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Investigation of immunomodulatory properties of Human Wharton's Jelly-derived Mesenchymal Stem Cells after lentiviral transduction

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ABSTRACT

Human Wharton's Jelly-derived Mesenchymal Stem Cells (hWJ-MSCs) are considered as an alternative for bone-marrow-derived MSCs. These cells have immunosuppressive properties. It was unclear whether the WJ-MSCs would sustain their immunomodulatory characteristics after lentiviral transduction or not. In this study, we evaluated immunomodulatory properties of WJ-MSCs after lentiviral transduction. HWJ-MSCs were transduced with lentiviral particles. Expression of transduced and un-transduced hWJ-MSCs surface molecules and secretion of IL-10, HGF, VEGF and TGF-β was analyzed. Cell proliferation and frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T regulatory cells was measured. There was no difference between the surface markers and secretion of IL-10, HGF, VEGF and TGF-β in transduced and un-transduced hWJ-MSCs. Both cells inhibited the proliferation of PHA stimulated PBMCs, and improved the frequency of T regulatory cells. These findings suggest that lentiviral transduction does not alter the immunomodulatory function of hWJ-MSCs. However, lentiviral transduction may have a wide range of applications in gene therapy.

1. Introduction

Due to immunomodulatory properties of mesenchymal stem cells (MSCs) and the ability of these cells to differentiate into different lineages of cells, MSCs have recently emerged as favorable cellular vehicles for gene therapy and clinical applications [1–6]. Although human bone marrow (BM) represents the main source of MSCs, aggressive BM sampling and short lifetime of BM-MSCs limit their application in cell therapy [7–9]. Human Wharton's Jelly-Mesenchymal Stem Cells (hWJ-MSCs) could be an appropriate substitute for BM MSCs, and can be obtained by a less aggressive approach without hurting the mother or her child [10,11]. Animal studies have shown that hWJ-MSCs have no tumorigenicity, and are more immature compared to BM-MSCs [12,13]. The latter property enables them to proliferate faster with greater longevity [14]. Moreover, they can differentiate to a wider range of cells in comparison with BM-MSCs [15,16]. Studies have revealed that the phenotype of hWJ-MSCs does not change up to 50 passages, and they cause no problem with rejection and ethical issues [14,17,18]. On the other hand, the immunomodulatory function of hWJ-MSCs remains intact compared to BM-MSCs, and they do not express MHCII on their surface even in the presence of inflammatory cytokine such as TNF-α and IFN-γ [19,20]. Among different gene delivery methods, lentiviral vectors have significant advantages over the other vector systems. The viruses are able to infect both dividing and non-dividing cells and insert large genetic segments in the host chromatin. In this way, this genetic segment is more likely to be transcribed and sustain stable long-term transgene expression [21–23]. This strategy has been used for gene transfer into hWJ-MSCs; however, it is not clear whether lentiviral transduction can change the immunomodulatory properties of hWJ-MSCs. The remaining intact immunomodulatory properties of hWJ-MSCs after lentiviral transduction is important for avoiding graft rejection problems in experimental research and clinical

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applications. So, in this article, we evaluated the expression of surface molecules of transduced hWJ-MSCs, which are involved in immune rejection and gene expression of immunomodulatory cytokines. We analyzed the ability of transduced hWJ-MSCs to inhibit the PBMCs proliferation stimulated by PHA, and evaluated the frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T regulatory cells in co-culture with transduced and un-transduced WJ-MSCs by multicolor flow cytometry.

2. Materials and methods

2.1. Isolation and culture of hWJ-MSCs

Umbilical cord collection was approved by the Research Ethics Committee of Tarbiat Modares University, Tehran, Iran. A consent was signed by the mother to use the umbilical cord tissues before child delivery. A total of 20 human umbilical cords were collected from normal births, delivered by cesarean section and normal vaginal delivery after full-term pregnancy. The residual blood was fully washed by phosphate-buffered saline (PBS). Umbilical arteries and vein were removed, and the remaining tissue was chopped into 3–5 mm pieces. The pieces were seeded onto the surface of a 10 cm culture dish with Dulbecco's Modified Eagle Medium containing nutrient mixture F-12 (DMEM-F12; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 2.5 μg/ml amphotericin B (Sigma) for 10 days at 37 °C with saturated humidity and 5% (v/v) CO_2 to allow migration of cells from the explants. The media was changed every 3 days. The fragments were removed after 10 days in culture. Adherent cells were expanded upon reaching 80% confluence. Then, the cells were trypsinized with 0.025% trypsin containing 0.02% EDTA (Gibco) and passaged into a new flask for further expansion. The cells in passage #3 were used.

2.2. Lentivirus production, concentration and titration

Lentiviral particles were produced in HEK-293T cells by transient co-transfection involving a three-plasmid expression system as previously described [24]. Plasmid DNA (21 μg of lentiviral construct containing the GFP-carrying shuttle (pWPI, Trono lab, Switzerland), 21 μg of psPAX2 and 10.5 μg pMD2G per 10 cm dish) was transfected into cells using the calcium–phosphate method. Lentiviral particles were collected within 36, 48, 60 and 72 h after transfection from the supernatant of HEK-293-T cells, were centrifuged for 10 min at 3000 rpm to remove cell debris and passed through a 0.45 μm filter. We concentrated the lentivirus by precipitation with 50% PEG-8000 to a final concentration of 5% and to a final concentration of 0.15 M using 5 M NaCl overnight. For titration, HEK 293-T cells were transduced with different concentrations of viruses. Three days later, we evaluated GFP expression in HEK293-T by flow cytometry (Becton Dickinson, San Diego, CA, USA).

2.3. Transduction of hWJ-MSCs

1×10^5 hWJ-MSCs were seeded on 6 well plates in 1 ml DMEM-F12 supplemented with 10% FBS. MOI = 50 of viruses was added to cell cultures in presence of 8 μg/ml Polybrene (Sigma), and was put on rotator at 5 rpm. After 18 h, the medium was changed with fresh DMEM-F12 with 10% FBS. The next day, MOI = 30 of virus was again added to the cells in presence of 4 μg/ml Polybrene and was again put on rotator at 5 rpm. The GFP expression in transduced-hWJ-MSCs was measured 72 h post-transduction by flow cytometry. The GFP-positive cells were released with trypsin–EDTA, washed once with PBS containing 1% FBS, and sorted by DIVA cell sorter (Becton Dickinson, Heidelberg, Germany) at 72 h were subcultured for an additional 17 and 31 days, and then trypsinized and the number of GFP-positive cells analyzed by flow cytometry.

2.4. Flow cytometry

Transduced and un-transduced hWJ-MSCs were trypsinized after the third passage. The cells were washed twice with PBS and stained with the following monoclonal antibodies on ice according to the manufacturer's recommendations: PE-conjugated mouse anti-human CD31, PE-conjugated mouse anti-human CD44, PE-conjugated mouse anti-human CD34, PE-conjugated mouse anti-human CD73, PE-conjugated mouse anti-human CD45, PE-conjugated mouse anti-human HLA-DR, PE-conjugated mouse anti-human CD117, PE-conjugated mouse anti-human CD90, PE-conjugated mouse anti-human CD105, PE-conjugated mouse anti-human HLA-I, PE-conjugated mouse anti-human CD80, PE-conjugated mouse anti-human CD86 and PE-conjugated mouse anti-human CD40. PE-conjugated mouse IgG1 was used as isotype controls (all the antibodies were purchased from eBioscience). The cells were analyzed using flow cytometry (Becton Dickinson, San Diego, CA, USA). At least 20,000 events were recorded for each sample, and the data were analyzed using FlowJo™ software.

2.5. Ex vivo differentiation of Lentivirus transduced and un-transduced hWJ-MSCs

For osteogenic differentiation, hWJ-MSCs were seeded at 20,000 cells/ml in a 4-well plate and incubated in DMEM with 10 nM dexamethasone (Sigma), 50 μg/mL ascorbic acid 2-phosphate (Sigma) and 10 mM β-glycerol phosphate (Sigma) for 21 days. The medium was changed twice a week. After this period, the cells were fixed with 10% formalin for 10 min and stained with alizarin red (Sigma) for 2 min at room temperature.

For adipogenesis, the cells (passage 3) were incubated with DMEM supplemented with 50 μg/mL indomethacin (Sigma) and 100 nM dexamethasone (Sigma) for 3 weeks. After 21 days, the cells were stained with 0.5% Oil Red O (Sigma) in methanol for 2 min at room temperature.

2.6. Population doubling time (PDT)

For this purpose, passaged-3 of transduced and un-transduced cells were plated at 10^4 cells per well in six-well tissue culture plates in DMEM-F12 supplemented with 10% FBS. The cells were collected and counted on days 2, 4, 6, 8 and 10 as described previously [25]. The population doubling time between each passage was calculated according to the equation PDT = culture time (CT)/population doubling number (PDN). To determine PDN, the formulae PDN = $\log N/N0 \times 3.31$ was used. In this equation N stands for the cell number at culture end and $N0$ the number of the cells at culture initiation.

2.7. Secretion of immunomodulation factors

The concentration of IL-10, HGF, VEGF and TGF-β in the cell supernatant was measured by ELISA according to the manufacturer's instructions. ELISA kits were obtained from eBioscience. For this purpose, first we washed the cells with PBS and then fresh DMEM-F12 supplemented with 10% FBS was added. After 3 days, we collected the medium and used it for ELISA test.

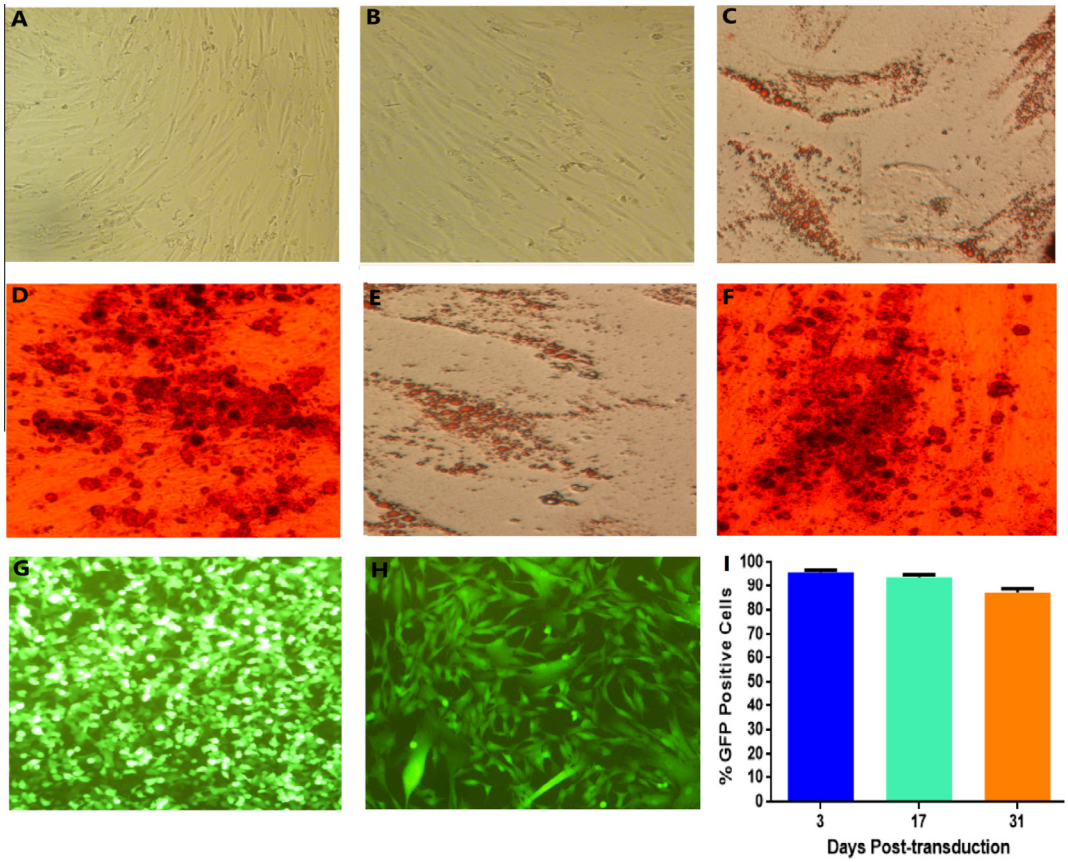

Fig. 1. Isolation, characterization and lentiviral transduction of hWJ-MSCs. (A) Wharton's Jelly-derived Mesenchymal Stem Cells after isolation-3rd passage. (B) Transduced Wharton's Jelly-derived Mesenchymal Stem Cells, (C) Oil Red O staining of un-transduced WJ-MSCs, intracellular lipid accumulation was stained bright red in adipocytes at day 21. (D) Alizarin Red S staining of un-transduced WJ-MSCs, calcium deposition was stained bright orange-red in osteocytes at day 21. (E) Oil Red O staining of transduced WJ-MSCs, intracellular lipid accumulation was stained bright red in adipocytes at day 21. (F) Alizarin Red S staining of transduced WJ-MSCs, calcium deposition was stained bright orange-red in osteocytes at day 21. (G) GFP expressions in HEK293T using fluorescence microscope. (H) GFP expressions in hWJ-MSCs using fluorescence microscope. hWJ-MSCs transduced by GFP-harboring lentiviral particles. (I) Post-transduction time course of GFP expression level in WJ-MSCs (d 3, 96%; d 17, 93%; d 31, 86%) ($p < 0.100$). Bars and whiskers represent the mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8. HWJ-MSCs and PBMCs co-culture experiments

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque density gradient centrifugation (density, 1.077 ± 0.002) (Sigma) from the venous blood of healthy volunteer donors.

The effect of hWJ-MSCs on PHA-stimulated PBMCs was assessed after dye-loading of PBMCs with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) as described previously [20]. Briefly, PBMCs were incubated with 5 mM CFSE for 5 min and were washed three times with PBS. 1×10^5 CFSE-labeled PBMCs were co-cultured in 5:1 ratio of transduced and un-transduced hWJ-MSCs in 200 μl RPMI-1640 medium with 10%FBS on 96 well plates. Mitogen stimulations were achieved with 10 μg/ml PHA (Invitrogen), and proliferation was measured on day 4. The CFSE-labeled PBMCs were divided into three experimental groups: PBMCs alone (no PHA stimulation), PBMCs stimulated with PHA (10 μg/ml) and PBMCs stimulated with PHA co-cultured hWJ-MSCs. After 4 days, proliferation was analyzed by flow cytometry and the flow cytometry data were analyzed using FlowJo™ software. To compare the effects of experimental variables, the proliferation index (PI) was used, a statistic generated by FlowJo, which correlated with the number of cell divisions the PBMCs had undergone following CFSE loading.

For induction of regulatory T cells, 1×10^6 PBMCs were co-cultured in 12 well plates with transduced and un-transduced hWJ-MSCs in 1:5 ratio or without WJ-MSCs in the medium for 4 days. Frequency of regulatory T cells was analyzed by Human Regulatory T Cell Staining Kit #2 (eBioscience) and human PE-Cy7-conjugated CD127 (eBioscience) according to the manufacturer's protocol by flow cytometry, and the flow cytometry data were analyzed using FlowJo™ software.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism (version: 5.04). One-way ANOVA and *t*-tests were used to evaluate differences between groups, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Lentiviral transduction of hWJ-MSCs

As shown in Fig. 1G, the GFP-harboring lentiviral particles were produced in HEK-293T cells. After concentration and titration, we transduced hWJ-MSCs at a MOI = 50. GFP positive transduced hWJ-MSCs were measured 72 h post-transduction by flow cytometry, and the transduction rate was up to 87% positive (GFP positive cells) (Fig. 1H). To assess the stability of transduction of the transduced GFP construct, the time course and level of expression were

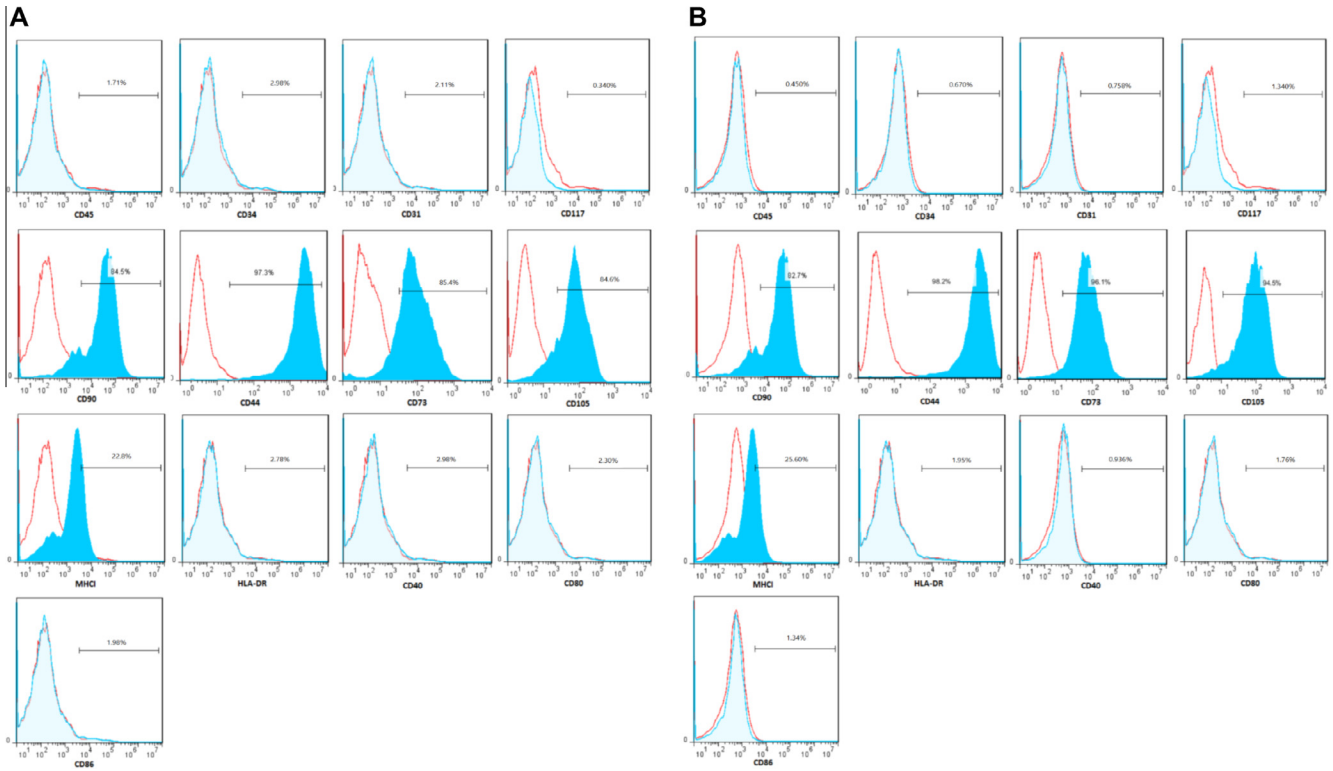

Fig. 2. Flow cytometry analysis of surface markers of un-transduced and transduced WJ-MSC. (A) Flow cytometry analysis of surface markers showing transduced WJ-MSC with lentivirus expressing CD90, CD44, CD73, CD105 and HLA-I but expressing CD31, CD34, CD45, CD117, CD80, CD86, CD40 and HLA-DR in a down regulated manner. (B) Flow cytometry analysis of surface markers showed that un-transduced WJ-MSC express CD90, CD 44, CD73, CD105 and HLA-I but express CD31, CD34, CD45, CD117, CD80, CD86, CD40 and HLA-DR in down regulated manner. Isotype control was used to obtain the auto fluorescence levels during flow cytometry (red curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Average percentage of CD markers expression in un-transduced and transduced WJ-MSCs as measured by FACS analysis. Values represent mean ± standard deviation.

CD markers	WJ-MSCs (%)		*P* value
	Untransduced	Transduced	
CD31	1.20 ± 0.123	1.88 ± 0.789	0.134
CD34	0.95 ± 0.204	2.67 ± 0.743	0.182
CD45	1.45 ± 0.376	1.62 ± 0.231	0.163
CD117	1.71 ± 0.412	1.05 ± 0.332	0.534
CD90	82.51 ± 1.489	83.42 ± 1.094	0.490
CD73	94.22 ± 1.820	83.22 ± 1.302	0.124
CD105	96.30 ± 1.020	84.63 ± 1.023	0.179
HLA-I	21.22 ± 2.894	20.32 ± 2.320	0.970
HLA-DR	1.20 ± 0.742	1.78 ± 0.270	0.100
CD80	0.82 ± 1.527	2.02 ± 1.123	0.189
CD86	0.89 ± 1.341	1.89 ± 1.341	0.100
CD40	0.93 ± 1.801	2.76 ± 1.908	0.165
CD44	97.1 ± 0.763	96.12 ± 0.751	0.300

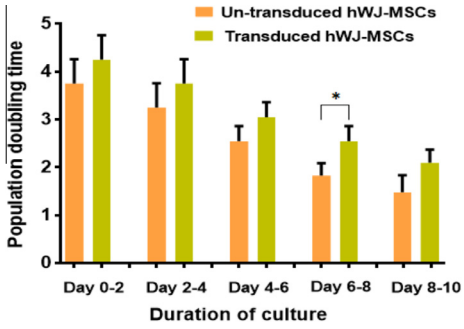

Fig. 3. The doubling times of un-transduced and transduced WJ-MSC. There was no difference in PDT at day 2 ($p < 0.200$), day 4 ($p < 0.200$), day 6 ($p < 0.142$) and day 10 ($p < 0.142$) but the PDT for transduced hWJ-MSCs was longer than that for un-transduced hWJ-MSCs at day 8 ($p < 0.028$). Bars and whiskers represent the mean ± SD.

determined. The GFP-positive cells FACS sorted at 72 h were sub-cultured for an additional 17 and 31 days, and then trypsinized and the number of GFP-positive cells analyzed by flow cytometry. As shown in Fig. 1I, the number of positive GFP-expressing cells was 96% by day 3, 93% by day 17 and 86% by day 31. After 31 days we did not see a significant change in the percentage of GFP-positive cells ($p < 0.100$).

3.2. Characterization of hWJ-MSCs

Ten days after initial plating and removal of tissue fragments from culture dish, fibroblast-like, spindle and star shaped cells became visible in the dish. After 20 days, hWJ-MSCs began to form colonies and became confluent. The hWJ-MSCs were passaged two times per week in 1:3 ratio, and no significant changes were observed in proliferation and morphology. After transduction, they became larger than un-transduced hWJ-MSCs in size and their expansion was slower than un-transduced cells. However, we did not observe any obvious changes in morphology of transduced cells, which were fibroblast-like, spindle and star-shaped cells (Fig. 1A and B). Both transduced and un-transduced cells were positive for CD73, CD105, CD90 and HLA-I, but were negative for CD34, CD31, CD45, CD117 and HLA-DR, indicating that the isolated cells were mesenchymal stem cells and were not derived from endothelial and hematopoietic cells. Moreover, these results showed that lentiviral transduction did not alter the nature of hWJ-MSCs, and they were mesenchymal stem cells even after lentiviral transduction.

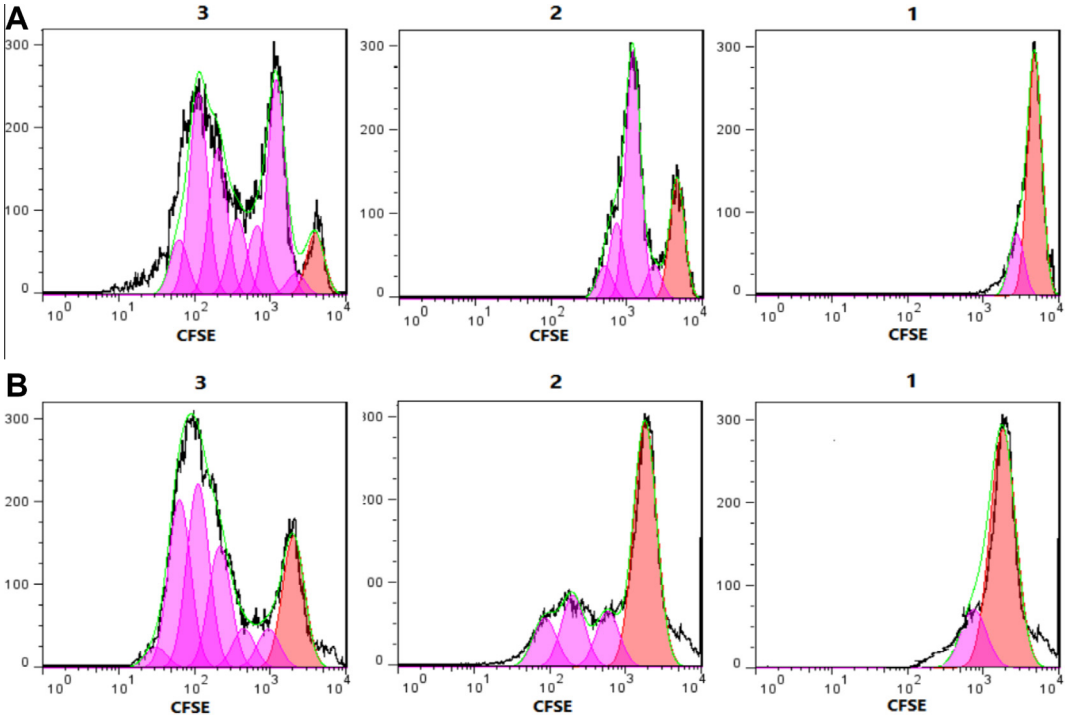

Fig. 4. Immune suppression by co-culture of PBMCs with hWJ-MSCs assessed by the CFSE method. PBMCs proliferation was analyzed by flow cytometry after 4 days. The proliferation index (PI; a statistic generated by FlowJo™ software) correlated with the number of cell divisions the PBMCs had undergone. (A) (i) PBMCs alone (no PHA stimulation) (PI, 1.68). (ii) PBMCs stimulated with PHA co-cultured with un-transduced hWJ-MSCs (PI, 2.21). (iii) PBMCs stimulated with PHA (10 μg/ml) (PI, 3.85) ($p < 0.001$). (B) (i) PBMCs alone (no PHA stimulation) (PI, 1.75). (ii) PBMCs stimulated with PHA co-cultured with transduced hWJ-MSCs (PI, 2.32). (iii) PBMCs stimulated with PHA (10 μg/ml) (PI, 3.78) ($p < 0.001$).

HLA-DR, CD80, CD86 and CD40 play a central role in the onset and maintenance of immune responses as well as in graft rejection reaction problems. We measured these surface molecules on transduced and un-transduced cells by flow cytometry (Fig. 2A and B).

Results showed that none of these surface molecules were expressed on transduced and un-transduced cells (Table 1), and the lentivirus did not switch on the expression of these markers. This is so important for lack of rejection reaction problems after transplantation.

3.3. Osteogenic and adipogenic differentiation

To prove that lentivirus has not altered the mesodermal property of hWJ-MSC, we cultured transduced and un-transduced cells under adipogenic and osteogenic differentiation process for 21 days. After 21 days, numerous lipid vacuoles were visualized in both the mentioned cells by Oil Red O. Moreover, Alizarin red staining of the cells indicated massive calcium depositions. These observations respectively showed adipogenic and osteogenic differentiation of both transduced and un-transduced hWJ-MSC. So, we found that lentivirus did not change the mesodermal property of cells, and these mesenchymal stem cells could differentiate into different lineage of cells (Fig. 1C–F).

3.4. Population doubling time (PDT)

The PDTs at days 2, 4, 6, 8 and 10 were 3.75 ± 0.506, 3.25 ± 0.506, 2.55 ± 0.310, 1.82 ± 0.262 and 1.47 ± 0.359 days for un-transduced hWJ-MSCs, and 4.25 ± 0.506, 3.74 ± 0.506, 3.5 ± 0.310, 2.55 ± 0.310 and 2.05 ± 0.173 days for transduced hWJ-MSCs, respectively (Fig. 3). Although there was no difference in PDT at day 2 ($p < 0.200$), day 4 ($p < 0.200$), day 6 ($p < 0.142$) and day 10 ($p < 0.142$) between un-transduced and transduced hWJ-MSCs. The PDT for transduced hWJ-MSCs was longer than that for un-transduced hWJ-MSCs at day 8 ($p < 0.028$).

3.5. Immunomodulatory effect of transduced and un-transduced hWJ-MSCs

Human WJ-MSCs can decrease the proliferation of PHA stimulated PBMCs at different ratios. We co-cultured PHA stimulated CFSE-labeled PBMCs and transduced and un-transduced hWJ-MSCs at 1:3 ratio to assess the effect of gene transduction via lentiviruses on immunomodulatory function of hWJ-MSCs. After 4 days culture of PHA stimulated PBMCs and transduced and un-transduced cells, the proliferation rate was analyzed by flow cytometry. Both transduced and un-transduced cells could significantly inhibit PBMCs proliferation. The co-culture of PHA stimulated PBMCs with un-transduced hWJ-MSCs produced a proliferation index of 2.21 compared to PBMCs stimulated with PHA, which yielded a proliferation index of 3.85 cells ($p < 0.001$). The co-culture of PHA stimulated PBMCs with transduced hWJ-MSCs yielded a proliferation index of 2.32 compared to PBMCs stimulated with PHA, which yielded a proliferation index of 3.78 ($p < 0.001$). Thus, we showed that lentivirus did not alter the immunomodulatory function of hWJ-MSCs, and the cells kept their nature after lentivirus transduction (Fig. 4A and B).

WJ-MSCs can improve the frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T regulatory (Treg) cells. To investigate whether transduced and un-transduced WJ-MSCs exerted immunomodulation on Treg cells, we co-cultured PBMCs with transduced and un-transduced cells and measured the frequency of T-reg cells by multicolor flow cytometry. Flow cytometry data revealed that the frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T regulatory cells was significantly increased in the presence of transduced (11%) and un-transduced cells (11.5%) in vitro for 4 days compared to those in the absence of WJ-MSCs in the medium (4.20%) ($p < 0.001$) (Fig. 5A–C).

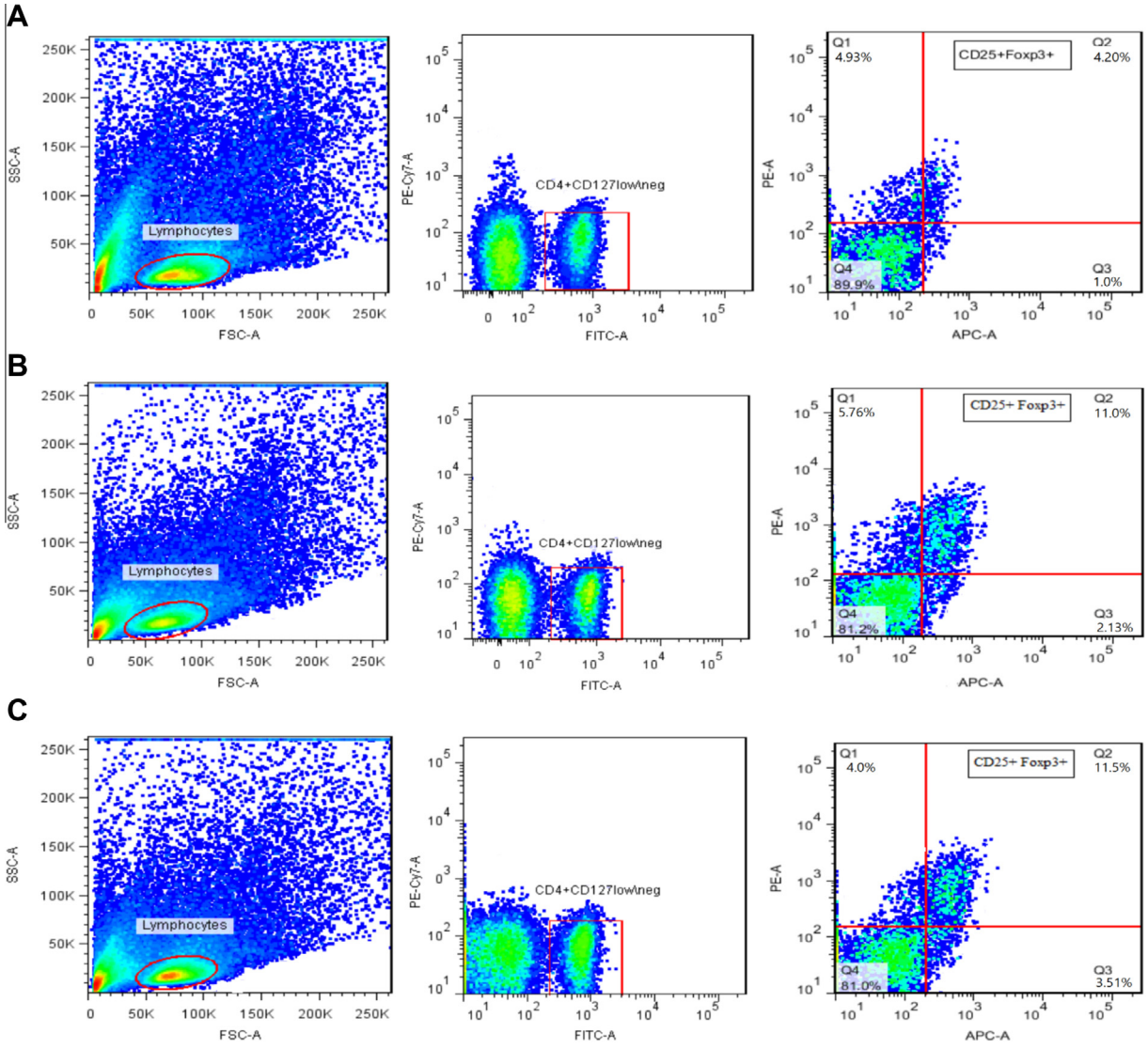

Fig. 5. WJ-MSCs improved the frequency of $CD4^{+}CD25^{+}$ $CD127^{low/neg}$ $Foxp3^{+}$ T regulatory cells in PBMCs. (A) Dot plot results of FITC-$CD4^{+}$ PE-$CD25^{+}$ PE-Cy7-$CD127^{low/neg}$ APC-$Foxp3^{+}$ Treg cells in lymphocytes population in the absence and (B) presence of transduced ($p < 0.001$) and (C) un-transduced ($p < 0.001$) WJ-MSCs by flow cytometry.

3.6. Increased immune suppression factors in hWJ-MSCs

IL-10, HGF, VEGF and TGF-β are immune suppression factors released from hWJ-MSCs to regulate immune responses. These factors are very important to avoid graft rejection problems. We analyzed the expression of these factors by ELISA in transduced and un-transduced hWJ-MSCs (Fig. 6). Both transduced and un-transduced hWJ-MSCs could secrete the same level of IL-10, HGF, VEGF and TGF-β, and insignificantly difference was observed in expression of these factors between these two cell groups.

4. Discussion

Transplantation of MSC represents a hopeful therapy for several degenerative disorders. MSCs are multipotent stem cells, which are capable of self-renewal and multiple differentiation. Since MSCs can enter the injury area and differentiate into different cell types, the use of these cells is an attractive approach for the treatment of several diseases [26–29]. MSCs have been isolated from various tissues, and the main source of them is bone marrow; however, some problems such as isolation, collection and ethical issues limit the application of BM MSCs. These cells are also present in Wharton's Jelly (WJ) of umbilical cord [9,10]. MSCs in Wharton's Jelly from umbilical cord proliferate faster than BM-MSCs. They are more primitive and have extensive multipotency compared with adult MSCs. The isolation method is safe, painless, and WJ is considered as an alternative source for hBM-MSCs [14–16]. These cells have immunosuppressive properties and inhibit all types of immune responses [19,20]. Recently, MSCs have been considered as a promising candidate for cell-based gene delivery because they can be efficiently transduced with target genes [30].

Lentiviruses form a subclass of retroviruses that can infect both dividing and non-dividing cells. They have recently been modified as gene delivery vehicles due to their capacity to integrate different genes into the genome of even non-dividing cells. The site of integration is random and unpredictable, which can make a problem in the integrity of cellular genes. However, several studies indicate that lentivirus vectors have a lower affinity than other retroviral vectors to integrate in places that potentially cause cancer. In addition, lentiviral vectors used in clinical trials for delivering genes did not cause an increase in mutagenic or oncologic events

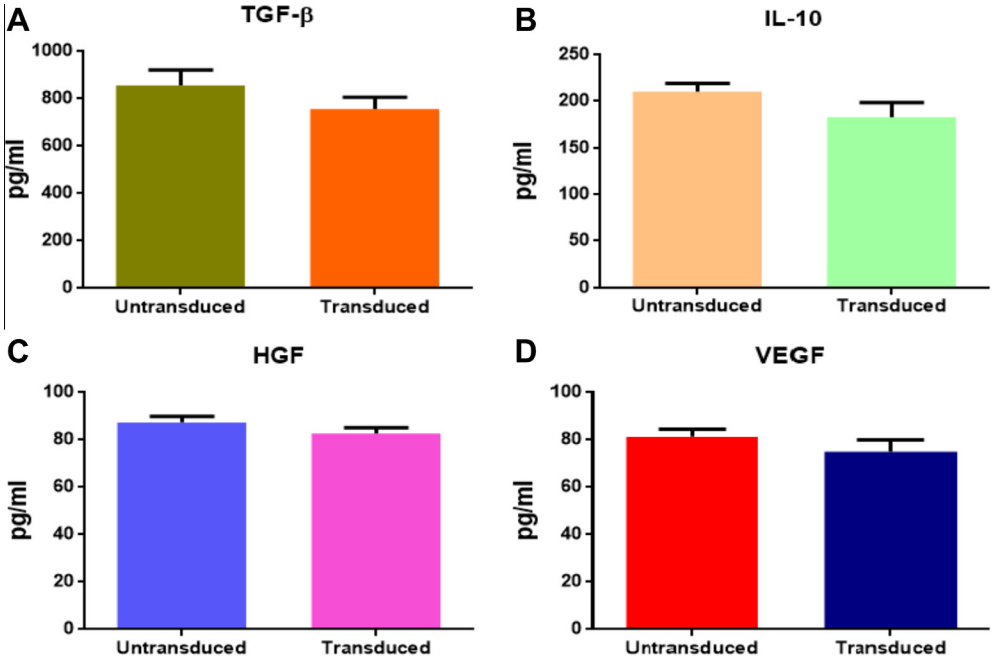

Fig. 6. Immune suppression factors in hWJ-MSCs. (A) TGF-β production in transduced and un-transduced hWJ-MSCs ($p < 0.400$). (B) IL-10 production in transduced and un-transduced hWJ-MSCs ($p < 0.879$). (C) HGF production in transduced and un-transduced hWJ-MSCs ($p < 0.687$). (D) VEGF production in transduced and un-transduced hWJ-MSCs ($p < 0.756$). Bars and whiskers represent the mean ± SD.

[21–23,31–33]. Recently, lentiviruses have been considered as good gene delivery vehicles into MSCs. It is so important for lentiviruses not to alter the nature and immunomodulation property of the cells when we use lentiviruses for delivering the genes into MSCs and want to transplant these cells in clinical trials. Qian et al. showed that the lentiviral transduced WJ-MSCs were capable of differentiating into adipogenic and osteogenic lineages, expressed stem cell markers such as Oct-4, Nanog, BMI-1 as well as nucleostemin and did not alter the nature of WJ-MSCs [30]. Fei-Ling YAP et al. showed that the transfected human BM MSCs retained their surface immunophenotypes and differentiation potential into adipocytes and osteocytes [34]. In this study, we investigated the immunomodulatory property of human WJ-MSCs upon lentiviral transduction. We successfully transduced WJ-MSCs with GFP-expressing lentiviral particles, and there was a positive transduction rate up to 87% (GFP positive cells). We also examined the stability of the transduction by monitoring expression levels of GFP over time. After initial sorting of the population for GFP positive cells, cells were subcultured on day 17 and 31. We did not see a significant change in the percentage of GFP-positive cells over this time and this result indicated that our transduction was stable. We did not see any sensible changes in morphology and growth pattern. However, transduced hWJ-MSCs became larger than un-transduced hWJ-MSCs in size, and their expansion was slower than un-transduced cells. Nevertheless this result, there was no difference in the doubling times of un-transduced and transduced WJ-MSC at day 2, 4, 6 and day 10 but the PDT for transduced hWJ-MSCs was longer than that for un-transduced hWJ-MSCs at day 8. The transduced cells could successfully differentiate into adipogenic and osteogenic lineages, and the lentivirus did not alter the mesodermal property of hWJ-MSCs. To reveal the effect of lentivirus on WJ-MSCs surface markers, we analyzed the expression of CD31, CD43, CD45, CD44, CD90, CD117, CD73 and CD105 by flow cytometry. There was no difference in the expression of these markers between transduced and un-transduced WJ-MSCs, and both cells were positive for CD73, CD105 and CD90 but negative for CD34, CD31, CD45 and CD117. The lentivirus did not affect the surface marker profile used to identify MSCs, and did not change the nature of WJ-MSCs. As already mentioned, it is important for the lentivirus not to alter the immunoregulation function of these cells. HLA-I and HLA-DR are major histocompatibility complex (MHC) molecules, and play a major role in graft rejection problems. CD40, CD80, and CD86 are co-stimulatory antigens implicated in the activation of both T and B cell responses, and have a central role in graft rejection reaction [19,20]. Studies indicated that HLA-I, HLA-DR, CD80, CD86 and CD40 were not changed on WJ-MSCs surface even in culture with inflammatory cytokines such as TNF-α and IFN-γ. In our study, we analyzed HLA-I, HLA-DR, CD80, CD86 and CD40 by flow cytometry. Transduced and un-transduced WJ-MSCs were positive for HLA-I but negative for HLA-DR, CD80, CD86 and CD40. To the best of our knowledge, this is the first study showing that lentivirus transduction does not alter the expression of HLA and co-stimulatory molecules on WJ-MSCs. IL-10, HGF, VEGF and TGF-β are immunomodulation factors that inhibit many types of immune responses, and play a pivotal role in the immunosuppressive capability of MSCs. MSCs secrete these factors to modulate the immune responses [19,20,35,36]. Transduced and un-transduced WJ-MSCs both expressed these factors, and there was no difference in IL-10, HGF, VEGF and TGF-β expression between them.

We showed that gene transfer by lentivirus did not affect the expression of immune suppression factors in WJ-MSCs. WJ-MSCs can inhibit the proliferation of stimulated PBMCs [19,37,38]. In addition, we examined the effect of transduced and un-transduced WJ-MSCs on proliferation of PHA stimulated PBMCs. Both cells could inhibit PHA stimulated PBMC proliferation. T regulatory cells are a component of the immune system that suppress immune responses of other cells. This is an important "self-check" built into the immune system to prevent excessive reactions, maintain tolerance to self-antigens, and abrogate autoimmune disease [39]. Recently, accumulating evidences have suggested that MSCs from human umbilical cords also display immunomodulatory function by suppressing the proliferation of activated T cells in vitro via cell contact and/or soluble factors, or via converting effector T cells into

Treg cells [40,41]. To investigate whether transduced WJ-MSCs exerted immunomodulation on Treg cells, we measured the frequency of Treg cells in co-culture with either transduced or untransduced WJ-MSCs by multicolor flow cytometry. We observed that transduced WJ-MSCs could significantly increase the frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T regulatory cells in PBMCs.

Taken together, we have shown that lentivirus did not alter the immunomodulatory function of WJ-MSCs, and the use of lentiviruses is still an effective method for transduction of MSCs and even for clinical application.

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