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In Vitro Generation of IL-35-expressing Human Wharton's Jelly-derived Mesenchymal Stem Cells Using Lentiviral Vector

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ABSTRACT

Human Wharton's Jelly-derived Mesenchymal Stem Cells (hWJ-MSCs) are easily available cells without transplant rejection problems or ethical concerns compared to bone-marrow-derived MSCs for prospective clinical applications. These cells display immunosuppressive properties and may be able to play an important role in autoimmune disorders. Regulatory T-cells (Treg) are important to prevent autoimmune disease development. Interleukin 35 (IL-35) induces the proliferation of Treg cell populations and reduces the activity of T helper 17 (Th17) and T helper 1 (Th1) cells, which play a central role in initiation of inflammation and autoimmune disease.

Recent studies identified IL-35 as a new inhibitory cytokine required for the suppressive function of Treg cells. We created IL-35-producing hWJ-MSCs as a good vehicle for reduction of inflammation and autoimmune diseases. We isolated hWJ-MSCs based on explant culture. HWJ-MSCs were transduced at MOI=50 (Multiplicity of Infection) with lentiviral particles harboring murine Interleukin 35 (mIL-35). Expression of IL-35 in hWJ-MSCs was quantified by an IL-35 ELISA kit.

IL-35 bioactivity was analyzed by inhibiting the proliferation of mouse splenocytes using CFSE cell proliferation kit. Frequency of $CD4^+CD25^+CD127^{low/neg}$ $Foxp3^+$ Treg cells was measured by flow cytometry. There was an up to 85% GFP positive transduction rate, and the cells successfully released a high level of mIL-35 protein (750 ng/ml). IL-35 managed to inhibit CD4+ T cell proliferation with PHA, and improved the frequency of Treg cells.

Our data suggest that transduced hWJ-MSCs overexpressing IL-35 may provide a useful approach for basic research on gene therapy for autoimmune disorders.

Keywords: IL-35; Lentivirus; Mesenchymal Stem Cells; Vector; Wharton's Jelly-Derived

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INTRODUCTION

Mesenchymal stem cells (MSCs) can differentiate into cells of different lineages. Moreover, due to potent immune regulatory function, MSCs have recently emerged as promising cellular vehicles for potential clinical applications.[1-4] Human bone marrow (BM) has been the main source of MSCs so far. A number of obstacles, including the invasive sampling procedure and reduced life span of human BM-MSCs concomitant with aging have limited the use of BM-MSCs for cell therapy in clinical applications.[5-7] Human Wharton's Jelly-derived Mesenchymal Stem Cells (hWJ-MSCs) can be obtained by a less invasive method as an attractive alternative source of MSCs without harming the mother or her infant.[8,9] HWJ-MSCs are similar to BM-MSCs in their morphological, biological and cytogenetic characteristics. Animal studies revealed that HWJ-MSCs have no tumorigenicity in vivo and are immature compared to BM-MSCs, which enables faster proliferation of them with a longer life span than BM-MSCs.[10-12] Moreover, embryo-like features, low antigenicity, easy accessibility, low ethical concerns and relative lack of rejection-associated problems compared to BM derived-MSCs indicate the potentially important role of WJ-MSCs in experimental research and clinical applications.[12-14]

Regulatory T cells (Tregs) form a subpopulation of T cells, which modulate the immune system, maintain tolerance to self-antigens and abrogate the autoimmune disease. Mouse models have suggested that modulation of Tregs can be effective in treatment of autoimmune diseases.[15,16]

Interleukin (IL)-35 belongs to IL-12 family of cytokines. It is composed of the IL-27 β chain (Ebi3) and the IL-12 α chain (p35).[17] IL-35 signals through a unique heterodimer of receptor chains, including IL-12Rβ2 and gp130 or homodimers of each of the mentioned chains, and uses the transcription factors STAT1 and STAT4 to transmit its signals.[18,19] IL-35 is expressed by forkhead box protein 3 ($Foxp3^+$) Tregs, peripheral $\gamma\delta$T-cells, CD8+ T-cells and placental trophoblasts. IL-35 has been recognized as an inhibitory cytokine specifically produced by Treg cells, which is required for maximal suppressive activity. Loss of IL-35 expression results in reduced suppressive capacity of Tregs in vivo.[20-23] On the other hand, IL-35 expands $CD4^+CD25^+$ T-cell population, expresses Foxp3 and produces elevated levels of IL-10. It can inhibit the promotion of T helper (Th)1 and Th17 differentiation and function,[24] which play a central role in initiation of inflammation and autoimmune diseases. The therapeutic effects of IL-35 has been shown in several preclinical animal models of allergic airway disease, autoimmune diabetes, cancer, collagen-induced arthritis (CIA), inflammatory bowel disease, lyme arthritis and melanoma.[22,25-30]

Considering the immunosuppressive function of WJ-MSCs and IL-35, they can collaborate with each other to improve the immunomodulatory and Treg cell functions, and can prevent the initiation of inflammation and subsequent autoimmune disease.

To our knowledge, this is the first case of IL-35 gene transduction into MSCs. We successfully transduced WJ-MSCs by Interleukin 35-harbouring lentiviral particles, and evaluated the expression of IL-35 in transduced hWJ-MSCs by RT-PCR and IL-35 ELISA kit. We also analyzed the IL-35 bioactivity and the effect of this cytokine on the frequency of Treg cells.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, six- to eight-weeks old, were purchased from Pasteur Institute of Iran. The use of animals in this study was approved by Tarbiat Modares University Institutional Animal Care and Use Committee.

Isolation and Culture of hWJ-MSCs

Human umbilical cord (UC) was obtained from full-term newborns delivered by cesarean section with informed consent of the donors (N=5). UC collection was approved by the Research Ethics Committee of Tarbiat Modares University, Tehran, Iran. UCs were maintained in a transfer medium of phosphate-buffered saline (PBS) containing penicillin (300 units/ml; Sigma), streptomycin (300μg/ml; Sigma) and amphotericin B (7.5 μg/ml; Sigma) at 4°C up to 24 h after collection. The whole cord was washed in sterile PBS three times to remove red blood cells; it was then immersed in 70% ethanol for 30s and immediately washed in PBS. Umbilical arteries and vein were removed, and the remaining tissue was minced into 3 -5 mm pieces, the segments were seeded onto 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), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) for 10 days. The culture was maintained at 37°C with saturated humidity and 5% (v/v) CO2. The medium was changed every 3 days after the initial plating. The pieces were removed after 10 days in culture. Adherent cells were trypsinized using 0.025% trypsin containing 0.02% EDTA (Gibco) and passaged into a new flask for further expansion upon reaching 80% confluence. In this study, we used the cells in passage 3. Finally, we cryogenically froze the WJ-MSCs (10% DMSO and 90% FBS) for later use.

Flow Cytometry

After the third passage, the cells were trypsinized (0.025 % trypsin and 0.02% EDTA), were twice washed by PBS and stained according to the manufacturer's recommendations for flow cytometry. The cells were labeled with the following antibodies: PE-conjugated mouse anti-human CD31, FITC-conjugated mouse anti-human CD44, FITC-conjugated mouse anti-human CD34, FITC-conjugated mouse anti-human CD73, PE-conjugated mouse anti-human CD45, FITC conjugated mouse anti-human HLA-DR, FITC conjugated mouse anti-human CD117, PE conjugated mouse anti-human CD90, FITC conjugated mouse anti-human CD105 and FITC conjugated mouse anti-human HLA-I. FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG1 were used as isotype controls (all the antibodies were purchased from e-Bioscience).

After incubation, the cell suspension was washed by PBS to remove any unlabeled antibodies. The cells were resuspended in PBS and analyzed using FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA). At least 20,000 events were recorded for each sample, and the data were analyzed using FlowJo™ software.

Differentiation of hWJ-MSCs

The cells in passage 3 of IL-35-transduced hWJ-MSCs and un-transduced hWJ-MSCs were plated at 20000 cells/ml in a 6-well plate and incubated in DMEM supplemented with 10% FCS until confluence is reached. The medium was replaced with an osteogenic medium, which contained DMEM containing 50 µg/mL ascorbic acid 2-phosphate (Sigma), 10 nM dexamethasone (Sigma) and 10 mM β-glycerol phosphate (Sigma). The medium was changed twice a week for 21 days. At the end of the cultivation 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 IL-35 transduced and un-transduced cells in passage 3 were incubated with a differentiation medium of DMEM, supplemented with 50 µg/mL indomethacin (Sigma) and 100 nM dexamethasone (Sigma). The medium was changed twice a week for 3 weeks. At the end of this period, the cells were stained with 0.5% oil red O (Sigma) in methanol for 2 min at room temperature.

Cloning of IL-35 cDNA in Shuttle of Lentiviral Vector

The murine IL-35 cDNA was amplified from plasmid pORF-mIL-35 (Invivogen) by the following primer: Forward:

5′CGCGGATCCCTGAGATCACCGGTAGGAGG -3′, reverse: 5′ TCCCCCCGGGGAGCTAG CTTTAGGCGG-3′.

To generate the GFP-harboring lentiviral particles (LvGFP), the GFP-carrying shuttle (pWPI, Trono lab, Switzerland) and mIL-35 cDNA were digested by XbaI and BamHI, and cDNA was subcloned into pWPI to generate a recombinant vector (pWPI- EF-1α –mIL-35-IRES-EGFP) (Figure 1A).

Lentivirus Production, Concentration and Titration

The lentivirus generation was accomplished as previously described [31]. Briefly, plasmid DNA (21 µg of lentiviral bearing the construct pWPI-mIL-35-EGFP, packaging plasmid psPAX2: 21 µg and envelope plasmid pMD2G: 10.5 µg per 10 cm dish) was transfected into HEK-293T cells using the calcium–phosphate method. Lentiviral particles were collected within 36, 48, 60 and 72 h post-transfection from the supernatant of HEK-293-T cells. The lentivirus was concentrated by precipitation with 50% PEG-8000 (Sigma) to a final concentration of 5% and by 5M NaCl (Sigma) to a final concentration of 0.15M overnight. For titration, we plated 60000 HEK 293T-cells and contaminated them with 1, 4 and 16 µl of virus. Three days later, the cells were detached and their fluorescence was analyzed by flow cytometry.

Transduction of hWJ-MSCs

HWJ-MSCs were trypsinized and seeded at a density of 1×10^5 cells in 1ml DMEM-F12 supplemented with 10 % FBS on six well plates. The virus was immediately added at MOI=50 in presence of 8 µg/ml Polybrene (Sigma) and was shaken on rotator

at 5 rpm. 18 hours later, the medium was changed with fresh DMEM-F12 supplemented with 10% FBS. After 12 hours, we again added virus at MOI=50 in presence of 4 µg/ml Polybrene and shook it on rotator at 5 rpm. GFP positive transduced hWJ-MSCs were measured 72 h post-transduction by flow cytometry, and the data were analyzed using FlowJo™ software.

The expression level and concentration of IL-35 protein in the cell supernatant was measured by mIL-35 ELISA kit (Bluegen Systems, China) according to the manufacturer's instructions. For this purpose, we first washed the cells with PBS, and fresh DMEM-F12 supplemented with 10% FBS was then added. Three days later, we collected the medium and used it for ELISA test.

RNA Extraction and RT–PCR (Reverse Transcription–PCR)

Total RNA was isolated from the transduced WJ-MSCs by RNX-Plus solution (CinnaGen, Tehran, Iran). Standard reverse transcription reactions were performed with 5µg total RNA using oligo (dT) 18 as a primer cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Subsequent PCR procedure was as follows: 2.5 µL cDNA, 1 × PCR buffer (AMS), 200 µM dNTP, 0.5 µM of each primer pair and 1 unit/25 µL reaction Taq DNA polymerase (Fermentas). The above-mentioned pair of primers was used to detect IL-35 gene expression.

IL-35 Protein Expression

To confirm IL-35 protein expression, we used intracellular staining flow cytometry. Briefly, IL-35 transduced hWJ-MSCs were fixed and permeabilized using intracellular fixation and permeabilization buffer set (eBioscience). Then, anti-IL-12/IL-35 p35 antibody was added and incubated in dark for 20-60 minutes.

After incubation, the cell suspension was washed with Flow Cytometry staining buffer, and the cells were resuspended in PBS and analyzed using FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA). Non-transduced cells were used as negative control cells.

Bioactivity Analysis of Recombinant IL-35

IL-35 can inhibit mitogen-induced CD4+ T cell proliferation. Thus, we examined the ability of recombinant IL-35 to inhibit proliferation of Phytohaemagglutinin (PHA)-stimulated CD4+ T cells. For this reason, mononuclear cells were isolated by Ficoll-hypaque density gradient centrifugation (density, 1.077±0.002) (Sigma) from mouse splenocytes. Mouse CD4+ T cells were isolated from splenocyte mononuclear cells using mouse CD4+ T cell isolation kit II (Miltenyi Biotec, Auburn, CA) following manufacturer's instructions, with purity in excess of 95%. The effect of recombinant IL-35 on PHA-stimulated mouse CD4+ T cells was assessed following dye-loading of CD4+ T cells with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) as described previously (32). Briefly, CD4+ T cells were incubated with 5 mM CFSE for 5 minutes and then washed three times with PBS. 1×10^5 CFSE-labeled CD4+ T cells were plated in 100 µl RPMI-1640 medium supplemented with 10% FBS and 100 µl IL-35 from transduced WJ-MSC cell supernatant. Mitogen stimulation was achieved with 10 µg/ml PHA (Invitrogen), and proliferation of the cells was measured on day 4. The CFSE-labeled CD4+ T cells were divided into four experimental groups: CD4+ T cells alone (no PHA stimulation), CD4+ T cells stimulated with PHA (10 µg/ml), CD4+ T cells stimulated with PHA and cultured with recombinant mouse IL-35, CD4+ T cells stimulated with PHA and cultured with hWJ-MSC supernatant as well as CD4+ T cells cultured with recombinant mIL-35. After 4 days, proliferation was analyzed by flow cytometry, and the flow cytometry data were analyzed using FlowJo™ software. To compare the effects of the experimental variables, the proliferation index (PI), a statistic generated by FlowJo and correlated with the number of cell divisions the CD4+T cells undergo following CFSE loading, was used.

For induction of T reg cells, 1×10^6 mouse splenocytes were cultured in 100 µl RPMI-1640 medium supplemented with 10% FBS and 100 µl IL-35 from transduced WJ-MSC cell supernatant, 100 µl WJ-MSC cell supernatant and 200 µl RPMI-1640 medium on 96 well plates for 4 days. Frequency of T reg cells was analyzed by Mouse Regulatory T-cell Staining Kit #2 (eBioscience) and human PE-Cy7-conjugated CD127 according to the manufacturer's protocol by flow cytometry, and the flow cytometry data were analyzed using FlowJo™ software.

Statistical Analysis

GraphPad Prism (version: 5.04) was used to perform statistical analyses. One-way ANOVA and t-tests were used to evaluate the differences between groups. $P<0.05$ was considered statistically significant.

RESULTS

Characterization of hWJ-MSCs

After five days, adherent single-spindle and fibroblast-like cells became visible around the tissue. On days 10-14 after initial plating, hWJ-MSCs began to form colonies and became confluent. After the hWJ-MSCs were passaged, they showed a strong proliferative ability and expanded without visible changes in either the growth pattern or morphology (Figure 1B). The hWJ-MSCs were positive for CD73, CD105, CD90 and HLA-I, but were negative for CD34, CD31, CD45, CD117 and HLA-DR, which indicates that the isolated cells were MSCs and were not derived from endothelial and hematopoietic cells (Figure 1G).

Osteogenic and Adipogenic Differentiation

After 21 days, IL-35 transduced hWJ-MSCs and untransduced hWJ-MSCs showed numerous lipid vacuoles visualized by oil red O. Moreover, when the mentioned cells were induced to differentiate into osteoblasts, massive calcium depositions was observed after Alizarin red staining (Figure 1C-F). Therefore, lentiviral particles harboring IL-35 gene did not change the mesodermal property of hWJ-MSCs.

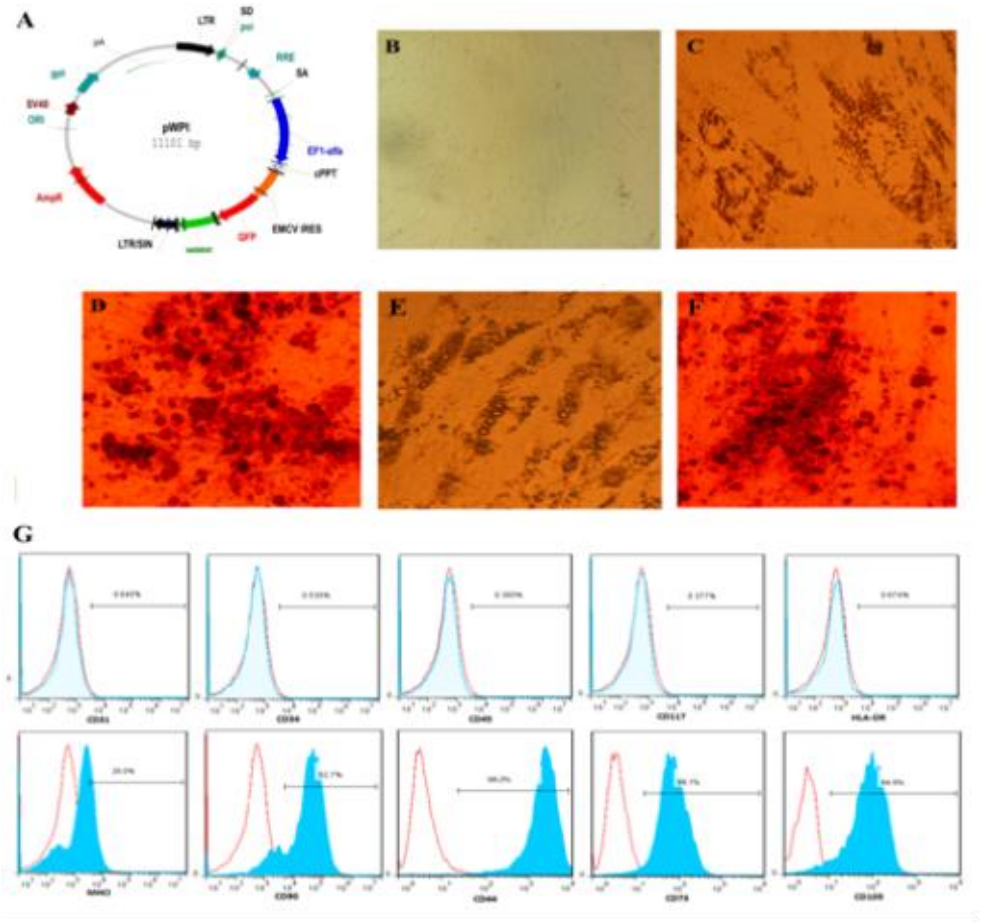

Figure 1. (A) The map of plasmid used in this study (www.addgene.org/12254). (B) Wharton's Jelly-derived Mesenchymal Stem Cells after isolation-3rd passage. (C) Oil Red O staining of un-transduced WJ-MSCs, intracellular lipid accumulation stained bright red in adipocytes at day 21, (D) Alizarin Red S staining of un-transduced WJ-MSCs, calcium deposition stained bright orange-red in osteocytes at day 21. (E) Oil Red O staining of IL-35 transduced WJ-MSCs, intracellular lipid accumulation stained bright red in adipocytes at day 21, (F) Alizarin Red S staining of IL-35 transduced WJ-MSCs, calcium deposition stained bright orange-red in osteocytes at day 21. (G) Surface antigen expression by hWJ-MSCs. WJ-MSCs were negative for CD31, CD34, CD45, CD117 and HLA-DR, and positive for CD73, CD44, CD90, MHCI and CD105, confirming that the cells were MSCs not hematopoietic or endothelial-derived cells.

Lentiviral Transduction of hWJ-MSCs

The open reading frame of mIL-35 gene was amplified by PCR and subcloned into the lentiviral vector. To demonstrate the insertion of IL-35 cDNA into GFP-carrying shuttle vector (pWPI), we digested the shuttle vector by XbaI and BamHI, and the 1315 bp fragment of mIL-35 was successfully cut out from the shuttle vector (Figure 2A, 1-3). Moreover, mIL-35 cDNA was detected by colony-PCR in shuttle vector. The cDNA was subsequently sequenced. No mutation was detected in cDNA sequence. We successfully produced IL-35-harbouring lentiviral particles in HEK-293T cells. After concentration and titration of the virus, we transduced hWJ-MSCs at MOI=50. GFP positive transduced hWJ-MSCs were measured 72 h post-transduction by flow cytometry, and there was an up to 85% positive transduction rate (GFP positive cells) (Fig. 2B-D). For IL-35 transduction into hWJ-MSCs, total RNA was isolated from the transduced WJ-MSCs, and IL-35 cDNA was successfully detected by RT-PCR (Figure 2A, 4).

MIL-35 Protein Expression and Secretion from hWJ-MSCs

IL-35 protein expression was confirmed by intracellular staining by flow cytometry, and IL-35 was successfully expressed in the transduced cells (Figure 2E). Secretion of IL-35 protein in the transduced WJ-kit, and the cells could successfully secrete IL-35 in culture media (750 ng/ml IL-35 protein compared to untransduced WJ-MSCs). IL-35 was not detected in untransduced WJ-MSCs (p<0.001) (Figure 3A).

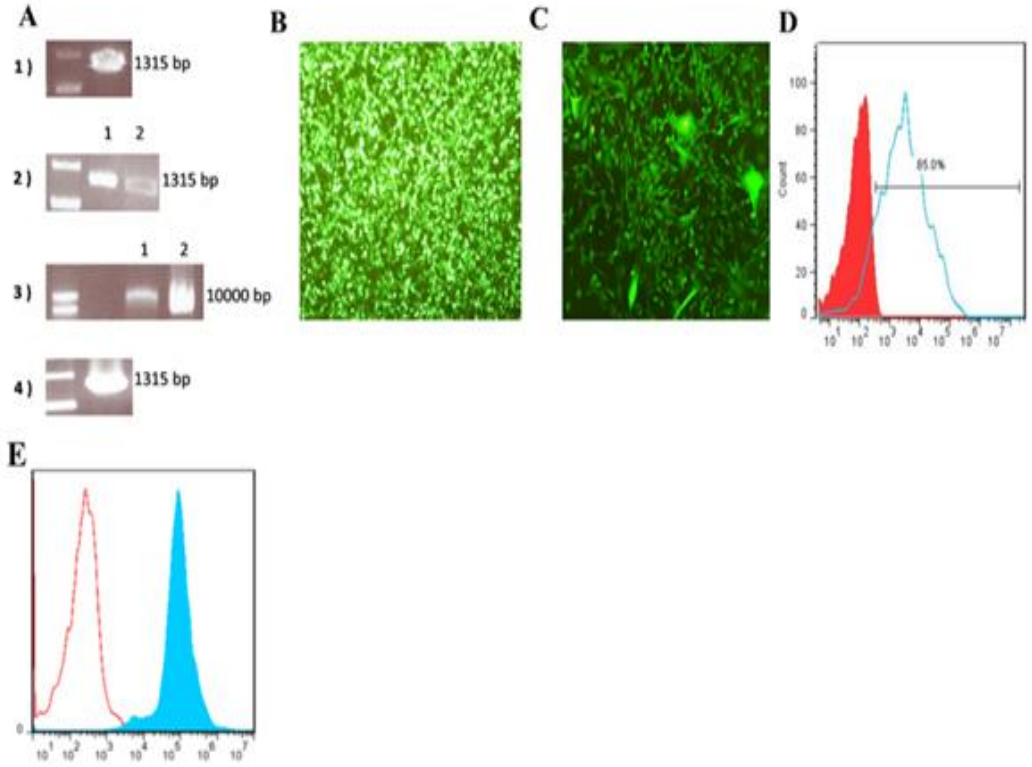

Figure 2. (A) Agarose gel electrophoresis of PCR product and constructs. (1) PCR product of IL-35 (2) Lane 1, undigested PCR product of IL-35. Lane 2, Double digestion of PCR product of IL-35 with XbaI and BamHI; (3) Lane 1, Double digestion of pWPI- EF-1α –IRES-EGFP construct XbaI and BamHI. Lane 2, undigested pWPI- EF-1α –IRES-EGFP construct; (4) IL-35 cDNA was successfully detected by RT-PCR from the transduced hWJ-MSCs. (B) GFP expression in HEK293T using fluorescence microscopy. HEK 293T cells transfected by final recombinant lentiviral construct, (C) GFP expression in hWJ-MSCs using fluorescence microscopy. HWJ-MSCs transduced by lentiviral vector harbouring IL-35, (D) FACS analysis of transduction rate. There was an up to 85% GFP positive transduction rate, (E) expression of IL-35 protein in hWJ-MSCs by Intracellular Staining flow cytometry.

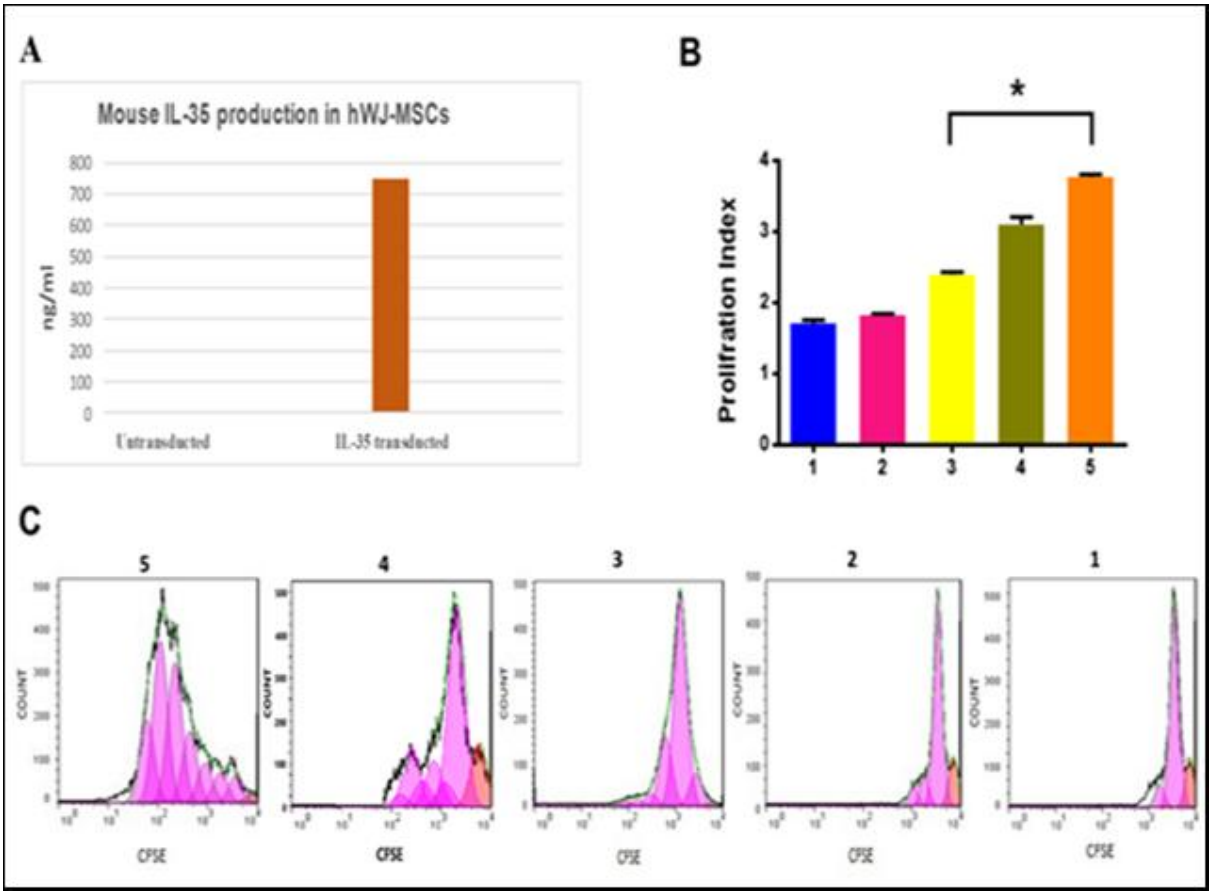

Figure 3. (A) IL-35 production in transduced and untransduced hWJ-MSCs (p<0.0001). (B) (C) Immune suppression by CD4+ T cells cultured with recombinant mIL-35 and assessed by the CFSE method. CD4+ T cell proliferation was analyzed by flow cytometry after four days. The proliferation index (PI; a statistic generated by FlowJo™ software) correlated with the number of CD4+ T cell divisions (1) CD4+ T cells alone (no PHA stimulation, PI, 1.80). (2) CD4+ T cells cultured with recombinant mIL-35 (no PHA stimulation, PI, 1.76). (3) CD4+ T cells stimulated by PHA and cultured with recombinant mIL-35 showed decreased CD4+ T cell proliferation (PI, 2.43) in comparison to CD4+ T cells stimulated with PHA (p<0.001). (4) CD4+ T cells stimulated with PHA and cultured with hWJ-MSCs supernatant (PI, 3) compared to CD4+ T cells stimulated with PHA (p<0.100). (5) CD4+ T cells stimulated with PHA, which proliferated extensively (PI, 3.74; no IL-35).

Bioactivity of Mouse IL-35

IL-35 can inhibit the mitogen-induced proliferation of CD4+T cells. We first isolated CD4+ T cells from splenocyte mononuclear cells using mouse CD4+ T cell isolation kit. The cells were then stained by CFSE. Proliferation was analyzed by flow cytometry four days after culture of the CD4+ T cells, recombinant IL-35 and PHA to demonstrate that the inhibitory function was due to IL-35 and not due to the factors released from hWJ-MSCs. We also cultured CD4+ T cells with hWJ-MSCs supernatant. Our recombinant IL-35 could significantly inhibit the proliferation of CD4+ T cells, which yielded a proliferation index of 2.43 compared to CD4+ T cells stimulated with PHA, yielding a proliferation index of 3.74 (p<0.001).

Supernatant of hWJ-MSCs also decreased the proliferation of CD4+ T cells (PI, 3) but was not significant compared to CD4+ T cells stimulated with PHA (PI, 3.74) (p<0.100) (Figure 3B,C).

IL-35 can improve the frequency and function of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T reg cells. To investigate whether our recombinant mIL-35 could affect the number of Treg cells, we cultured the splenocytes with IL-35 from transduced UC-MSC cell supernatant for four days and measured the frequency of Treg cells by multicolor flow cytometry. Flow cytometry data revealed that the frequency of $CD4^+CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T reg cells was significantly increased (p<0.001) compared to those in WJ-MSCs cell supernatant and RPMI-1640 medium

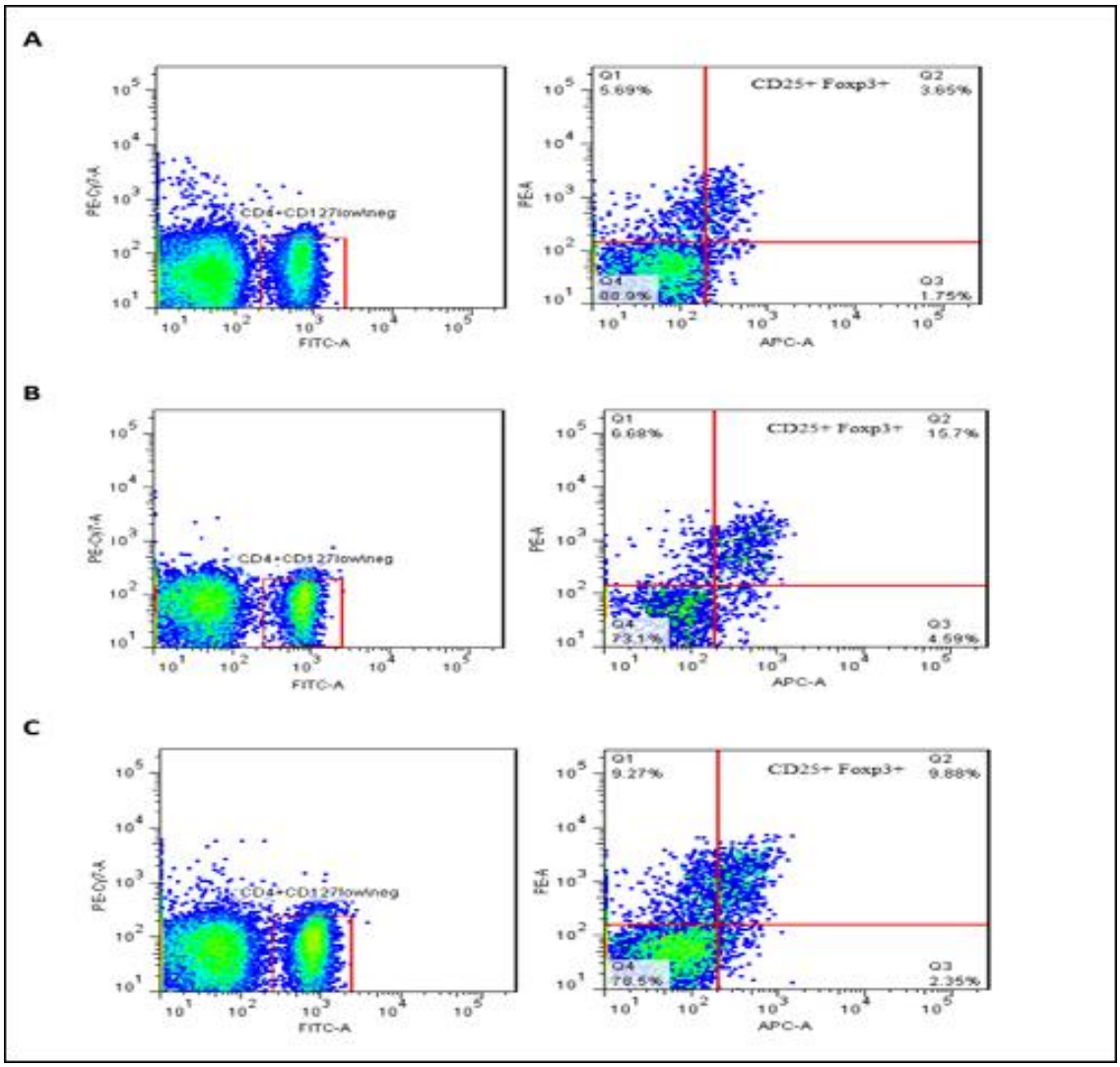

Figure 4. Our recombinant IL-35 improved the frequency of CD4+CD25+CD127low/negFoxp3+T reg cells in splenocytes. (A) Dot plot results of FITC-CD4+ PE-CD25+ PE-Cy7-CD127low/neg APC-Foxp3+ Treg cells in lymphocyte population in the RPMI-1640 medium (B) cultured with IL-35 from transduced WJ-MSC cell supernatant (p<0.001) and (C) WJ-MSC cell supernatant by flow cytometry.

(Figure 4A-C). Thus, we showed the bioactivity of our recombinant mIL-35.

DISCUSSION

Autoimmune diseases cause several health problems all over the world. Autoimmune diseases arise from an irregular immune response to antigens and constituents of the body's own tissues. This may be restricted to certain organs or involve a particular tissue. The immunosuppressive drugs are prescribed most typically for the treatment of autoimmune diseases to decrease the immune response.[33] As an alternative to conventional immunosuppression, cell therapy-based approaches have recently been tested with a view of restoring the integrity of immunoregulatory networks while preserving a pool of memory cells capable of responding to environmental pathogens. MSCs have immunosuppressive properties, and their potential for self-renewal and multilineage differentiation offers a potentially attractive therapeutic modality for

autoimmune diseases. MSCs inhibit virtually all types of immune responses in vitro and prevent the induction of disease in several experimental models of autoimmunity. In addition, MSCs are being considered as a candidate for cell-based gene delivery because of their efficiency to be transduced with different genes, and promise a major hope for tissue engineering and cell-based therapy.[1,2,4] Human bone marrow (hBM)-MSCs are considered as the main source for isolation of MSCs but disadvantages such as difficult collection, isolation and ethical issues limit the application of hBM-MSCs.[6,7]

MSCs could be isolated from different fetal tissues such as amnion, placenta, amniotic fluid, umbilical cord blood and Wharton's jelly of the umbilical cord. In comparison to hBM-MSCs, the umbilical cord is a convenient source of easily collected and isolated MSCs without requirement of invasive surgery. Recently, WJ-MSCs are reported to be a richer source of MSCs than UC blood. WJ-MSCs express a combination of MSC and embryonic cell markers. The therapeutic role of WJ-MSCs has been demonstrated in several preclinical animal models of human diseases, and can be used for treatment of different autoimmune disorders to provide a beneficial tool for basic research on gene therapy for autoimmune disorders.[9,12,13] In this study, we successfully isolated hWJ-MSCs from Wharton's jelly of the umbilical cord and characterized these cells by flow cytometry as well as osteogenic and adipogenic differentiation.

IL-35-transduced hWJ-MSCs successfully differentiated into adipogenic and osteogenic lineages, and lentiviral particles harboring IL-35 gene did not alter the mesodermal property of hWJ-MSCs. IL-35 can suppress the function of effector T cells such as Th1, Th2 and Th17, and may reduce the development of autoimmune and inflammatory diseases.[24] IL-35 has therapeutic effects in several autoimmune diseases such as autoimmune diabetes, collagen-induced arthritis (CIA), inflammatory bowel disease and lethal autoimmunity.[26,27,29,30] Several studies indicate that lentivirus vectors have a lower affinity than other retroviral vectors to integrate in sites that potentially cause cancer. Moreover, lentiviral vectors used in clinical trials for gene delivery do not increase the mutagenic or oncologic events.[34-37] Hui Qian et al showed that the lentiviral transduced WJ-MSCs were capable of differentiating into adipogenic and osteogenic lineages, were able to express stem cell markers such as Oct-4, Nanog, BMI-1 as well as nucleostemin and did not alter the nature of WJ-MSCs.[38] Due to this finding and the importance of IL-35 in different diseases in combination with MSCs in therapy of autoimmune diseases in future animal models, we transduced mIL-35 into hWJ-MSCs by lentiviral vector. These cells successfully released IL-35 in culture media, and there was up to 85 % GFP positive transduction rate of cells. We did not see any sensible changes in morphology and growth pattern. Collison et al showed that IL-35 was able to inhibit CD4+ T cell proliferation.[39] Because of this finding, we analyzed the ability of our recombinant mIL-35 to inhibit CD4+ T cells proliferation by flow cytometry. Our recombinant mIL-35 was biologically active and inhibited the proliferation of mouse CD4+ T cells. Treg cells play an important role in maintaining immune homeostasis. Treg cells suppress the function of other T cells to limit the immune response.[15] IL-35 induces the proliferation of Treg cells and improves their function.[20-22] To investigate the effect of this recombinant mIL-35 on the frequency of Treg cells, we measured the frequency of Treg cells in mouse splenocytes cultured with IL-35 using multicolor flow cytometry. We observed that mIL-35 could significantly increase the frequency of $CD4^+$ $CD25^+$ $CD127^{low/neg}$ $Foxp3^+$ T reg cells in splenocytes. In conclusion, to our knowledge, this is the first successful case of IL-35 transduction into WJ-MSCs, which may be an effective approach to use these cells as a good vehicle for gene therapy in autoimmune disorders.

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