 0.05) and P10 (0.27 ± 0.06) AMSCs was ~2-fold higher than untreated P5 (0.15 ± 0.03) and P10 (0.13 ± 0.03) AMSCs, respectively (Fig. 3C). Treatment of MSCs with 5-aza-dC did not induce HLA-G5 expression (Table 2).

3.3. HDAC inhibition does not modify HLA-G expression

MSCs were treated with VPA (a known HDAC inhibitor) [33] and HLA-G1, -G3 and HLA-G5 transcripts were evaluated by semi-quantitative RT-PCR. The relative HLA-G expression levels were obtained by dividing band intensity values of HLA-G to that of endogenous reference β2M gene. Relative HLA-G1 expression in VPA treated P5 (0.08 ± 0.02) and P10 BMSCs (0.05 ± 0.002) was not statistically significantly different from untreated P5 (0.07 ± 0.002, $p > 0.88$) and P10 (0.06 ± 0.002 $p > 0.49$) cells, respectively (Fig. 4A). Relative HLA-G3 expression in treated P5 (0.04 ± 0.01) and P10 (0.01 ± 0.001) BMSCs was not statistically significantly different from untreated P5 (0.03 ± 0.008, $p > 0.62$) and P10 (0.02 ± 0.001, $p > 0.44$) cells, respectively (Fig. 4B). Similarly, there was no upregulation of HLA-G1 (Fig. 4C), HLA-G3 (Fig. 4D), or HLA-G5 (Table 2) mRNA in AMSCs treated with VPA.

Table 2
Effects of 5-Aza-dC and VPA treatment on HLA-G5 expression in MSCs. Cells were treated with 5-aza-dC or VPA for 72 h and HLA-G5 mRNA level was evaluated with RT-PCR. Results are relative expression levels obtained by dividing band intensity values of HLA-G5 to that of endogenous reference β2M gene. Means ± SEM from 3 independent runs are illustrated.

Treatments groups	BMSCs	AMSCs
Untreated control	0.007 ± 0.001	0.006 ± 0.00
5-aza-dC	0.009 ± 0.00	0.009 ± 0.001
VPA	0.006 ± 0.004	0.006 ± 0.00

5-aza-dC: 5-aza-2′-deoxycytidine, VPA: valproic acid.

4. Discussion

MSCs are attractive therapeutic candidates in regenerative medicine because of their immunosuppressive and multi-lineage differentiation properties. The immunosuppressive properties of MSCs are mediated by HLA-G [34] and other soluble factors like IDO [5], PGE_2 [11], and TGF-β1 [35], amongst others. However, the level of HLA-G expression is very low and sometimes undetected in adult MSCs [36,37]. We hypothesized that the low level of HLA-G expression in MSCs could be due to epigenetic control mechanisms that are known to play a central role in transcriptional regulation of gene expression. Indeed, HLA-G activation due to epigenetic changes has been demonstrated in some human cancer cell lines [23] and human embryonic stem cells [38]. Thus we investigated methylation-mediated repression of HLA-G expression in adult MSCs.

Although numerous studies concerning HLA-G expression and its tolerogenic role have been published, the mechanisms

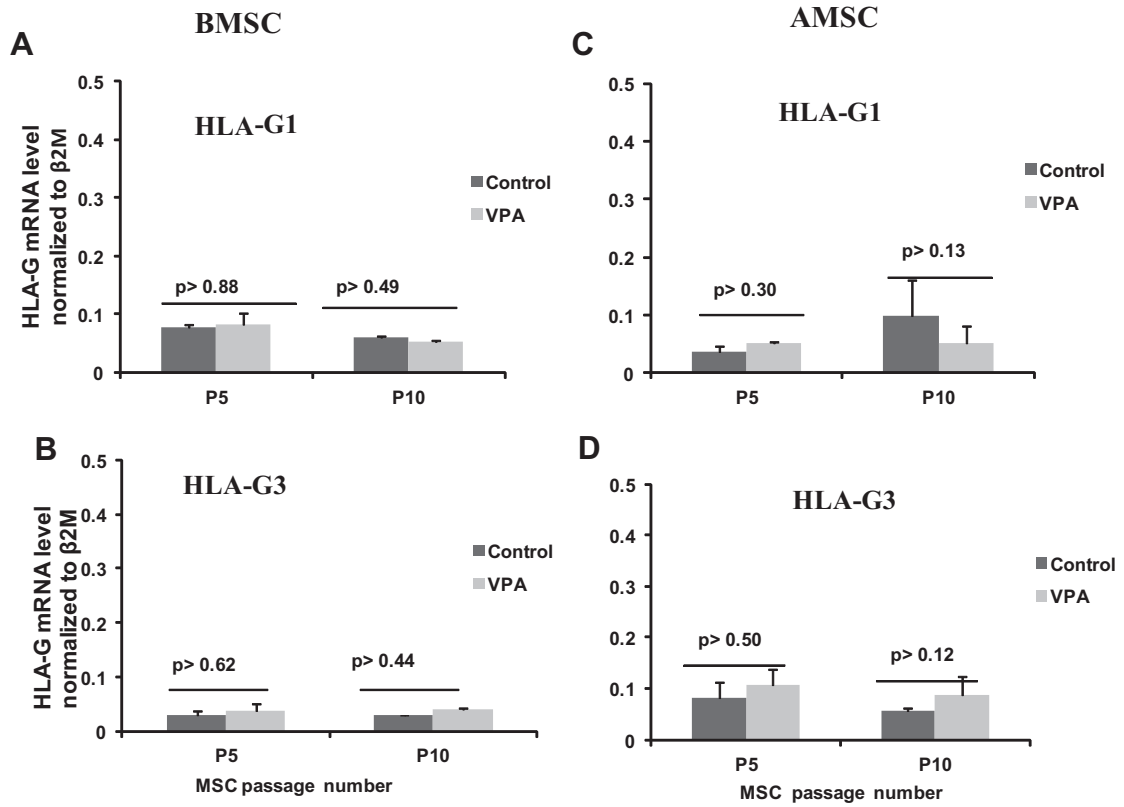

Fig. 4. RT-PCR evaluation of HLA-G expression in MSCs treated with valproic acid (VPA). Bar graphs showing the relative HLA-G transcript expression levels in BMSCs (A: HLA-G1, B: HLA-G3) and AMSCs (C: HLA-G1, D: HLA-G3). Relative expression levels were obtained by dividing band intensity values of HLA-G transcript to that of endogenous reference β2M gene. Means ± SEM from 3 independent runs are illustrated.

controlling the constitutive and inducible expression of HLA-G is not well understood. DNA methylation is one of the major epigenetic mechanisms responsible for gene repression through promoter hypermethylation [39]. DNA methylation status is maintained by DNA methyltransferase that catalyze methylation of cytosine-phosphate-guanine (CpG) islands [39]. Treatment with 5-aza-dC, an inhibitor of DNA methyltransferases [40], has been shown to activate HLA-G in some cancer cell lines [24]. Herein, we showed for the first time that MSC treatment with 5-aza-dC resulted in upregulation of full length HLA-G1 and truncated HLA-G3 isoforms in both AMSCs and BMSCs. Since HLA-G is expressed in several isoforms [20], we utilized HLA-G1, -G3 and -G5 isoform specific primers to detect the membrane bound (HLA-G1 and -G3) and soluble (HLA-G5) isoforms. HLA-G1 and -G3 were detected at baseline and found to be upregulated by 5-aza-dC treatment in both early passage (P5) and late passage (P10) AMSCs and BMSCs. HLA-G1 expression in MSCs has been reported previously [26]; however, our study demonstrated for the first time that HLA-G3 can be activated in MSCs by treatment with DNA demethylating agents. Although previous studies showed MSCs express HLA-G5 [41], we could not detect expression at baseline or after 5-aza-dC treatment suggesting an isoform specific phenomenon. The apparent discrepancies between previous studies and our results could be explained by differences in the experimental procedures. Our results are in agreement with those of Giuliani et al. who also reported the absence of HLA-G5 in adult MSCs [26]. Although, HLA-G1 is a widely known tolerogenic molecule, the role of HLA-G3 has also been demonstrated in some HLA-G3 transfected cell lines [16]. However, the immunosuppressive role of HLA-G3 in MSCs requires further evaluation.

Epigenetic regulation mediated by the opposing activities of HDACs and histone acetyltransferases modulate acetylation level of histones [22]. In general, histone acetylation promotes transcriptional activation while deacetylation silences gene transcription [42]. In contrast to the upregulation observed with demethylation, exposure of MSCs to HDAC inhibitor valproic acid (VPA) [33] did not induce HLA-G expression in MSCs. This suggested that HLA-G silencing is not HDAC-mediated and that epigenetic regulation is specific in MSCs. Indeed, previous studies have shown that exposure of different cell lines with HDAC inhibitors such as sodium butyrate or trichostatin resulted in HLA-G transcript activation in only M8 melanoma cell lines [24]. However, exposure of the same cell types with demethylating agent 5-aza-dC induced HLA-G transcription in most of the cell lines tested [24]. Thus, HDAC-mediated repression of HLA-G is a more restricted regulatory mechanism than methylation process.

Clinical trials involve large doses of MSCs ($1–5 \times 10^6$ cells/kg body weights) [7,8], necessitating *in vitro* expansion to obtain an adequate number of MSCs for clinical application. On the other hand, HLA-G expression dramatically decreases during serial passaging [34], correlating with the observed diminution of immunosuppression in late passage MSCs [26]. Hence, in order to utilize large-scale *in vitro* expanded MSCs as therapeutics, stable expression of HLA-G is required. Sustained expression of HLA-G in MSCs can be achieved by genetic engineering [27]; however, this strategy is hampered by potential health risks. Thus, DNA demethylating agents may be useful alternatives to achieve sustained HLA-G expression. Indeed, HLA-G upregulation was observed in both early (passage 5) and late passage (passage 10) MSCs treated with 5-aza-dC. In addition, previous studies indicated that exposure of MSCs with 5-aza-dC stimulated osteogenic differentiation of MSCs with significant therapeutic implication [43].

In conclusion, our findings suggest that HLA-G transcriptional regulation is under the control of DNA methylation not HDACs in MSCs that treatment with demethylating agents may allow for more stable immunosuppressive properties independent of passage number making them more attractive therapeutic candidates.

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