博　士　論　文

Biological characterization and potential application of umbilical cord Wharton' s jelly-derived mesenchymal stem cells

（臍帯ワルトンゼリー由来間葉系幹細胞の生物学的性状とその応用に関する研究）

He Haiping

何 海萍

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I. Abstract

Umbilical cord Wharton's jelly is a rich source of mesenchymal stem cells (WJ-MSCs). My research presentation is consisted of two parts; To investigate the significance of Stage-specific embryonic antigen (SSEA) 4 in WJ-MSCs, and to study the immunosuppressive effect of WJ-MSCs on activated T cells.

SSEA4 has been reported as a stem cell marker in bone marrow MSCs. In my study, SSEA4 was expressed for a long-term culture. In contrast, $SSEA3^{+}$ disappeared rapidly in early passages. No differences in growth and differentiation ability to osteocytes and adipocytes were observed between the sorted $SSEA4^{+}$ cells and $SSEA4^{-}$ cells. Furthermore, I found that SSEA4 expression was significantly correlated with FBS concentration in the culture medium. Conclusively, SSEA4, which may display altered expression profiles in response to culture conditions, may not be an essential marker of WJ-MSC multipotency.

Next, in the study of immunosuppressive effects of WJ-MSCs, WJ-MSCs efficiently inhibited the responder T cells proliferation triggered by autologous or allogeneic dendritic cells in mixed lymphocyte reaction (MLR). Moreover, even 3^{rd} party-derived WJ-MSCs also strongly suppressed allogeneic MLR. The inhibitory effects of WJ-MSCs on T cell proliferation were attenuated by the blockade of cell-cell contact using the transwell chamber or the addition of culture supernatant of WJ-MSCs, but were reversed by the addition of indoleamine 2, 3-dioxygenase (IDO) inhibitor, 1-methyltryptophan (1-MT), in a dose dependent manner. In vivo experiments, to treat xenogeneic-GVHD mice model with WJ-MSCs are now undergoing.

In conclusion, WJ-MSCs are feasible alternative source of other MSCs for

regenerative medicine and immunotherapy.

Abbreviations

UC; Umbilical cord

WJ; Wharton's jelly

MSCs; Mesenchymal stem cells

CB; Cord blood

BM; Bone marrow;

PB-MNCs; Peripheral blood mononuclear cells;

CB-MNCs; Cord blood mononuclear cells;

DCs; Dendritic cells;

iPS; Pluripotent stem cells

HSCT; Hematopoietic stem cells transplantation

mAb; Monoclonal antibody;

SSEA; Stage-specific embryonic antigen

OCT4; Octamer-binding transcription factor 4

Klf4; Kruppel-like factor 4

Sox2; Sex determining region Y-box 2

Rex1; Ring-exported protein

PI; Propidium;

FBS; Fetal bovine serum

WJe-MSCs; Wharton's jelly-derived MSCs obtained by an explant method

WJc-MSCs; Wharton's jelly-derived MSCs obtained by an collagenase method

ISCT; International Society for Cellular Therapy

ES cells; Embryonic stem cells

GSL; Glycosphingolipid

RT-PCR ; Reverse transcription polymerase chain reaction

FACS; Fluorescence activated cell sorting

FCM; Flow cytometry

MLR; Mixed lymphocyte reaction

CFSE; Carboxyfluorescein diacetate N-succinimidyl ester

IDO; Indoleamine 2, 3-dioxygenase

1MT; 1-methyltryptophan

pGE2; Prostaglandin E_2

HLA-G5; Human leukocyte antigen 5

TGF; Transforming growth factor

IL; Interleukin

TNF-α; Tumor necrosis factor-alpha

IFN-γ; Interferon-gamma

PHA-L; Phytohemagglutimin

GM-CSF; Granulocyte macrophage colony-stimulating factor

IVIS; In vivo imaging system

NOG mice; NOD/Shi-scid/IL-2Rγnull mice

GVHD; Graft versus host disease

Ip; Intraperitoneal injection

R; Responder cells

S; Stimulator cells

II. Introductions

1.What are mesenchymal stem cells?

Mesenchymal stem cells (MSCs) originate in the human embryo and are considered to be multipotent stem cells. MSCs are a heterogeneous subset of stromal stem cells, which can be isolated from bone marrow[1], mobilized peripheral blood[2], cord blood[3], umbilical cord[4,5], placenta[6], adipose tissue[7], dental pulp[8], even the fetal liver[9] and lung[10], as shown in Figure 1. MSCs have the ability to differentiate into adipocytes, osteocytes [11], cardiomyocytes[12], hepatocytes [13], neurogenic cells[14,15] and skeletal muscle[16]. MSCs have a distinct ability of self-renewal, while maintaining their multipotency.

International Society of Cell Therapy (ISCT) proposes minimal criteria to define human MSCs[17,18]. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen (HLA) -DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*, although some directional differentiation abilities are dependent on the original source. In addition, MSCs also generated great interest for their ability to display immunomodulatory capacities[19,20]. Now, MSCs have been proposed as a potential candidate for regenerative medicine and immunotherapy.

2. Biomarkers of Multipotent MSCs

There is no single specific marker, which is defined as multipotent MSCs. As described above, MSCs are positive for CD73 (ecto-5'-nucleotidase), CD90 (Thy-1), CD105 (endoglin) , CD44, CD29, CD13, CD146, CD106, CD54 , CD271, and CD166[21,22,23] (Figure 1), but do not express CD34 (hematopoietic maker), CD14

(monocytes／macrophages), LFA-1, CD11a (lymphocytes), CD45 (leukocytes), RBCs (glycophorin A), CD31 (endothelial cells) , CD40, CD80, CD86 (costimulatory molecules), and CD31,CD18, CD56 (adhesion molecules). MSCs express low for HLA-Class I molecules and lack of HLA-DR. Furthermore, ESCs markers such as *Oct4, Nanog, Sox2, and KLF4* are expressed at low level[24-26]. It suggests that MSCs are primitive stem cells between ESCs and mature adult stem cells. However, these molecules are the transcription markers, therefore, how to precisely isolate multipotent MSCs using specific marker remain challenge.

SSEA3 and SSEA4 are epitopes on the related glycosphingolipids (GSLs), GL-5 and GL-7, respectively, GSLs consist of a carbohydrate moiety or a chain linked to ceramide[27]. However, the role of SSEA3 and SSEA4 in MSCs remains controversial. Gang et al reported that $SSEA4^+$ cells proliferated predominantly when the culture was initiated from primary BM cells[28] and Wakao et al. showed that SSEA3 is a multipotent stem cell marker on MSCs defined as multilineage-differentiating stress-enduring (Muse) cells[29,30]. In contrast, Brimble et al. demonstrated that both SSEA-3 and SSEA-4 are not essential for human ES cell multipotency, as proven by glycosphingolipid inhibitors[31]. At present, how to precisely isolate multipotent MSCs using some of specific marker remains discussion.

3. Multipotency of MSCs

MSCs have a great capacity of self-renewal while maintaining their multipotency. MSCs have trilineage differentiation potential which can be differentiated into osteoblasts, adipocytes, chondrocytes, muscle cells (mesoderm)[17,18], neuron-like cells[14,15], epithelial cells (ectoderm)[32,33] and hepatic like cells(endoderm)[14,34] (Figure 1). The capacity of

differentiation was related to the age of the donor, the cells source, the individuals, and also the induction methods. But until now, it is not clear whether this variation is due to a different amount of "true" progenitor cells in the culture or variable differentiation capacities of independents' progenitors.

4. Immunomodulatory properties of MSCs

Immunosuppressive effect has now become most enthusiastic property of MSCs for clinical use.

First, MSCs, themselves, are shown the low immunogenicity owing to the lack of HLA-DR and low express HLA-Class I molecules. MSCs have not only immunomodulation, but also escape immune rejection[35]. Furthermore, MSCs lack both CD80 and CD86 proteins[36,37], which are co-stimulatory molecules inducing T cells activation and survival.

Second, MSCs have the immunosuppressive effects *in vitro* and *in vivo*. Recently, several reports demonstrated that immunosuppressive effect of MSCs is mediated by cell-cell contact among the target cell MSCs and the secreted soluble factors from MSCs such as indoleamine 2,3-dioxygenase (IDO)[38], Galectin-9[39], human leukocyte antigen G5 (HLA-G5)[40], transforming growth factor (TGF)-β1, and prostaglandin E_2 (PGE2)[41](Figure 2). Among these factors, IDO is considered the most effective molecule in human, which is inducible by IFN-γ and catalyzes the conversion from tryptophan to kynurenine[42]. The depletion of tryptophan from the environment can suppress T cells proliferation. These immunomodulatory effects of MSCs have been studied mainly in BM-MSCs.

5. Umbilical Cord Wharton's jelly derived MSCs

Among stem cells, embryonic stem (ES) are the leading candidate for tissue engineering due to their highly self-renewal and pluripotent differentiating ability into all germ layers *in vitro* and *in vivo*. However, in addition to ethical restriction, their clinical application is also severely limited by technical difficulties in purification and concerns for formation of teratoma.

In contrast, adult stem cells (such as skin, BM, and adipose) could have wider application in clinic. Especially BM-MSCs can be applied for autologous use. Recently many clinical applications using autologous BM-MSCs were performed for cardiac infarction, graft versus host disease (GVHD), Crohn's disease etc. However, their application was sometimes limited by cells numbers, decreased growth and differential capacities with increasing age[43,44].

During pregnancy, the fetus and mother was connected by an umbilical cord (UC) which prevents umbilical vessels from compression, torsion, and bending while providing a good blood circulation. UC consists of two umbilical arteries (UCA) and one umbilical vein (UCV), both embedded within a specific mucous connective tissue, known as Wharton's jelly (UCWJ), which is then covered by amniotic epithelium (Figure3 A).

McElreavey et al first reported the isolation of fibroblast-like cells from the human UC Wharton's jelly (WJ) on 1991[4]. The UC derived cells have the similar of surface phenotype, plastic adherence and multipotency with other type of MSCs. My colleagues, Ishige, Nagamura-Inoue et al reported firstly in Japan that MSCs can be isolated from WJ, UCA and UCV, respectively. Hsieh et al compared the gene expression profiles of

BM-MSCs and WJ-MSCs and concluded that WJ-MSCs were more primitive and more similar to ES cells than BM-MSCs[45,46]. There are many protocols to obtain MSCs from various compartments of UC including WJ, arteries, vein, UC lining and sub-amnion, and UC perivascular stem cells (HUCPVC) or the whole UC. Among these compartments of UC, WJ is most accessible and obtained larger amount of tissue rather than other compartments. The collection procedure is a non-invasive process and these cells could be isolated without ethical problem. These are the advantages of WJ-MSCs, and it is the reason why I chose WJ-MSCs.

WJ-MSCs can be considered either for autologous or allogeneic use. Autologous WJ-MSCs might be applied for gene therapies in genetic disease, regenerative or anti-inflammatory therapy in newborn injuries. On the other hand, allogeneic WJ-MSCs can be expanded and cryopreserved as banking for the people in need. Only disadvantage to be taken care is that it is necessary to confirm the baby's health as WJ-MSCs donor. At birth, we do not know the WJ-MSCs donor is grown normally, while the adult stem cells donor can be confirmed the health at the donation. In case of Japanese CB banks, CB banks shall chase the baby's health after birth. It is important to know the advantages and disadvantages of WJ-MSCs in the steps of clinical applications.

WJ-MSCs seemed the attractive feasible source of MSCs for me and I liked to study more about this.

6. The objectives of my study

The identification of specific markers for stem cells in MSCs always remains discussion. SSEA4 has been reported as a stem cell marker in BM-derived MSCs[28], but whether $SSEA4^+$ cells have growth and differentiation advantages over $SSEA4^-$ cells

remains unknown in WJ-MSCs. To investigate the significance of SSEA4 in WJ-MSCs, I investigated the SSEA4 and SSEA3 expressions by different method of collections, and sorting by the expression of SSEA4, and the culture medium including fetal bovine serum (FBS) concentrations.

Another issue is the immunosuppressive effect of WJ-MSCs on activated T cells. Recently, it is reported that MSCs have the ability to migrate to inflammatory tissues and suppress adverse immune reactions. In fact, BM-MSCs have been already applied for the patients to treat acute graft versus host disease (aGVHD) in hematopoietic stem cells transplantations (HSCT). However, the mechanism by which WJ-MSCs exert their immunosuppressive effects is not completely understood. To study the immunosuppressive properties of WJ-MSCs on activated T cells, I investigated the effective conditions in which WJ-MSCs show the immunosuppressive effect in mixed lymphocyte reactions (MLR) *in vitro* and in xenogeneic GVHD in mice.

III. Materials and Methods

1. Isolation and culture of adherent cells

The present study was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo, Japan and the NTT Medical Center hospital. Informed consent was obtained from mothers planning to have deliveries. UCs and CBs were collected aseptically at full-term cesarean section. The UCs were transferred after collection and the process was initiated within 24 h of delivery. The UC surface was rinsed with phosphate-buffered saline (PBS; Gibco–BRL, CA, USA) containing antibiotics and antifungal reagents Anti–Anti (Antibiotic–Antimycotic, 100X; Gibco–BRL). After removing two arteries and one vein, the remaining WJ tissues were minced into 1–2 mm^3 fragments and divided into two groups for the explant and collagenase treatment methods (Figure 3A). In the explant method, the minced fragments were aligned and attached at regular intervals in 10-cm culture dishes. After the fragments were semi-dried and firmly attached to the bottom, the culture medium was gently poured into the dishes[5, 47]. In the collagenase treatment group, the minced WJ tissues were incubated in 1 mg/ml collagenase type I solution (Sigma–Aldrich, St. Louis, MO, USA) in α-MEM (Gibco–BRL) with shaking at 37°C for 2–3 h[47,48]. The cells were then washed with α-MEM supplemented with 10% fetal bovine serum (FBS) and seeded in 10-cm tissue culture dishes with the culture medium as described above [5]. The culture medium was refreshed once a week until fibroblast-like adherent cells reached 80%–90% confluence by 3 to 4 weeks of culture. Both WJe-MSCs and WJc-MSCs were spindle-shaped fibroblast-like cells (Figure 3B). The first harvested master cells were defined as passage 0 (P0). The adherent cells and tissue fragments were rinsed once with

PBS and detached using 10% trypsin solution (TrypLE Express; Invitrogen, Carlsbad, CA, USA) followed by washing with α-MEM supplemented with 10% FBS. In the explant method, the cells and tissue fragments were filtered to remove the tissue fragments. The harvested cells, other than those undergoing further analysis, were immediately cryopreserved in 10% DMSO/5% dextran 40 solution. For serial cultures, the cells were inoculated at 2×10^5 cells per 10-cm culture dish and counted at each passage.

2. Flow cytometry analysis and sorting

Standard flow cytometry (FCM) techniques were used to determine the typical cell surface markers of WJ-MSCs. WJ-MSCs were stained with the following mouse monoclonal antibodies (mAbs): phycoerythrin (PE)-conjugated anti-human CD73 (BD, CA, USA), CD271 (Miltenyi, Germany), and HLA-ABC (BD); fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 (BD), CD105 (eBioscience, CA, USA), HLA-DR (BD), and CD45 (BD), FITC-, PE-, and Alexa-conjugated anti-mouse IgGs (BD) were used as isotypic controls. Dead cells were identified by staining with propidium iodide (PI). To detect the ES cell markers in WJ-MSCs, Alexa-conjugated mouse anti-human SSEA4 (BD) and FITC-conjugated rat anti-human SSEA3 (BD) together with the MSCs markers CD73 or CD105 mAbs were used. The stained cells were acquired with a FACS Caliber (BD) and analyzed by FlowJo (Tomy Digital Biology, Co. Ltd., Japan). For cell sorting, WJe-MSCs were stained with Alexa-conjugated anti-human SSEA4 and PE-conjugated anti human-CD73 antibodies. The cells were acquired with a FACS Aria cell sorting system (BD) and sorted by SSEA4, SSEA3, and

CD73 expression (Figure 3C).

3. Proliferation assays of sorted SSEA4^{+} and SSEA4^{-} MSCs

To evaluate the proliferative abilities of sorted SSEA4^{+} and SSEA4^{-} WJe-MSCs, the sorted cells were plated at 1×10^{4}/well in six-well plates (Greiner Bio-one, German) and cultured in αMEM supplemented with 10% FBS. The cells were harvested every week and the cell numbers were counted with trypan blue (Gibco–BRL) for 9 weeks.

4. SSEA4 expression in WJ-MSCs with different FBS concentrations

To evaluate whether culture conditions influenced the SSEA4 expression, WJe-MSCs (P0) were cultured at 1×10^{5}cells/well in 6-well plates (n = 3) in αMEM with the indicated FBS concentrations. After 1 week, the SSEA4, SSEA3, and CD73 expressions were analyzed by flow cytometry. To study the influence of the proliferation of WJe-MSCs on SSEA4 expressions, I explored the time-course experiment to see the relationship been SSEA4 expression and WJe-MSCs growth curve. WJe-MSCs were plated in 6-well plates with indicated concentrations of FBS, and counted the cell number to figure the growth curve on indicated days. The cells were analyzed by flow cytometry to analyze the expression of SSEA3, SSEA4, and CD73.

Furthermore, to analyze the influence of FBS on SSEA4 WJe-MSCs, SSEA4^{+} and SSEA4^{-} WJe-MSCs were cultured in 12-well plates with different concentrations of FBS followed by flow cytometry.

5. SSEA4 expression in BM-MSCs with different FBS concentrations

To clarify that the phenomena of SSEA4 expression is limited to the WJe-MSCs, I studied the SSEA4 expression in BM-MSCs obtained from BM-mononuclear cells (MNCs). Frozen BM-MNCs were purchased from Lonza Walkersville Inc, MD. BM-MNCs (8×10^5/well) were seeded in 6-well plates and cultured in the 37°C with 5%CO_2. On day 0 to day21, the proportion of CD45, SSEA4, and CD73 positive or negative cells were analyzed by flow cytometry. To see the influence of FBS on BM-MSCs, I continued to culture BM-MNCs in αMEM supplemented with 10% FBS and obtained the MSCs. BM-MSCs at p2 were plated in 6-well plates to figure the growth curve and analyzed the incidence of CD45, SSEA3, SSEA4, and CD73 by flow cytometry, as described in WJe-MSCs.

6. RNA isolation and RT-PCR analysis

Total RNAs were extracted from WJ-MSCs at P3 and from sorted $SSEA4^+$ and $SSEA4^-$ MSCs using TRIzol® Reagent (Invitrogen Corp, Carlsbad, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the PrimeScript RT-PCR Kit (Takara Shuzou, Shiga, Japan) according to the manufacturer's instructions. The ES markers, *Nanog, Oct4, Klf4, Sox2* [37] and glyceraldehyde-3-phosphate dehydrogenase as the control were amplified from the synthesized cDNAs by PCR with the primer pairs shown in Table 1. The amplification conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. The PCR products were separated by electrophoresis on 2.0%-agarose gels and visualized by staining with ethidium bromide[5].

7. Adipogenic differentiation

WJe-MSCs were cultured at 2×10^4 cells/well in 6-well plates in αMEM supplemented with 10% FBS. When the cells achieved 80% confluence, the medium was replaced with adipogenesis induction medium[45], consisting of 100 uM indomethacin (Sigma–Aldrich), 1 uM dexamethasone (Sigma–Aldrich), 0.5 mM IBMX (Sigma–Aldrich), and 10 ug/ml insulin (Sigma–Aldrich). The medium was refreshed every 3 days. After 3 weeks, the cells were fixed with 10% formaldehyde, washed with PBS and 60% isopropanol, and stained with Oil Red O (Sigma–Aldrich).

8. Osteogenic differentiation

WJe-MSCs at 2×10^4/well (P2) and $SSEA4^+$and $SSEA4^-$ WJe-MSCs were cultured in 24-well plates in αMEM supplemented with 10% FBS. On the following day, the medium was replaced with osteogenic induction medium including 10 nM dexamethasone (Sigma–Aldrich), 10 mM β-glycerol phosphate (Sigma–Aldrich), 100 μM ascorbic acid (Sigma–Aldrich), and 50 ng/ml human BMP2 (rhBMP2; Peprotech, NJ, USA)[50,51]. Human BM-derived MSCs were used as the positive control. The induction medium was refreshed every 3 days. After 5 weeks, the cells were fixed with 2.5% glutaraldehyde for 15 min at room temperature followed by rinsing with PBS. The bone matrix was stained with 2% Alizarin Red S solution (Sigma–Aldrich) with pH adjusted to approximately 4.1–4.3 with 1% ammonium hydroxide (Sigma–Aldrich)[51].

9. Mixed lymphocyte reaction assays

9.1. Mononuclear cells preparation

CB were obtained at the collections of UC in NTT Medical Center hospital and partially CB were purchased from RIKEN BRC in National Bioresource Project (NBRP). PB were obtained from healthy volunteers after informed consent. Frozen–thawed or fresh CB mononuclear cells (CB-MNCs) or fresh peripheral MNCs (PB-MNCs) were used as responder cells or as stimulator cells. Protocol of MNCs preparations was written elsewhere. Briefly, fresh CB or PB were poured onto the Ficoll-Paque PLUS (GE, PA), followed by centrifugation at 1200rpm for 20min. MNCs layer was collected and washed twice with PBS, and MNCs counts were calculated as the sum of lymphocytes and monocytes, measured by an automated hemocytometer (Sysmex 2100, Kobe, Japan).

9.2. Stimulator cells preparation

Mature dendritic cells (DCs) were used as stimulator cells. 1×10^7 cells of PB-MNCs or CB-MNCs were plated in 10cm diameter-tissue culture dish. These cells were cultured for 5 days in the RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% FBS containing10 ng/ml granulocytemacrophage colony-stimulating factor (GM-CSF, Peprotech Inc.) and 250ng/ml IL4 (Peprotech Inc). On day 5, the medium was replaced with fresh medium containing 10ng/ml tumor necrosis factor-α (TNF-α) (Peprotech, Inc.) and cultured for 2 days. On day 7, mature DCs were harvested using lifter. Then DCs were adjusted to 2×10^5 /ml in RPMI 1640 and 10% FBS medium and irradiated at 50Gy.

9.3. Responder cells preparation with CFSE

The CB-MNCs or PB-MNCs were stained with 2.5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE; Vybrant CFDA SE Cell Tracer Kit; Invitrogen) in PBS for 9 min at 37°C. The CFSE labeling was stopped by the addition of 50% FBS containing medium, then the cells were washed extensively with 50% of FBS containing medium. The CFSE-labeled responder cells were counted the cell concentration and adjusted to $2x10^6$/ml and kept on ice for use.

9.4. MLR preparation

WJ-MSCs were plated at $2x10^5$/ml in 24-well plates 2days before the day of MLR. On the day of MLR, the cells were suspended in RPMI 1640 supplemented with 10% FBS and inoculated in 24-well plates as follows: CFSE labeled-CB-MNCs or PB-MNCs responder, irradiated DCs, and WJ-MSCs at a 10:1:1 ratio. To stimulate the reactions, I added 12.5ng/ml of anti-human CD3 mAb (OKT3). After 4 days of culture, the cells were harvested and stained with APC- anti-CD4 or APC-anti-CD8 antibodies. The CFSE fluorescence intensities of responder T cells were measured separately for $CD4^+$ responder cells and $CD8^+$ responder cells. As positive controls, the responder cells were treated with phytohemagglutinin-L (PHA-L; Sigam-Aldrich). In CFSE histograms, when the cell divides, the fluorescence should be apportioned equally to daughter cells to make sub-peaks, roughly half the parental intensity. Parental intensity should be the standard line and immunosuppressive effect of WJ-MSCs were evaluated by the blockade of the division of the initial cells (Figure 4).

9.5. Separation culture using Transwell chamber

To evaluate whether WJ-MSCs-mediated immunosuppression requires cell-cell contact, I separated the MLR from WJ-MSCs by transwell chamber. The WJ-MSCs (2×10^5/ml) were cultured in the lower chamber 2 days before MLR. On the day of MLR, CFSE-labeled CB-MNCs (2×10^6/ml) with irradiated DC cells (2×10^5/ml) were put in the upper chamber at a ratio of 10:1 in the presence of anti-CD3 mAb. To see the inhibitory effect of WJ-MSCs on PHA-L stimulated T cells, I also cultured CB-MNCs (2×10^6/ml) in the upper chamber and WJ-MSCs in the lower chamber in the presence of PHA-L (5μg/ml). As the control, MLR was performed in the absence of MSCs. The floating cells were harvested after 4 days of culture flowed by the further FCM analysis.

9.6. WJ-MSCs supernatants preparation

To evaluate the soluble factors secreted from WJ-MSCs, I cultured WJ-MSCs at 2×10^5/ml in RPMI+10% FBS medium in 24-well plates. When MSCs were 90% confluent, the culture medium was collected. The medium was once centrifuged at 1500rpm 5 min and the sub-supernatant was used as the WJ-MSCs supernatant. WJ-MSCs supernatant was added to MLR at a ratio of 3:1. As the control, fresh medium was added to MLR. The floating cells were harvested after 4 days of MLR culture followed by the FCM analysis.

9.7. IDO inhibitor (1MT)

IDO is one of the representative soluble molecules. To evaluate the role of IDO, I added IDO inhibitor, 1-methyl-DL-tryptophan (1-MT, Sigma-Aldrich,St-Louis, MO) at

the indicated concentrations in MLR. 1MT was dissolved in NaOH at 1 M followed by dilution with RPMI to 20mM stock solution. After 4 days of MLR culture, the cells were harvested and analyzed by FCM.

9.8. Influence of increased passages of WJ-MSCs on immunosuppressive effect

In order to evaluate the influence of increased passages on the immunosuppressive effect of WJ-MSCs in MLR, I compared the inhibitory effects of WJ-MSCs in p10 with those in p1.

10. Treatment of xenogenic GVHD mice model using WJ-MSCs

10.1. NOG mice

Six-weeks-old NOD/Shi-scid/IL-2Rγnull (NOG) mice were purchased from SLC Japan (Tokyo, Japan) and were handled according to the guidelines of the Institute of Medical Science, University of Tokyo. The experiments were approved by the Committee for Animal Research at the institution.

10.2. WJ-MSCs injection into xeno–GVHD model mice

To study the immunosuppressive effect of WJ-MSCs on activated T cells in vivo, I established xenogeneic GVHD mice model. In this model, NOG mice were irradiated at 2.5 Gy and injected the activated T cells, which were cultured in the coated flask with anti-CD3 and CD28 mAb in Alys505N IL2 medium supplemented with 5% FBS, (Cell Science Inc., Japan) at the presence of 700u/ml of IL2. One tenth of activated T cells was

transduced with firefly luciferase gene (luc) for *in vivo* monitoring xeno-GVHD activity and mixed with non-transduced activated T cells on the day of injection. The high titer lenti viral vectors (HIV-EF1a-Luciferase) were prepared according to the procedure described previously[52]. Injected activated T cells can be tracked by *In vivo* bioluminescence imaging (BLI) (IVIS Imaging System 100; Xenogen, CA). WJe-MSCs were washed with PBS and injected to NOG mice at a dose of $1x10^6$/mice, twice a week up to 5 times totally.

NOG mice injected with 2×10^7 luc-transduced T cells on Day 0 were divided into 3 groups according to the initial injection day of WJ-MSCs; control group (n = 4):T cells, treatment group with $1x10^6$ WJ-MSCs on Day -1 (n = 3): T cells + MSCs (Day -1), and treatment group with $1x10^6$ WJ-MSCs from Day 0 (n = 3): T cells + MSCs (Day 0) (Figure 5).

10.3. In vivo monitoring of xeno-GVHD mice with or without WJ-MSCs

These mice were weekly monitored by IVIS® imaging system for T cell expansion. Whole-body images were obtained and analyzed with Living Image 2.50 software (Xenogen). Briefly, *In vivo* bioluminescence imaging (BLI) was performed using a cooled CCD camera system. The inoculated mice received a subcutaneous injection of 75 mg/kg D-luciferin (Sigma-Aldrich) and were placed in the light-tight chamber of the CCD camera system under isoflurane anesthesia. Beginning 10 min after the injection, photographic and luminescence images in the ventral projections were acquired. The exposure time for luminescence imaging was set at 300 sec.

10.4. Assessment of acute GVHD

Recipient NOG mice were weighed every other day for the signs of acute graft versus host disease (aGVHD), and observed general appearance of the fur, and mobility. Overall survival was figured out by Kaplan-Meir Method. Mice were sacrificed when the mice lost the weight at the reduction rate > 20%, or appearance of either limited mobility or disruption of general appearance.

10.5. Immunohistological analysis

The immunohistological analysis was performed for three groups of recipient mice. The liver, lung, intestine and skin of the sacrificed mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. The samples were processed for immunostaining with anti-human CD45 antibody by standard procedure.

11. Statistical analysis

Differences between groups were analyzed with JMP 6.0.2 software (SAS Institute, Cary, NC, USA). Statistical analyses were performed with Turkey-Kramer tests, and a *P*-value of 0.05 was regarded as statistically significant.

IV. Results

A. Significance of SSEA4 in WJ-MSCs

1. Collection efficiency and WJe-MSCs and WJc-MSCs biomarkers

First, I compared the characteristic between WJe-MSCs and WJc-MSCs to use the further analysis. There was no significant difference between WJe-MSCs and WJc-MSCs in the collected cell numbers at P0, even though the collected cell numbers of WJe-MSCs varied (Figure 6A). The median number of collected WJe-MSCs from 1 g of WJ was 2×10^6 (range, from 9.1×10^4 to 10.3×10^6; n = 23) and of collected WJc-MSCs was 1.7×10^6 (range, from 9.2×10^4 to 7.5×10^6; n = 20). Further, we compared their surface markers as defined by the ISCT. Both WJe-MSCs and WJc-MSCs were plastic adherent cells (Figure 3B) and positive for CD73, CD90, CD105, and HLA-ABC with a small percentage of cells also positive for CD271 and negative for CD34, CD45, and HLA-DR (Figure 6B). In addition, WJe-MSCs and WJc-MSCs expressed the ES-related genes, *Nanog, Oct4, Klf4, Rex1, and Sox2* (Figure 6C).

2. Expression of SSEA4 and SSEA3 in WJ-MSCs

Because $SSEA4^+$and $SSEA3^+$ cells have been considered as representative of immature cells, I periodically monitored SSEA4, SSEA3 with CD73 expressions in WJe-MSCs and WJc-MSCs during P0–P9. The percentages of $SSEA4^+$ cells at P0 were similar in WJe-MSCs and WJc-MSCs. At P0, the WJe-MSCs included 32.4% ± 17.5% $SSEA4^+CD73^+$; 62.8% ± 18.9% $SSEA4^-CD73^+$; and 1.3% ± 1.8% $SSEA4^+CD73^-$, whereas the WJc-MSCs included 26.1% ± 16.1%, 70.4 ± 16.2%, and 0.7 ± 0.8% cells, respectively (n = 8, P =0.21). However, the percentage of WJe-MSCs $SSEA4^+$cells

decreased after the first passage and recovered to the original level by P7, whereas the incidence of WJc-MSCs SSEA4$^+$ cells was relatively stable until P9 (Figures 7A,C). In contrast, the percentage of SSEA3$^+$ cells among both WJe-MSCs and WJc-MSCs was highest at P0 that declined and disappeared by P5 (Figures 7B,D). At P0, the percentage of SSEA3$^+$cells among WJe-MSCs was 6.7% ± 6.3% and among WJc-MSCs was 6.1% ± 6.1% (n = 6).

Explant method does not require non-human collagenase derived from clostridium histolyticum and save the time for lysing the tissue with enzyme in the procedure. This non-human reagent might influence on the quality when WJ-MSCs for clinical use. Also WJe-MSCs and WJc-MSCs did not show significant difference on cells number and surface markers. Therefore, I chose WJe-MSCs for my further study.

3. Comparison of SSEA4$^+$ and SSEA4$^-$ cells sorted from WJe-MSCs

Further, I sorted SSEA4$^+$ and SSEA4$^-$ cells from WJe-MSCs at P4 using FACS Aria and analyzed the SSEA4 expression in the sorted cells every week. The mean purity of SSEA4$^+$CD73$^+$ was 89% and that of SSEA4$^-$CD73$^+$ was 97.7%. The SSEA4$^+$ MSCs and SSEA4$^-$ MSCs were of similar sizes (Figure 8A).

The percentage of SSEA4$^+$ cells derived from the sorted SSEA4$^+$ WJe-MSCs decreased rapidly in the first week and then increased gradually until week 4 (Figures 8B). Interestingly, SSEA4$^+$ MSCs were present in the SSEA4$^-$ WJe-MSCs, and the incidence of SSEA4$^+$ cells in the subsequent cultures was similar to that in the SSEA4$^+$ sorted cells. After 4 weeks, the incidence of SSEA4$^+$ cells from both the SSEA4$^+$ and SSEA4$^-$ MSCs decreased to less than 10%. The incidences of SSEA4$^+$ cells derived from

SSEA4^{+} WJe-MSCs and SSEA4^{-} WJe-MSCs were 8.5% ± 8.8% and 8.5% ± 8.8% at week 1 and 43.5% ± 21.6% and 55.1% ± 29.4% at week 4, respectively (n = 3) (Figure 8C). In addition, there was no difference in proliferation ability between SSEA4^{+} and SSEA4^{-} WJe-MSCs (Figure 8D).

RT-PCR analysis showed that the sorted SSEA4^{+} and SSEA4^{-} WJe-MSCs expressed *Nanog, Oct4, and Klf4* (Figure 8E).

4. Adipocyte differentiation

To determine the differentiation ability of SSEA4^{+} WJe-MSCs, I induced sorted SSEA4^{+} and SSEA4^{-} WJe-MSCs into adipocytes with induction medium. I observed the accumulation of Oil Red O-stained lipid drops in SSEA4^{+}, SSEA4^{-}, and non-sorted WJe-MSCs, whereas there were no changes in WJe-MSCs cultured without induction medium (Figure 9A).

5. Osteogenic differentiation

I also compared the osteogenic differentiation abilities of SSEA4^{+} and SSEA4^{-} WJe-MSCs. There was no difference between SSEA4^{+} and SSEA4^{-} WJe-MSCs after histochemical staining with Alizarin red, even though WJe-MSCs were difficult to differentiate into osteoblasts, as previously reported. Induction of osteogenic differentiation in WJe-MSCs required a relatively high concentration (100 nM/ml) of BMP2 and a longer culture period of 5 weeks (n = 3) (Figure 9B).

6. Expression of SSEA4/3 in medium supplemented with different FBS

concentrations

I examined SSEA4/3 expression after 1 week in cultures containing 0.1%, 1, 10, and 20% FBS, which contains GSL. The cultured WJe-MSCs were all positive for CD73, and proliferation of WJe-MSCs was associated with FBS concentration (Figures 10A and B). The SSEA4 expression was positively correlated with the FBS concentration (Figure 10C), whereas SSEA3 expression was negatively correlated (Figure 10D). The WJe-MSCs were 15.8% ± 6.2% $SSEA4^+CD73^+$ and 5.8% ± 1.9% $SSEA3^+CD73^+$ in 0.1% FBS medium; 24.5% ±9.8% and 2.7% ±1.0% in 1% FBS; 41.5% ± 13.1% and 0.7% ± 0.5% in 10% FBS; and 48.3% ± 12.0% and 0.6% ± 0.4% in 20% FBS, respectively (n = 3).

To see that the increase in SSEA4 expression upon increasing FBS concentration is caused by the change in expression alone but not because of increased WJe-MSCs proliferation, I analyzed SSEA4 expression associated with growth curve with different FBS concentrations. In consistent with the prior data, SSEA4 expression was correlated with FBS concentration, while SSEA3 was inversely correlated (Figure 10E,F,G). The higher FBS concentration accelerated the proliferation of WJe-MSCs with higher expression of SSEA4 (Figure 10E, F). The incidence of $SSEA4^+$ and $SSEA3^+$ cells were highest on day 3, followed by the decline from day 3 to 7, still during proliferation. The possibility of the substrate shortage for SSEA4 and SSEA3 during culture could be denied, because I replaced the fresh medium on day4. The data are representative of three independent experiments.

Next, when the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs were cultured in 1%, 5%, and 10% FBS for 1 week, SSEA4 expression in the sorted $SSEA4^+$ WJe-MSCs was 4.74%

and 17.4% in 1% and 10% FBS medium, respectively, whereas SSEA4 expression in the sorted $SSEA4^{-}$ WJe-MSCs was 2.69% and 5.08% in 1% and 10% FBS, respectively (n = 3) (Figures 10H,I).

Furthermore, to clarify whether the phenomena of SSEA4 expression influenced by FBS concentration were limited to WJe-MSCs, I performed the same experiments using BM-MSCs derived from frozen-thawed MNCs. As shown in Figure 11A, BM-MNCs before culture included hematopoietic cells with low incidence of MSCs and also low incidence of SSEA4+ cells (Day 0). As expected, the proportion of $CD45^{+}$ hematopoietic cells expression declined during the passages, instead $CD45^{-}$ cells increased (Figure 11A). The latter cells expressed $CD45^{-}CD73^{+}$MSCs (Data not shown). However, in α-MEM with 10% FBS, I could not observe the predominant growth of $SSEA4^{+}$ cells regardless of CD45 expression (Figure 11A). After I established BM-MNCs, I studied the experiment about the correlation between FBS concentration and SSEA3/4 expression. In consistent with the results in WJ-MSCs, the SSEA4 expression in BM-MSCs (p2) was correlated with the FBS concentration, whereas SSEA3 expression was inversely correlated (Figure 11B,C,D). Day0 in Figure 11 B-D means the initiation day of P2 BM-MSCs . The relationship between the growth curve and SSEA4/3 expression was also consistent with the results in WJ-MSCs.

B. Immunosuppressive effects of WJ-MSCs

1. Mixed lymphocyte reactions

1.1. Immunosuppressive effects of WJ-MSCs on activated T cells in MLR

Next, I studied the immunosuppressive effect of WJ-MSCs on activated T cells in mixed lymphocyte reaction (MLR). T cells proliferated upon the stimulation by allogeneic DCs, while they were inhibited the proliferation by WJ-MSCs (Figure 12A,B). Autologous setting was mixed CB-MNCs as responder cells, DCs, and WJ-MSCs from the single donor. Allogeneic setting was consisted of the responder cells, DCs from different donor, and WJ-MSCs from the same donor of responder cells or the same donor of DCs. If the responder cells, DCs, and WJ-MSCs were from all different donors, I defined 3^{rd}-party setting.

In MLR, I found that WJ-MSCs efficiently inhibited the responder T cells derived from the same donor of WJ-MSCs, triggered by autologous; or allogeneic dendritic DCs (Autologous and allogeneic MLR) (Figure 13A,B). The responder T cells which proliferated in MLR included both $CD4^+$ and $CD8^+$ T cells, which were inhibited their proliferation by WJ-MSCs, respectively. Moreover, 3rd party-derived WJ-MSCs also strongly suppressed allogeneic responder CD4 and CD8 T cells proliferation triggered by allogeneic DCs (3rd party MLR) (n=3). The data were representative of three independent experiments. It indicated that WJ-MSCs immunosuppressive effect was not restricted by human leukocyte antigen (HLA).

1.2. Immunosuppressive effects of WJ-MSCs on separated activated T cells

WJ-MSCs have the immunosuppressive effect even in the 3^{rd} party setting. I

speculated that there might be some soluble factors secreted from WJ-MSCs upon the MLR stimulation. Next, I put the transwell chamber between the responder cells/DCs and WJ-MSCs. The proliferation of $CD4^+$ and $CD8^+$ T cells was determined by cell division. Compare with the MLR, WJ-MSCs decreased T cells division when separated from MLR, but it was not like the direct contact of WJ-MSCs and activated T cells (Figure 14A). WJ-MSCs also decreased T cells division upon PHA stimulation, whereas their inhibitory effects were distinctly attenuated by the blockade of cell to cell contact using the transwell chamber (Figure 14B).

1.3. Influence of WJ-MSCs supernatant on separated activated T cells

Next, I evaluated the immunosuppressive effect of WJ-MSCs supernatant on activated T cells stimulated by allogeneic DCs. After 4 days of culture, the WJ-MSCs supernatant slightly inhibit the $CD4^+$ and $CD8^+$ T cells division (Figure14C).

1.4. Influence of IDO inhibitor on immunosuppressive effects of WJ-MSCs

According to the above results, the immunosuppressive effect by WJ-MSCs may be induced by the soluble factors. IDO plays a critical role to immunosuppressive effect of BM-MSCs. Therefore I added the IDO inhibitor, 1MT at the dose of 0.1mM, 0.5mM, and 1mM to MLR co-cultured with WJ-MSCs. The proliferation activity of $CD4^+$ and $CD8^+$ T cells division was assessed by CFSE staining. The percentage of proliferating $CD4^+$ T cells after initial division was 29.7% at the dose of 0.1mM, 67.6% at 0.5mM IDO and 78.8% at 1mM IDO, respectively. The percentage of proliferation $CD8^+$ T cells after initial division was 31.3% at the dose of 0.1mM IDO, 59.5% at 0.5mM IDO and 66.4% at

1mM ID. The inhibitory effects of the WJ-MSCs on MLR were reversed by the addition of IDO inhibitor in a dose-dependent manner (Figure 15).

1.5 Influence of increased passages on immunosuppressive effects of WJ-MSCs

To see the influence of the increased passages on the immunosuppressive effect of WJ-MSCs, I compared the WJ-MSCs in the late passage (p10) with those in the early passage (p1) on the inhibition of MLR. After 4 days co-culture , the percentage of proliferating $CD4^+$ T cells after initial division was 32.9% and 36.4% and the percentage of 16.7% and 18.6% of $CD8^+$ T cells in R+S+WJe-MSCs P1 and P10, respectively. I demonstrated that the WJ-MSCs in the late passage could suppress MLR as well as those in the early passage (Figure 16).

2.Treatment of xeno-GVHD mice with WJ-MSCs

To see the immunosuppressive effect of WJ-MSCs on activated T cells in vivo, I induced xeno-GVHD in NOG mice by the injection of expanded human T cells that partially carried a luciferase transgene. Quantitative analysis using a xenogen IVIS 200 Imaging System visually demonstrated the elevation and spread of signals of xenogeneic-responsive T cells after injection. I found that WJ-MSCs suppressed the total luciferase activities of xenogeneic-reactive T cells and the mice survived relatively longer than those without WJ-MSCs injection, when the WJ-MSCs were injected to the mice on the day 0 of activated T cells injection(n=3). However, in the case of injection of WJ-MSCs on -1 day of activated T cells injection, the WJ-MSCs stimulated the xenogeneic-GVHD (n=3) (Figure 17A). Overall survival in the Tcells+MSCs (Day-1)

mice appeared inferior to that in the Tcells+MSCs (Day0) (Figure 17 B,C).

Immunohistological analysis of the recipient mice organs was performed to detect the human T cells stained by anti-human CD45 mAb. In the lung, liver, intestine and skin, human $CD45^+$ T cells were detected more in the T cells +MSCs (Day -1) group and T cells group rather than T cells +MSCs (Day 0) group. The histological examination demonstrated that the intestine in T cells +MSCs (Day -1) group showed effacement and blunting of the villous architecture, mucous cell depletion, and sloughing of epithelial cells, with patchy mucosal ulceration, while other two groups did not show (Figure 18).

V. Discussions

A. Significance of SSEA4 in WJ-MSCs

To gain insight into the role of SSEA3 and SSEA4 in UC WJ-MSCs, I examined the SSEA3 and SSEA4 expressions on WJ-MSCs obtained by different methods and compared the differentiation abilities of $SSEA4^+$ and $SSEA4^-$ cells.

First, I compared SSEA3 and SSEA4 expression in WJe-MSCs and WJc-MSCs. The explant method has several advantages. It does not require non-human collagenase derived from Clostridium histolyticum and save the time for lysing the tissue with enzyme in the procedure. The disadvantages of the explant method are that collecting adequate cell numbers is dependent on the amount of WJ tissue fragments that attach firmly to the bottom of the dish, which in turn, is dependent on the individual researcher's ability. Although the collagenase process from cutting the tissue to plating can be unified; I found that some samples are more sensitive to collagenase, resulting in reduced viability. We did not find any significant differences between WJe-MSCs and

WJc-MSCs in cells number, MSCs surface markers as defined by the ISCT, or ES cell-related gene expression at P0. In addition, the incidence of $SSEA4^+$ and $SSEA3^+$ at P0 was similar between the two types. However, SSEA3 disappeared rapidly in the early culture passages, as described previously[53, 31]. It is known that SSEA3 disappears more rapidly from the cell surface compared with SSEA4 if GSL synthesis is blocked by inhibitors, and a similar phenomenon has also been detected during ES cell differentiation[31, 54]. Interestingly, the incidence of WJe-MSCs $SSEA4^+$cells was reduced after the first passage, and by P7, the original levels were recovered. In contrast, the incidence of WJc-MSCs $SSEA4^+$ cells was relatively stable until P9. The reason for the depression of SSEA4 expression in WJe-MSCs at P1 is unknown. However, $SSEA4^+$ MSCs in both WJe-MSCs and WJc-MSCs did not proliferate predominantly in my culture medium. As previously reported, osteogenic differentiation was difficult with WJ-MSCs, requiring a longer induction period, additional cytokines such as BMP2, and specific FBS concentrations. However, both sorted $SSEA4^+$ and $SSEA4^-$ WJ-MSCs eventually differentiated into osteocytes and adipocytes in a similar manner, and there were no differences in ES-marker gene expression between the $SSEA4^+$ and $SSEA4^-$ MSCs. Interestingly, $SSEA4^+$ cells appeared even from the $SSEA4^-$ MSCs, and the incidence of $SSEA4^+$ cells derived from the $SSEA4^-$ MSCs demonstrated a similar transition pattern as those derived from the $SSEA4^+$ MSCs. This result suggested that the culture medium may have been the source of SSEA4 antigens.

The role of SSEA3 and SSEA4 in MSCs remains controversial. Rovstoskaya et al. also suggested SSEA4 marked adipogenic progenitor lacking osteogenic capacity[55]. In ES cell study, Ramirez et al. demonstrated that both SSEA3 and SSEA4 are markers of immature

ES cells, but particularly SSEA3 together with OCT4 and TRA-1-60 were good tracers for validating pluripotent stem cells, whereas SSEA4 was expressed for long during the differentiation of ES cells[53]. Gang et al. reported that $SSEA4^+$ cells proliferated predominantly when the culture was initiated from primary BM cells, which were mostly hematopoietic cells[28]. But in my culture condition, the incidence of $SSEA4^+$ cells, which were characterized as MSCs, was not increased dramatically as reported by Gang's group. Their results might be induced by the special cocktail of the medium, consisted of MCDB-201, 10% FBS, ITS, linoleic acid–bovine serum albumin, dexamethasone, ascorbic acid, hPDGF-BB, and hEGF, followed by the medium with relatively high FBS concentration. In other papers, Schrobback et al. assessed the SSEA4 in human articular chondrocytes, osteoblasts in BM-derived MSCs and characterized their differentiation potential. But their results showed that SSEA4 levels in these cells were unrelated to the cells' chondrogenic and osteogenic and proliferation potentials *in vitro*[56]. Suila et al. reported that SSEA4, and not SSEA3, was expressed on the surface of cord blood-derived MSCs, whereas SSEA3 was expressed at very low levels in cord blood hematopoietic stem cells[57]. They also suggested that FBS contains detectable amounts of globoseries GSLs and showed that the SSEA3 was influenced and upregulated by culturing with FBS overnight, even though they did not demonstrate an influence on SSEA4 expression. In my study, I demonstrated that SSEA4 expression significantly correlated with FBS concentration, whereas SSEA3 appeared to be negatively correlated with FBS concentration. The possibility that FBS stimulated the proliferation of WJe-MSCs, resulting in the increase of SSEA4 expression, cannot be denied completely. But the fact that the SSEA4 expression was declined during the proliferation of

WJe-MSCs and BM-MSCs in each medium did not support this hypothesis. Regardless of cell growth or cell concentration, consistently, the SSEA4 expressions was clearly associated with FBS concentration not only in WJ-MSCs but also in BM-MSCs. Reversely to the SSEA4 expression, SSEA3 expression appeared to be negatively correlated with FBS concentration. The reason why the data of SSEA3 elevation upon the higher FBS concentration are not coincident with Suila's data remained unresolved. I add a partial speculation that SSEA4 is derived from SSEA3, thus the increase of SSEA4 means the waste of SSEA3, or MSCs with high concentration of FBS are differentiated accompanied with decrease of SSEA3. But I need the scrupulous attention to analyze SSEA3 by flow cytometry in various FBS concentration. We also found that SSEA4 could be induced from pure $SSEA4^{-}$ WJ-MSCs. This suggests the presence of substrate for SSEA4 in $SSEA4^{-}$ cells and also the influence of FBS on the SSEA4 expression in $SSEA4^{-}$ cells. Brimble et al. reported that the depletion of these two molecules by the addition of GSL synthesis inhibitors apparently did not affect the ES cells pluripotency[31]. In conclusion, these results indicate that SSEA4 may display altered expression profiles in response to culture medium including FBS and may not be an essential marker of WJ-MSCs multipotency.

B. Immunosuppressive effects of WJ-MSCs

Concerning the immunomodulatory effects of MSCs, they have the ability to migrate into inflammatory sites and to suppress adverse immune reactions. In fact, BM-derived MSCs have already been applied to treat aGVHD with promising efficacy[20 58]. However, the mechanism by which MSCs including WJ-MSCs exert their immunosuppressive

effects is not completely understood. Here, I demonstrated that WJ-MSCs have the immunosuppressive effect in autologous, allogeneic and 3rd party MLR setting. indicating that this effect is not MHC-restricted. In addition, I observed the MLR-inhibitory effect of WJ-MSCs was partly mediated by soluble factors included in their culture supernantant and diffusible through transwell chambers, although its activity was more potent in the presence of cell to cell contact. The MLR-inhibitory effect of WJ-MSCs was reversed by the addition of IDO inhibitor, 1-MT in a dose dependent manner, suggesting that soluble IDO may play an important role in MLR inhibition.

In recent papers, BM-derived MSCs secreted soluble factors such as HLA-G, TGF-β, PGE2, IDO, and IL6 and additional factors may contribute to their immunosuppressive activity, especially in the presence of direct contact between MSCs and activated T cells. Furthermore, I am now trying to confirm the immunosuppressive effect of WJ-MSCs in immunodeficient mice developing xenogeneic GVHD. However, in some experiments, administration of WJ-MSCs resulted in attenuation of GVHD and life extension of mice, but in others, they caused exacerbation of GVHD. It is recently suggested that the inflammatory environment, affected by the level of cytokines such as IFN-γ and TNF-α, might alter the biologic feature of MSCs from immunosuppression to immunostimulation. In recent papers, BM-MSCs-mediated immunosuppression requires preliminary activation by immune cells through the secretion of the proinflammatory cytokines[59,60], such as tumor necrosis factor-alpha (TNF-α)[61], interferon-gamma (IFN-γ)[62], interleukin-10(IL10)[63], IL-1α or IL-1β[37] (Figure 2). Actually, to stabilize the suppressive function of WJ-MSCs, priming those with IFN-γ and TNF-α is recommended prior to their administration.

VI. Conclusions

WJ-MSCs could be efficiently obtained by explants method. SSEA4 may display altered expression profiles in response to culture medium including FBS and may not be an essential marker of WJ-MSCs multipotency. WJ-MSCs inhibited T cells proliferation, and IDO is one of the critical immunosuppressive factors in WJ-MSCs. In order to do the regenerative and immunosuppressive therapy using WJ-MSCs effectively and safely, further investigations of the mechanisms on multipotency and immunomodulatory effect are necessary.

VII. References

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VIII. Figures and Tables

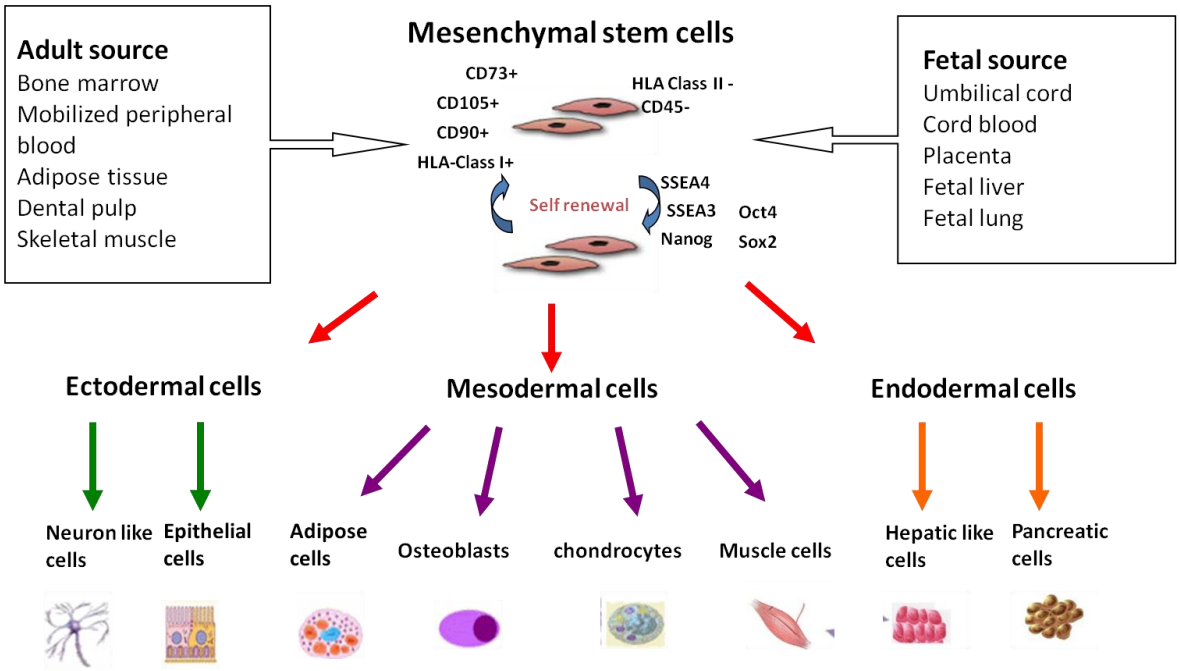

Figure 1: Characteristics of mesenchymal stem cells.
Mesenchymal stem cells (MSCs) can be isolated from adult or fetal tissue sources. MSCs are positive CD73, CD105, CD90, and HLA class I, but negative for CD45, HLA Class II. MSCs have the potentials of self-renew and multipotency to differentiating into not only mesodermal cells, but also ectodermal and endodermal cells in the previous papers.

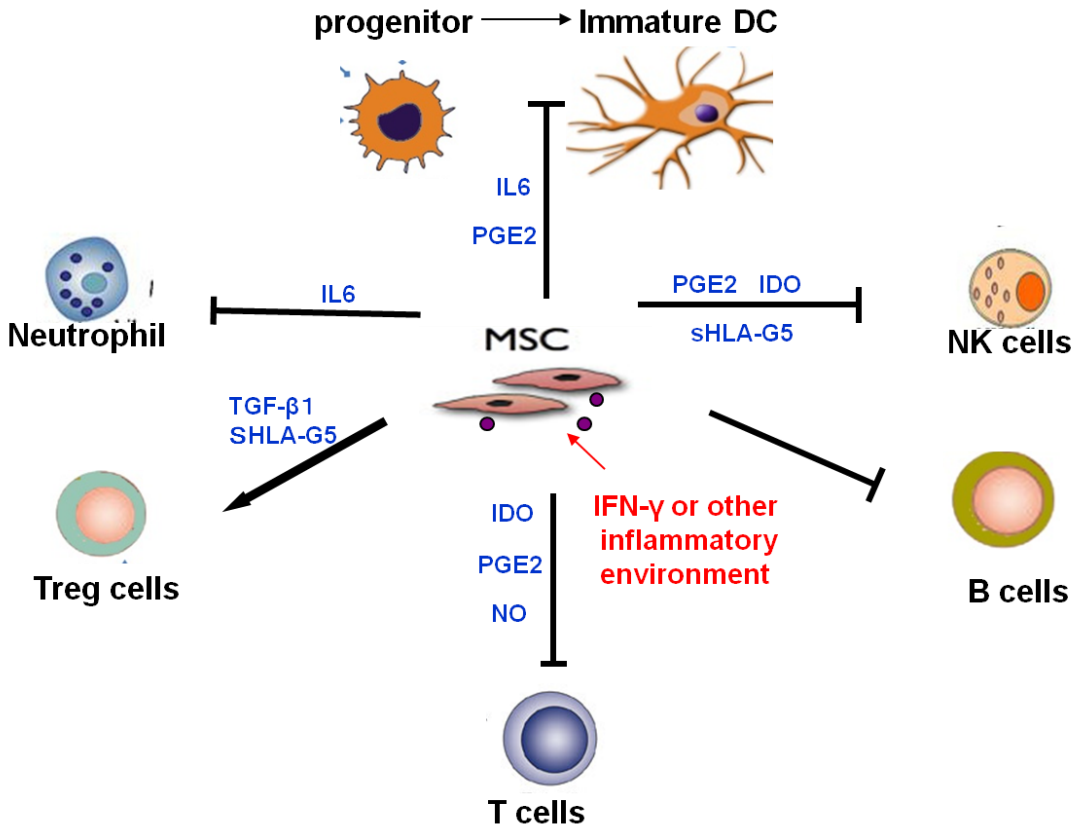

Figure 2: Potential mechanisms of the MSCs interactions with immune cells.
MSCs immunosuppressive effects are mediated by cell-cell contact with target cell and MSCs secreted soluble factors such as IDO, PGE2, NO etc. Production of these mediators regulates the proliferation and function of a variety of immune cells including regulatory T (Treg) cells, B cells, Natural Killer (NK) cells, and neutrophils.

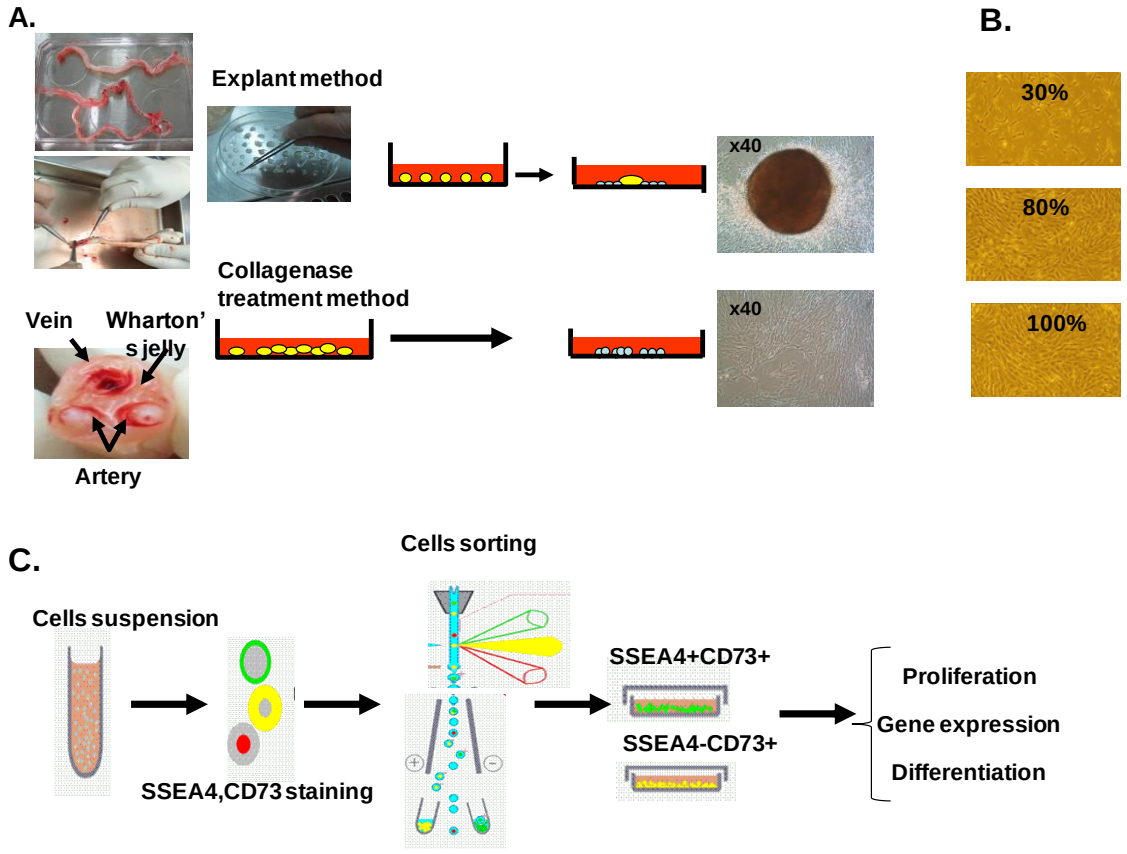

Figure 3: Umbilical cord Wharton's jelly (WJ)-derived mesenchymal stem cells (MSCs).

A. MSCs were collected from WJ tissue by the explant method (WJe-MSCs) and by the collagenase-treatment method (WJc-MSCs). Photographs of migrating cells from the minced tissue in the explant method and adherent cells from the scattered cells in the collagenase-treatment method are shown. **B.** Morphology of WJ-MSCs at the indicated confluences. **C**. $SSEA4^{+}$ and $SSEA4^{-}$WJ-MSCs were sorted using FACS Aria.

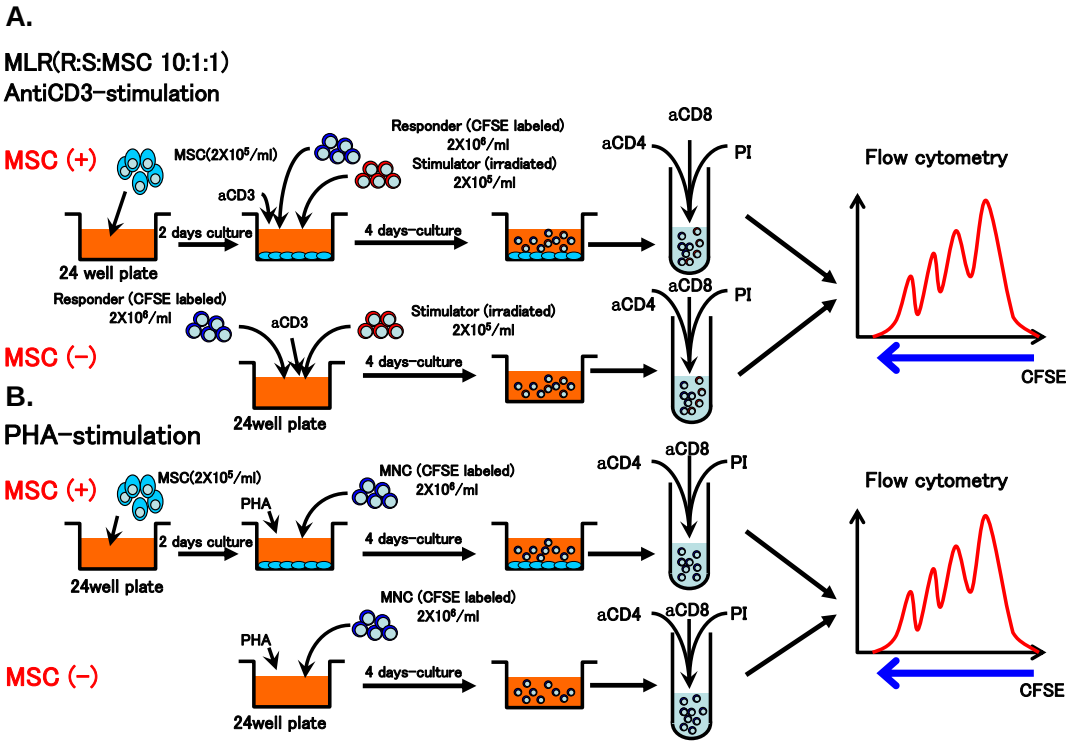

Figure 4: The method of MLR and CFSE labeled responder cells analyzed by flow cytometry.

A. WJ-MSCs cultured in 24well plate, 2 days ahead of MLR performed with or without WJ-MSCs .The CFSE labeled $CD4^+$ and $CD8^+$ T cells division checked by flow cytometry. **B.** Responder cells performed with or without WJ-MSCs .The CFSE labeled CD4 and CD8 responder cells division checked by flow cytometry.

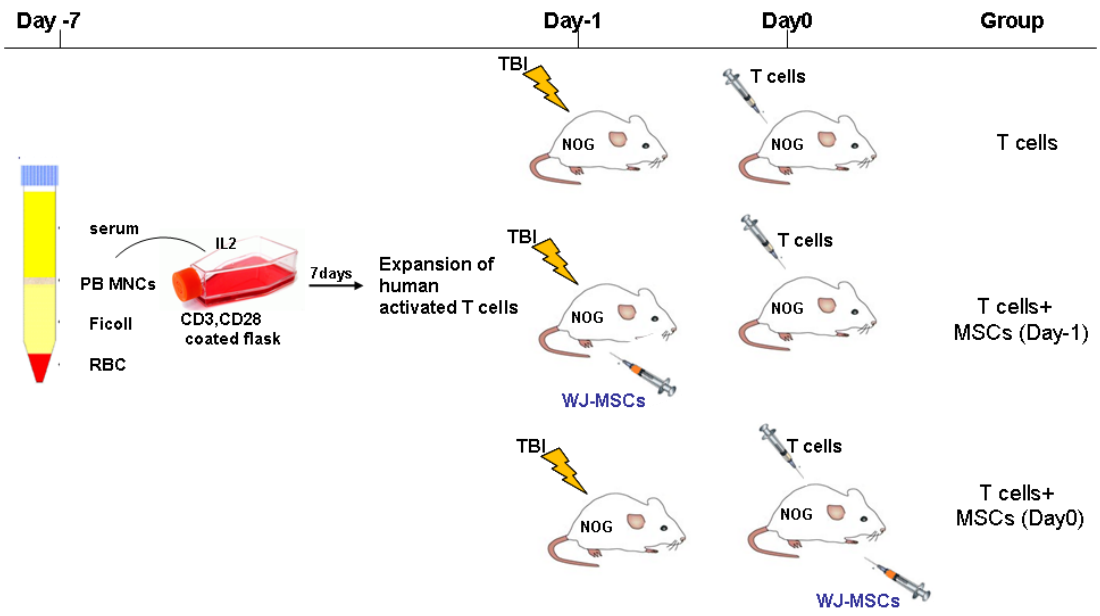

Figure 5: The protocol of xeno-GVHD model.
Activated T cells were expanded in anti-CD3 and CD28 mAb coated flask and cultured for 7 days in Alys505N IL2 medium supplemented with 5% FBS. After 2.5 Gy total body irradiation (TBI), NOG mice were received the injection with 2 x 10^7 luc-transduced T cells on Day 0. According to the initial injection of WJ-MSCs, the mice were divided into 3 groups; control group (n = 4):T cells, treatment group with $1x10^6$ WJ-MSCs on Day -1 (n = 3): T cells + MSCs (Day -1), and treatment group with $1x10^6$ WJ-MSCs from Day 0 (n = 3): T cells + MSCs (Day 0).

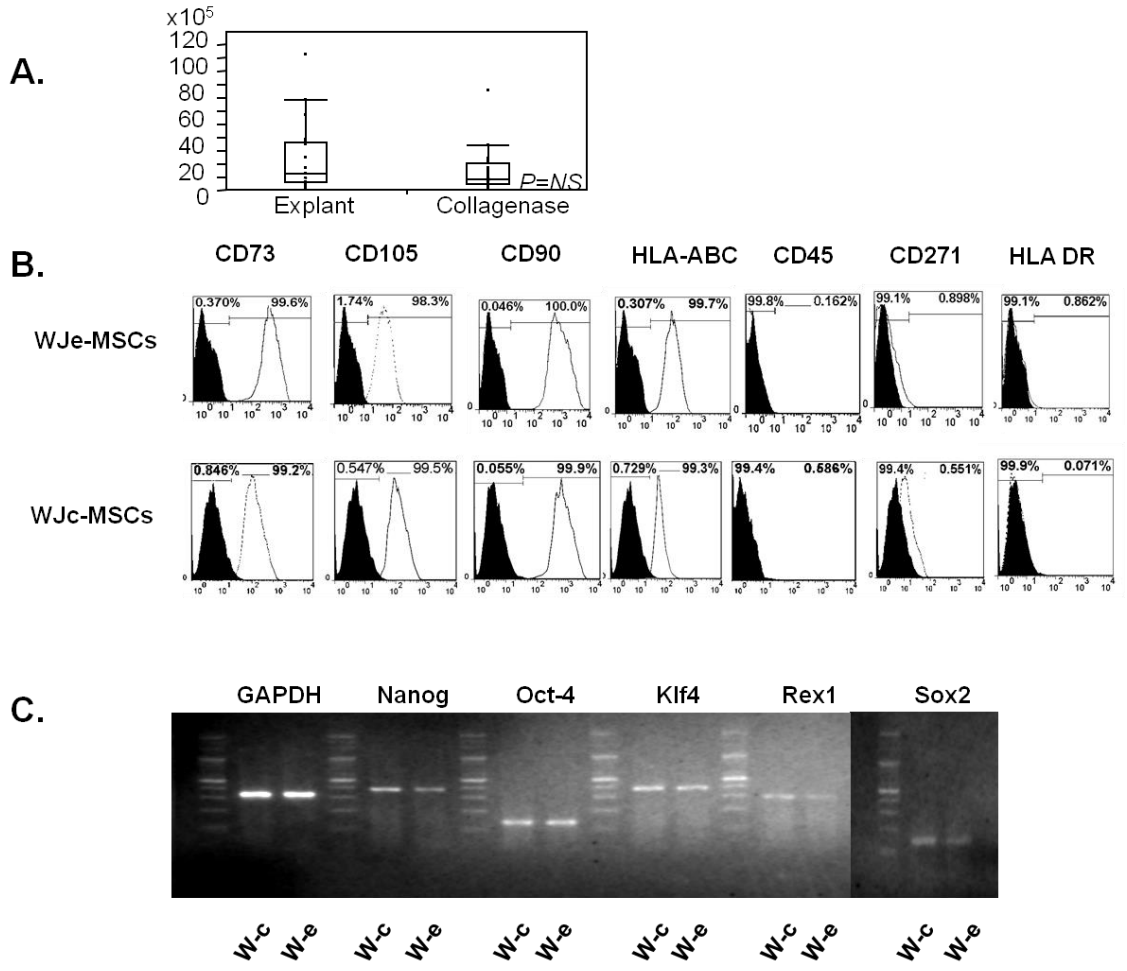

Figure 6: Characterization of Wharton's jelly (WJ)-derived mesenchymal stem cells (MSCs) collected by the explant (WJe-MSCs) and the collagenase-treatment methods (WJc-MSCs).

A. Median cell numbers collected by the explants method (n=3) and the collagenase-treatment method are shown (n = 20). **B.** Both WJe-MSCs and WJc-MSCs were positive for CD73, CD90, CD105, CD271, and HLA class I and negative for CD45 and HLA class II. **C.** Expression of embryonic stem cell-related markers Nanog, Oct4, Klf4, Rex1, and Sox2 are shown. The data are representative of three independent experiments.

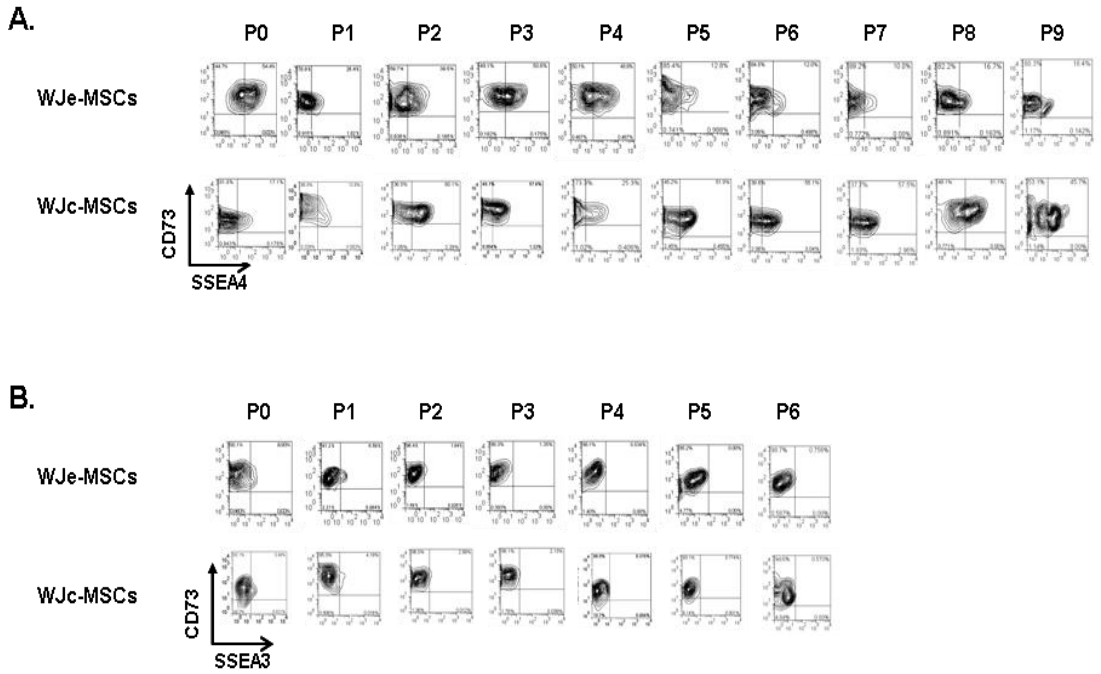

Figure 7: Stage-specific embryonic antigen SSEA4 expression during passages.
A. SSEA4 and CD73 expressions were monitored in Wharton's jelly (WJ)-derived mesenchymal stem cells (MSCs) collected by the explant (WJe-MSCs) and the collagenase-treatment methods (WJc-MSCs) during P0–P9. **B.** SSEA3 and CD73 expressions were periodically monitored in WJe-MSCs and WJc-MSCs during P0–P6.

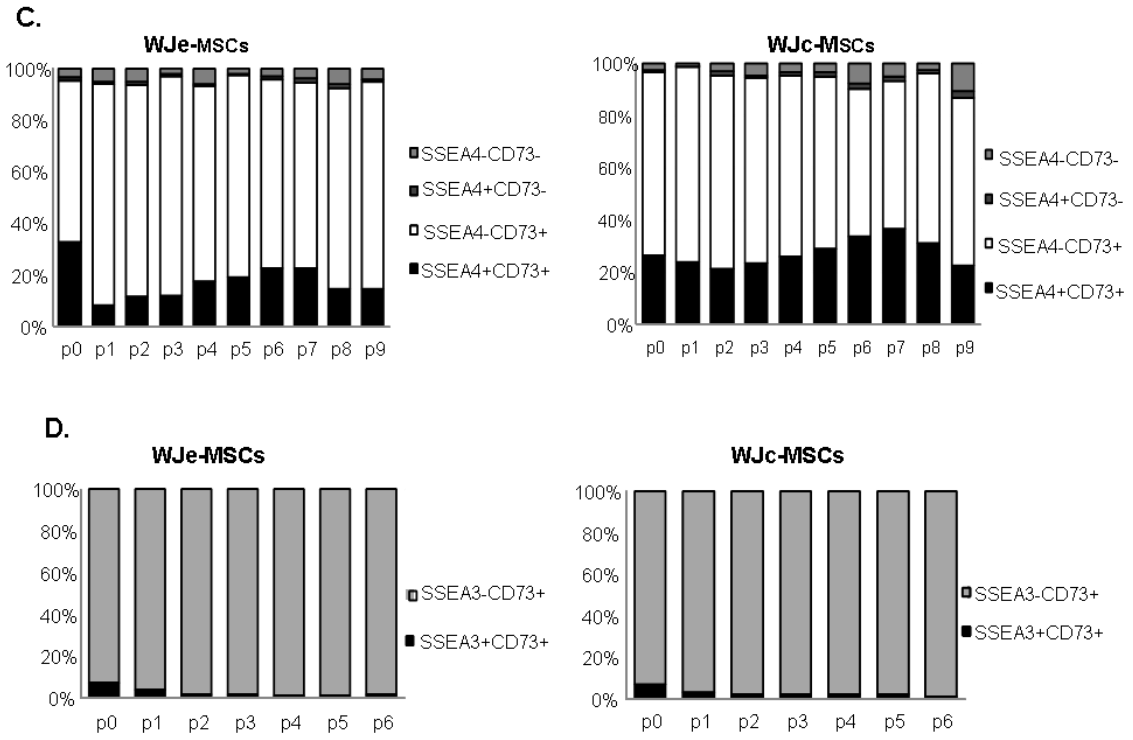

Figure 7: Stage-specific embryonic antigen SSEA4 expression during passages. *(Continued)*

C. The mean incidence of SSEA4 and CD73 in WJe-MSCs and WJc-MSCs is shown. **D.** The mean incidence of SSEA3 in $CD73^{+}$ WJe-MSCs and WJc-MSCs is shown. The data are representative of eight independent experiments.

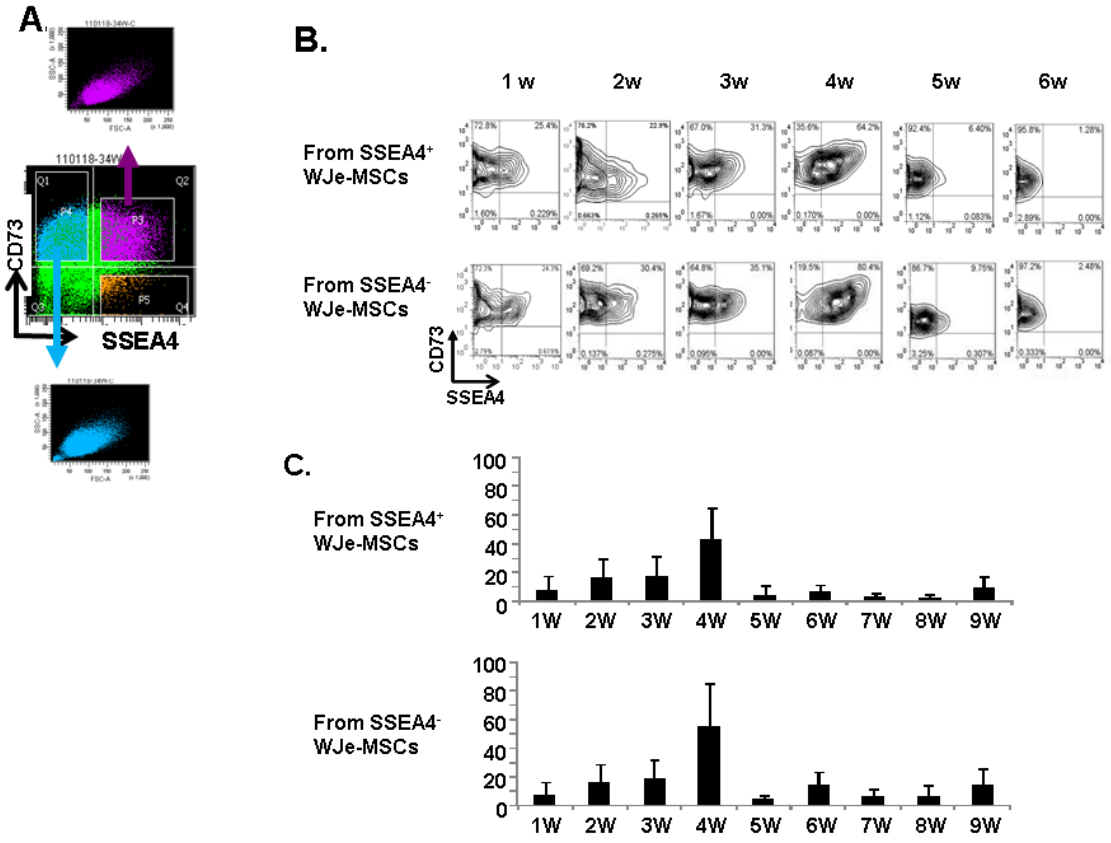

Figure 8: Sorted stage-specific embryonic antigen SSEA4$^+$ and SSEA4$^-$ Wharton's jelly-derived mesenchymal stem cells collected by the explant method (WJe-MSCs). **A.** SEA4$^+$CD73$^+$ WJe-MSCs and SSEA4$^-$CD73$^+$ WJe-MSCs were sorted by FACS Aria. **B.** SSEA4 and CD73 expression in sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs during passages 1–6 weeks (w) are shown. **C.** The percentages of SSEA4$^+$ cells in the sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs during weeks 1–9 are shown (n = 3).

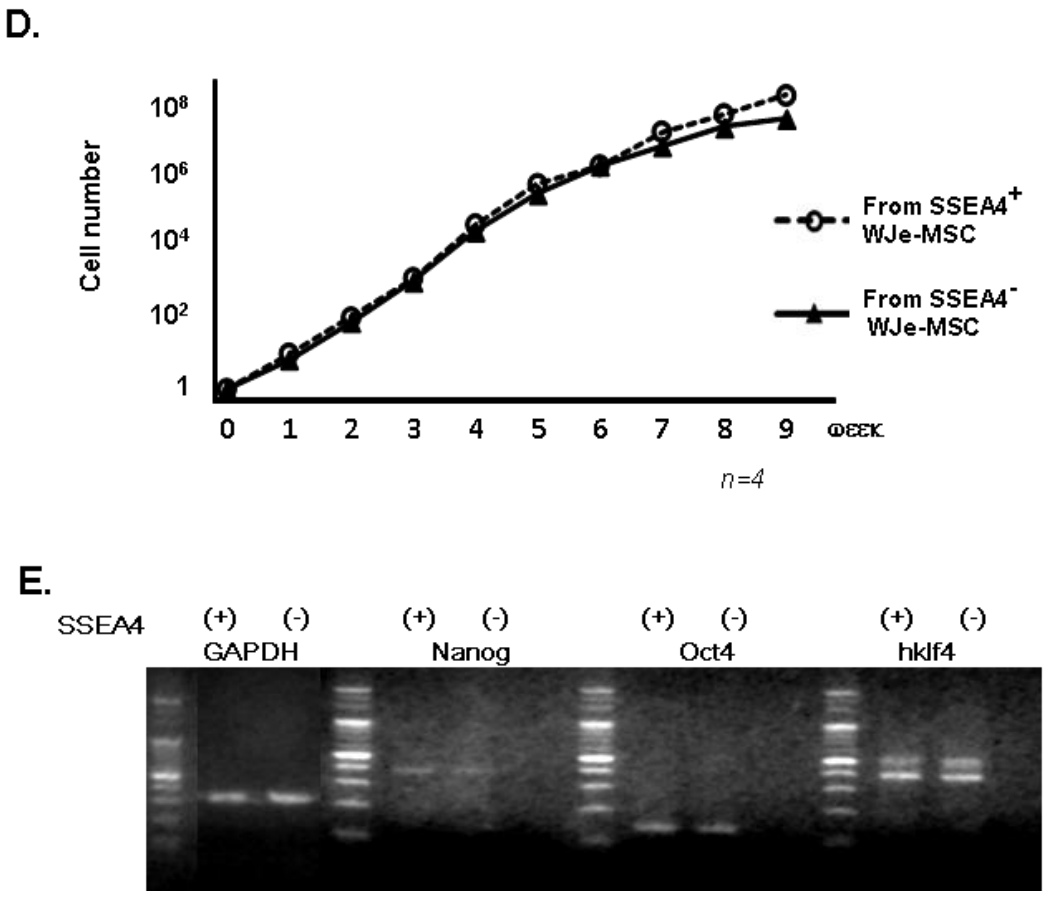

Figure 8: Sorted stage-specific embryonic antigen SSEA4$^+$ and SSEA4$^-$ Wharton's jelly-derived mesenchymal stem cells collected by the explant method (WJe-MSCs). *(Continued)*

D. Growth curves of the sorted SSEA4$^+$ (open circles) and SSEA4$^-$ (closed triangles) WJe-MSCs over 9 weeks are shown. **E.** Gene expression analysis of the sorted SSEA4$^+$ and SSEA4$^-$ WJe-MSCs showed that both were positive for *Oct4, Nanog, and Klf4*.

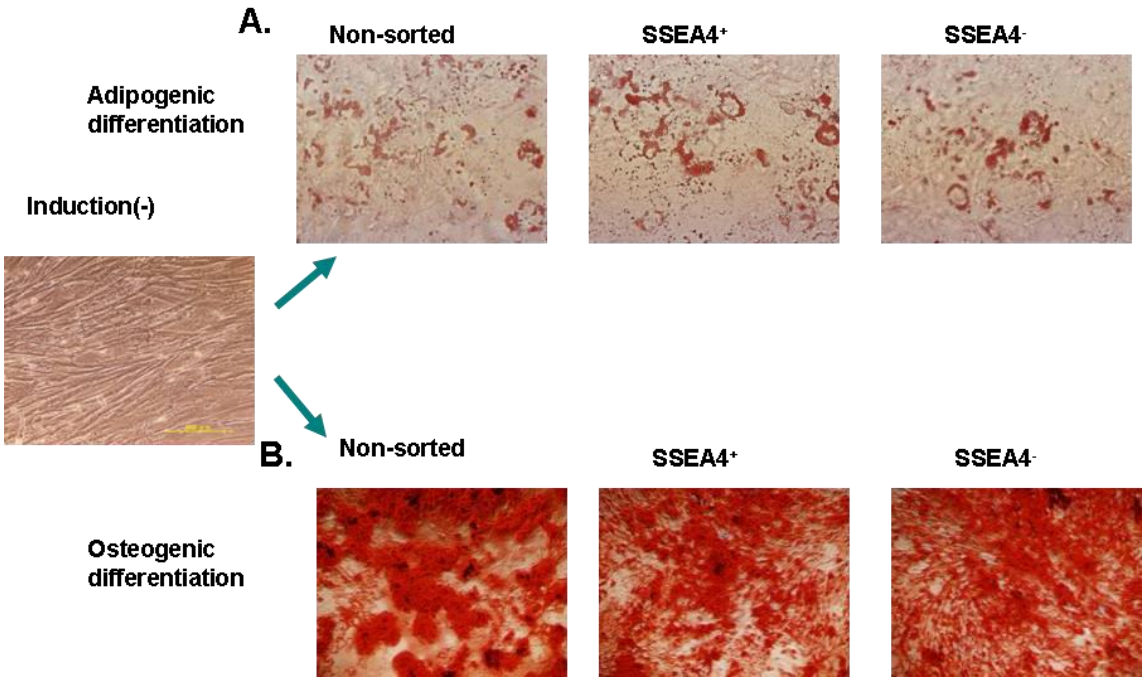

Figure 9: Adipogenic and osteogenic differentiation of sorted stage-specific embryonic antigen SSEA4$^+$ and SSEA4$^-$ Wharton's jelly-derived mesenchymal stem cells collected by the explant method (WJe-MSCs).
A. Adipogenic differentiation of non-sorted and SSEA4$^+$ and SSEA4$^-$ sorted WJe-MSCs is shown by Red O-stained lipid drops at 3 weeks. **B.** Osteogenic differentiation of unsorted and SSEA4$^+$- and SSEA4$^-$-sorted WJe-MSCs are shown.

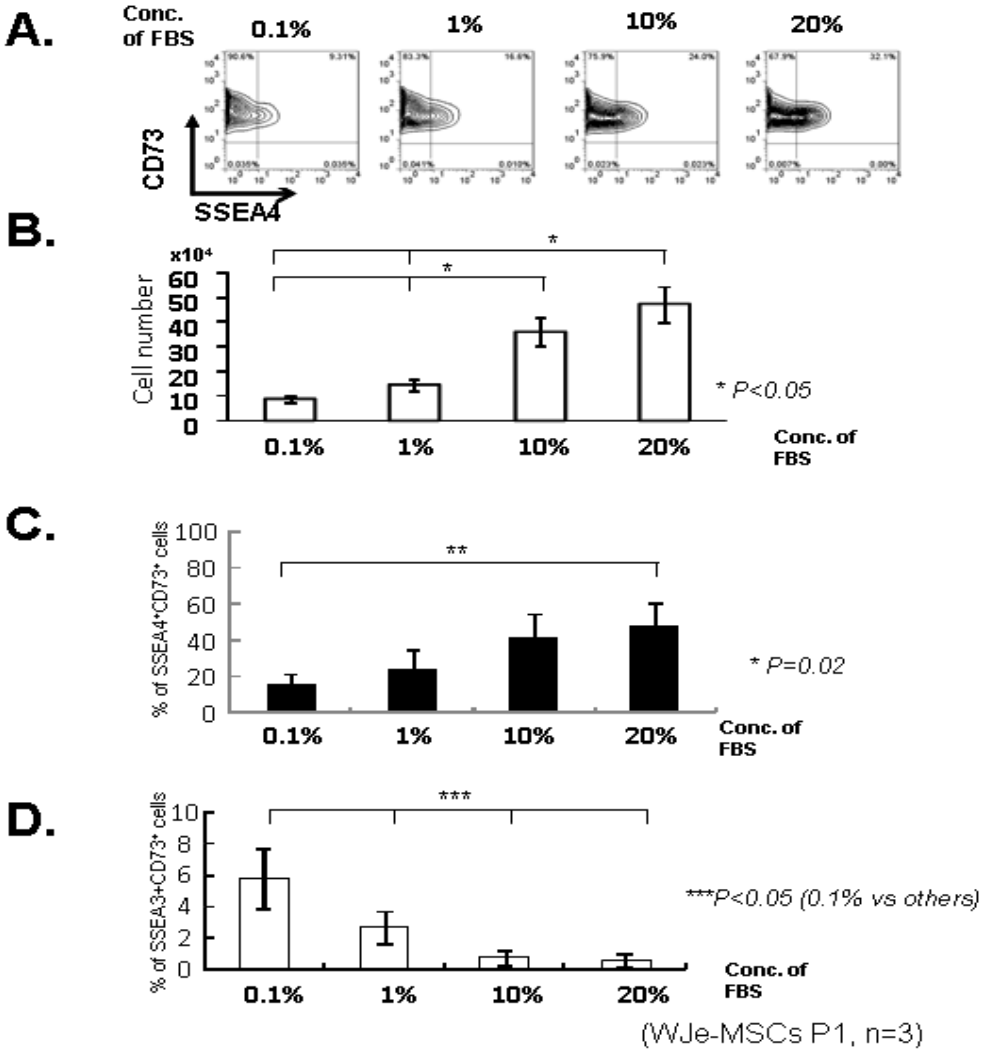

Figure 10: Influence of fetal bovine serum (FBS) on stage-specific embryonic antigen SSEA4 and SSEA3 expression.

A. Wharton's jelly-derived mesenchymal stem cells collected by the explant method (WJe-MSCs) were cultured for 1 week in medium containing 0.1%, 1%, 10%, and 20% FBS, and SSEA4 expression patterns were analyzed by flow cytometry. The data are representative of three independent experiments. **B.** WJe-MSCs cell numbers in medium with different concentration of FBS were determined. **C, D.** Percentages of $SSEA4^+$ and $SSEA3^+$ cells, respectively, among the WJe-MSCs in medium with different concentrations of FBS were determined.

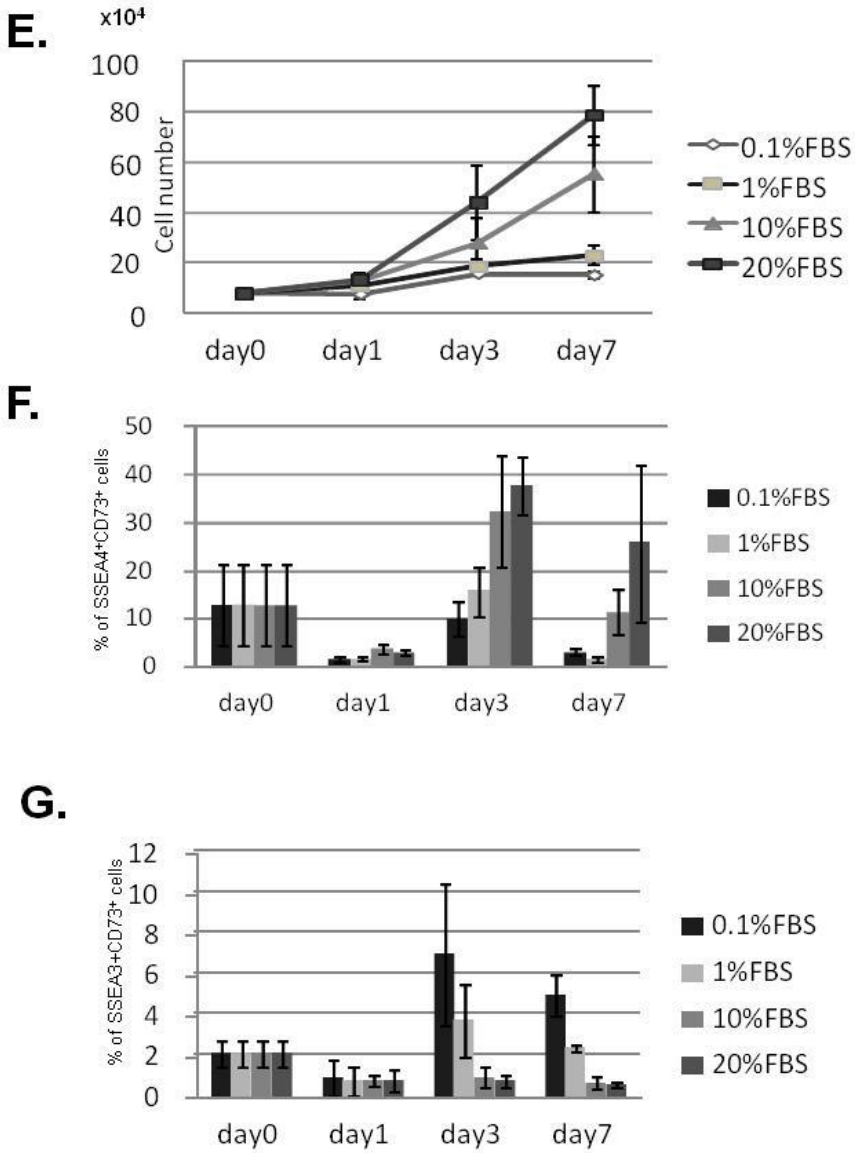

Figure 10: Influence of fetal bovine serum (FBS) on stage-specific embryonic antigen SSEA4 and SSEA3 expression. *(Continued)*

E. Growth curves of WJe-MSCs with different concentration of FBS. **F, G.** Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of WJe-MSCs, respectively.

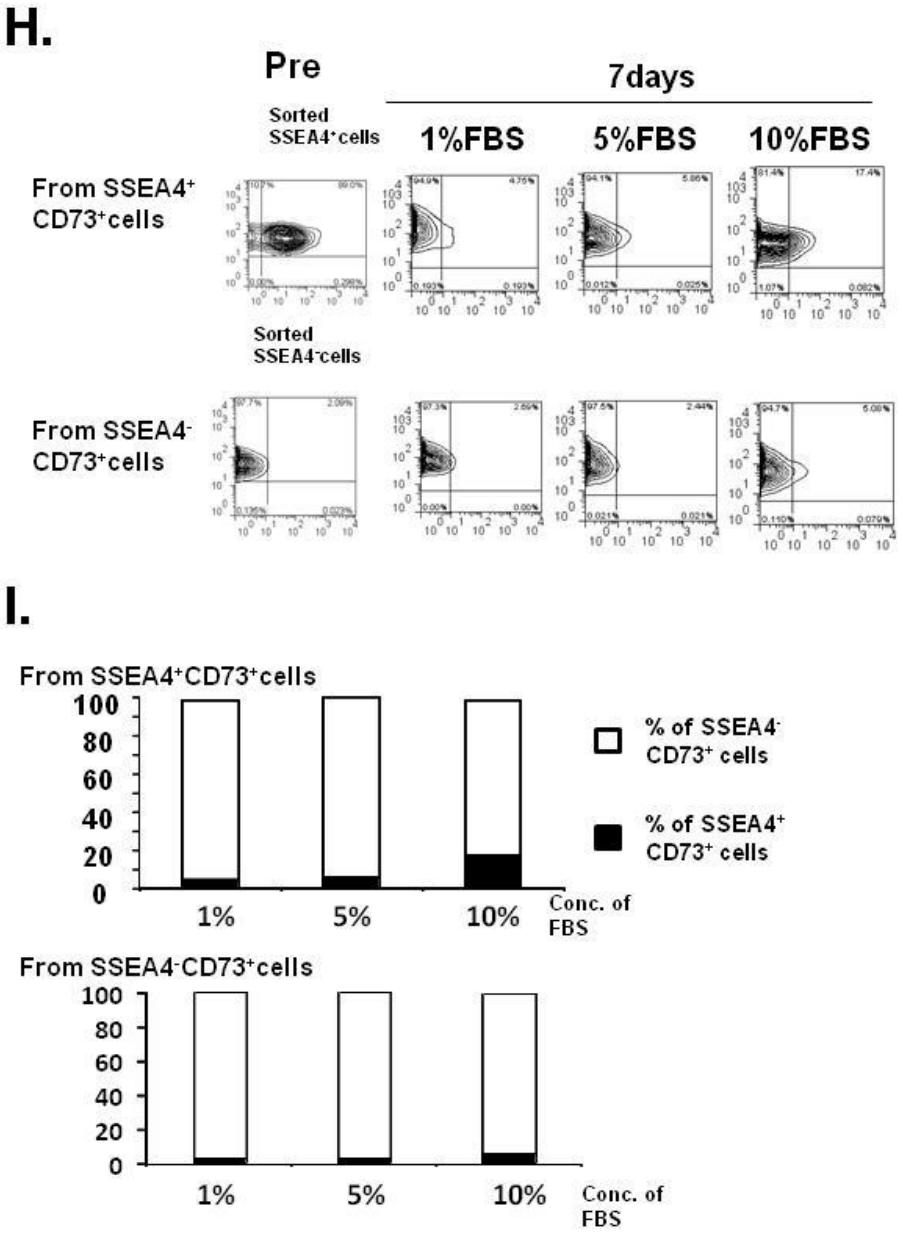

Figure 10: Influence of fetal bovine serum (FBS) on stage-specific embryonic antigen SSEA4 and SSEA3 expression. *(Continued)*

H, I. Flow cytometry patterns and percentages of SSEA4 expression in the sorted $SSEA4^+$ and $SSEA4^-$ WJe-MSCs in medium with 1%, 5%, and 10% FBS were determined.

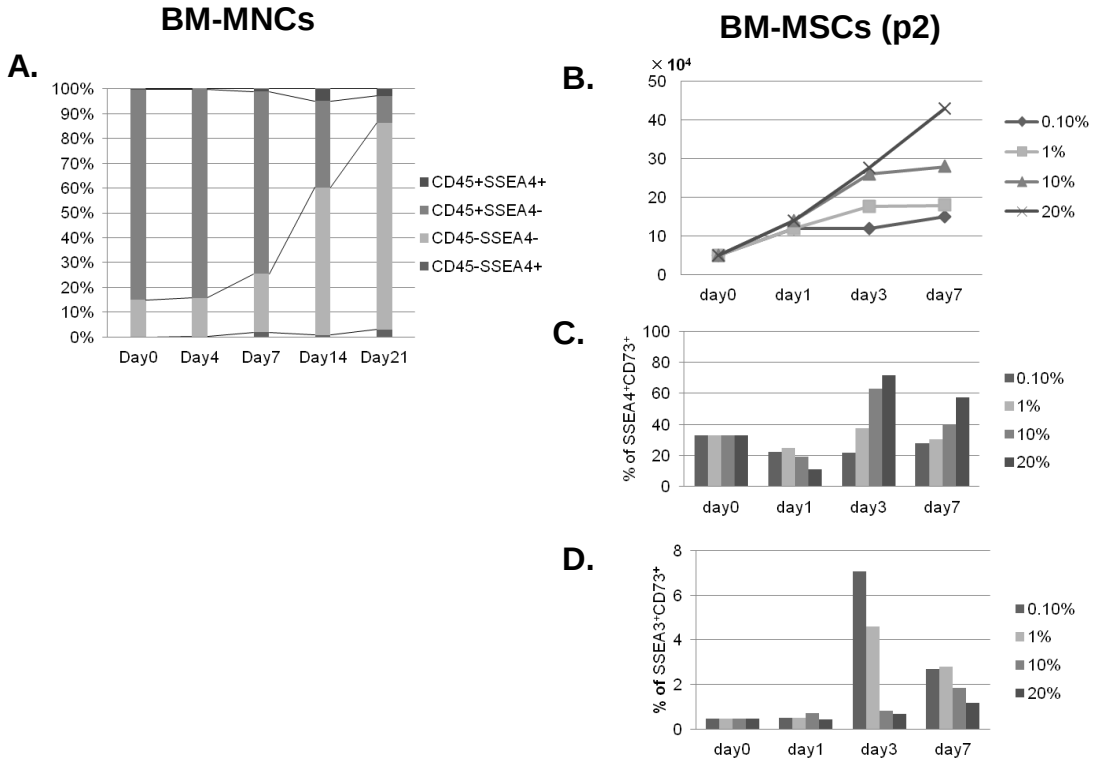

Figure 11: Influence of fetal bovine serum (FBS) on SSEA4 and SSEA3 expression in bone marrow-derived mesenchymal stem cells (BM-MSCs).
A. Shift of the proportion of $CD45^+$ hematopoietic cells and $CD45^-$ cells with or without SSEA4 expression of BM-MNCs during the passages. **B**. Growth curves of BM-MSCs (p2) with different concentration of FBS. **C,D**. Percentage of $SSEA4^+$ and $SSEA3^+$ cells during the proliferation of BM-MSCs, respectively. The data are representative of two independent experiments.

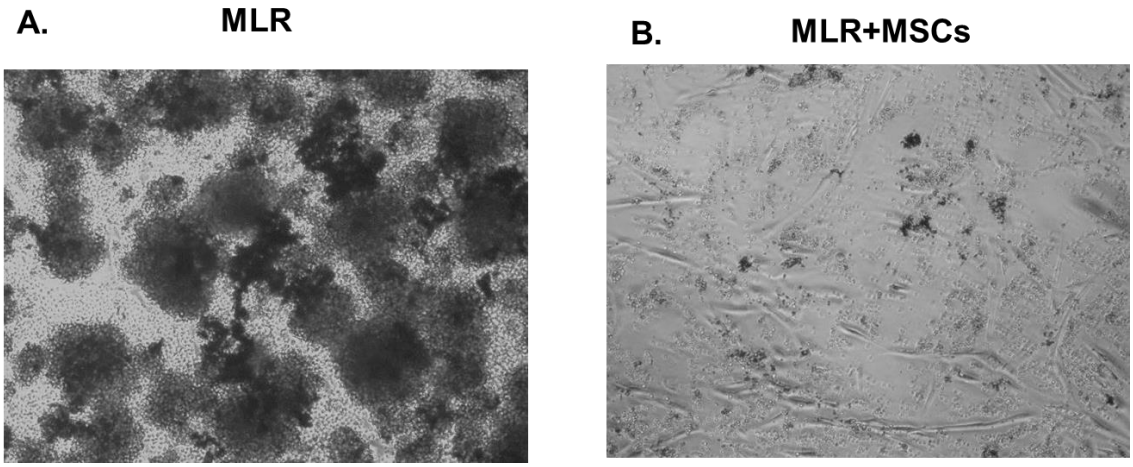

Figure 12: Photomicrograhs of MLR with or without WJ-MSCs.

Responder T cells were stimulated with allogeneic DCs plus anti-CD3 mAb and cultured without **(A**) or with WJ-MSCs **(B)**. After 4 days, a photomicrograph was taken.

	1	2	3	4	5
R: MNCs	CB	CB	CB	CB	PB
S: DCs	Auto-CB	Allo-PB	Allo-CB	Allo-CB	Allo-CB
WJ-MSCs	Auto	Auto	Allo	3rd	3rd

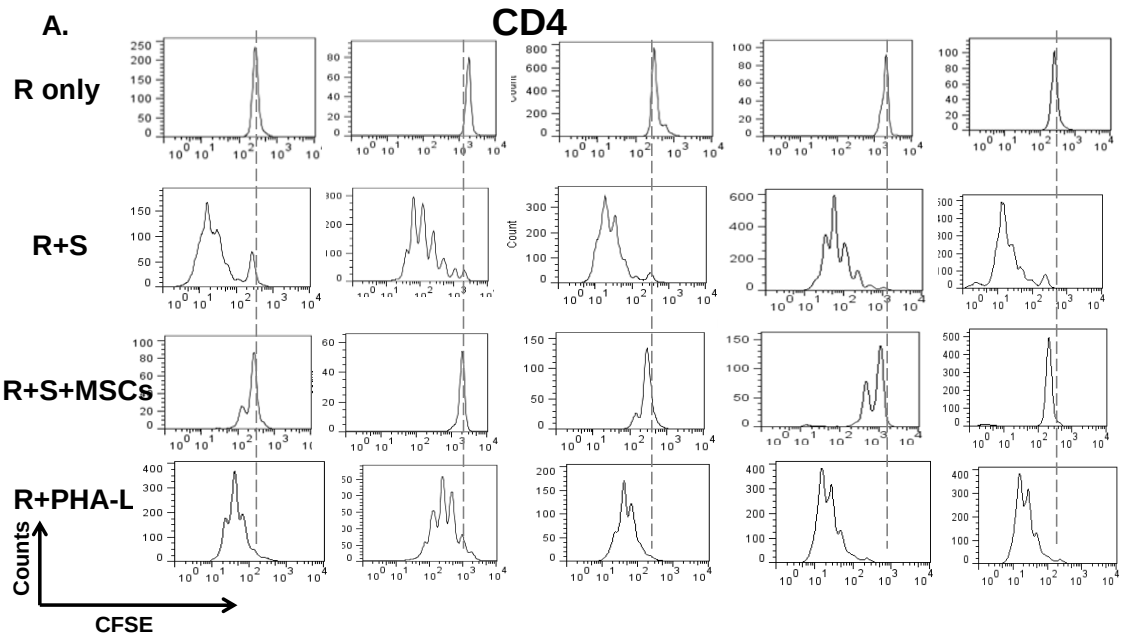

Figure 13: WJ-MSCs suppressed T cells proliferation in autologous, allogeneic and 3rd party MLR.

A. CFSE-labeled MNCs (R) were cultured in 4 different conditions; 1. Control, 2. Co-culture with DCs (S) plus anti-CD3 mAb, 3. Co-culture with DCs (S) and WJ-MSCs, 4. Stimulation with PHA-L. After 4 days, cells divisions defined by CFSE intensity were analyzed by flow-cytometry in a CD4 plot. The donor of each MLR component was denoted in the upper panel.

(R: responder cells; S: stimulator cells; Auto: autologous; Allo: allogeneic; PB: peripheral blood; CB: cord blood; MNC: mononuclear cells; DC: dendritic cells)

	1	2	3	4	5
R: MNCs	CB	CB	CB	CB	PB
S: DCs	Auto-CB	Allo-PB	Allo-CB	Allo-CB	Allo-CB
WJ-MSCs	Auto	Auto	Allo	3rd	3rd

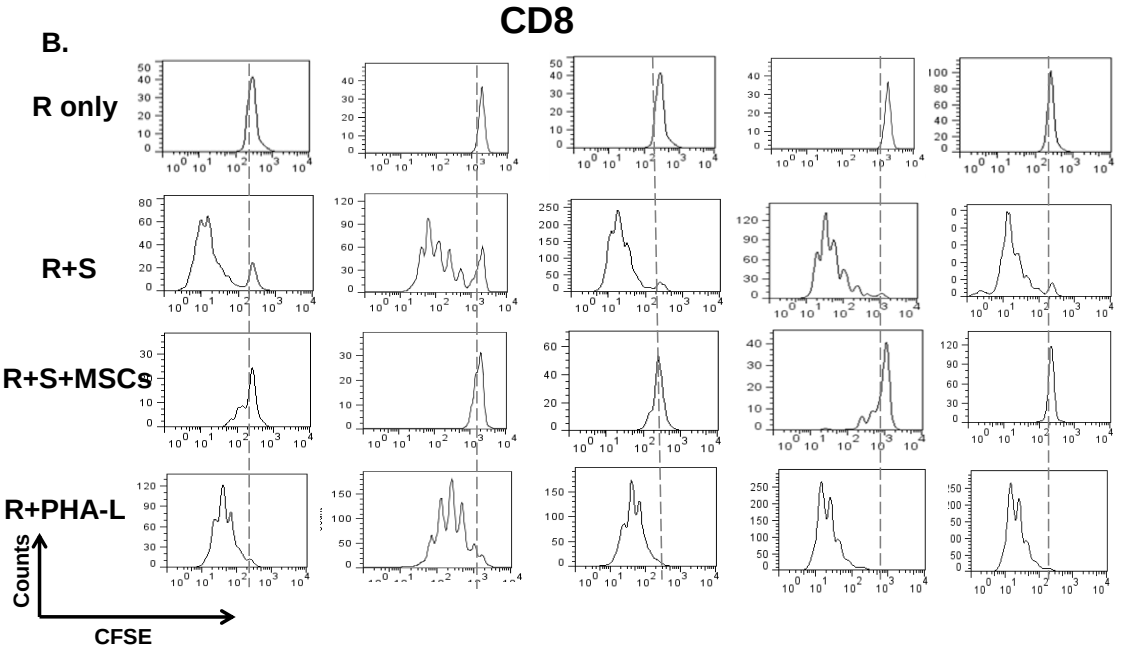

Figure 13: WJ-MSCs suppressed T cells proliferation in autologous, allogeneic and 3rd party MLR. *(Continued)*

B. CFSE-labeled MNCs (R) were cultured in 4 different conditions; 1. Control, 2. Co-culture with DCs (S) plus anti-CD3 mAb, 3. Co-culture with DCs (S) and WJ-MSCs. 4. Stimulation with PHA-L. After 4 days, cells divisions defined by CFSE intensity were analyzed by flow-cytometry in a CD8 plot. The donor of each MLR component was denoted in the upper panel.

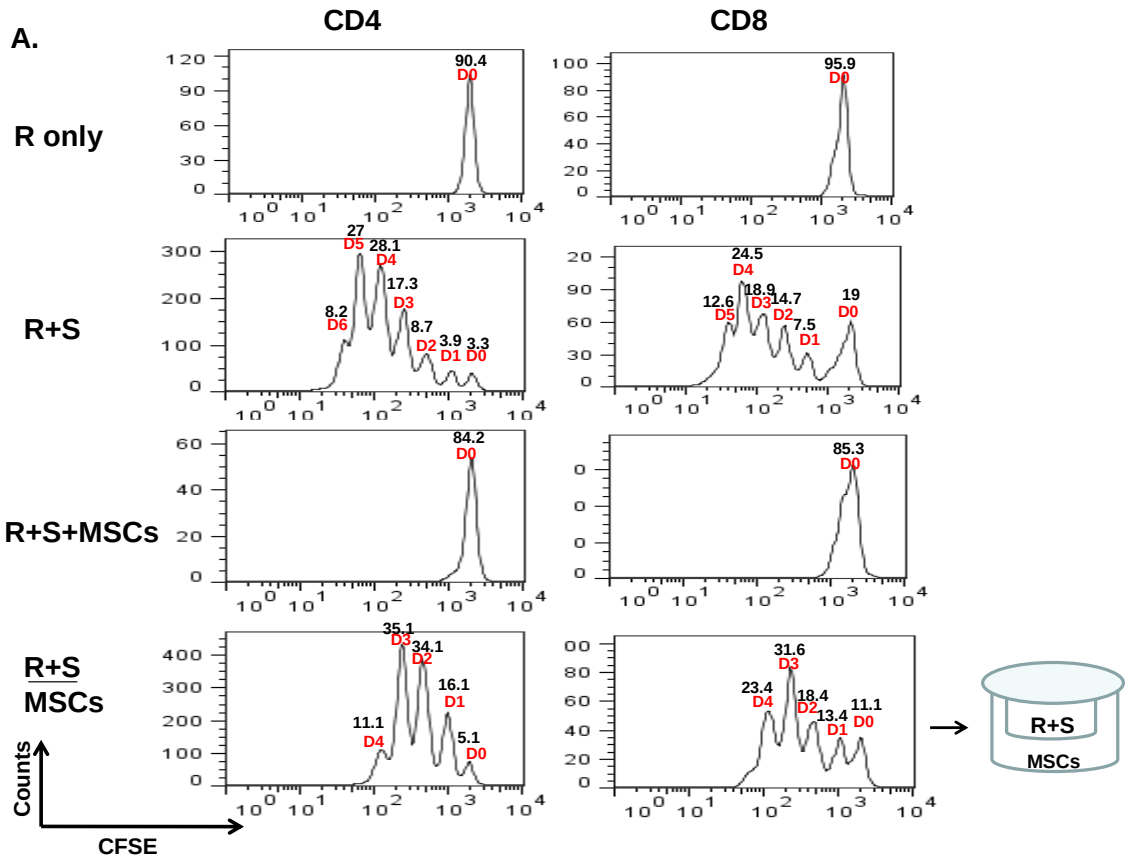

Figure 14: WJ-MSCs partially suppressed alloantigen-stimulated T cells responses in mutually separated condition.

A. CFSE-labeled CB MNCs (R) were mixed with PB DCs (S), and then subjected to the co-culture with WJ-MSCs in the presence or absence of transwell chambers. After 4 days, $CD4^+$ or $CD8^+$ T cells divisions were analyzed by flow-cytometry. The data are representative of three independent experiments.

(R: CB-MNCs; S: Allo PB-DCs; MSCs: Auto WJ-MSCs;)

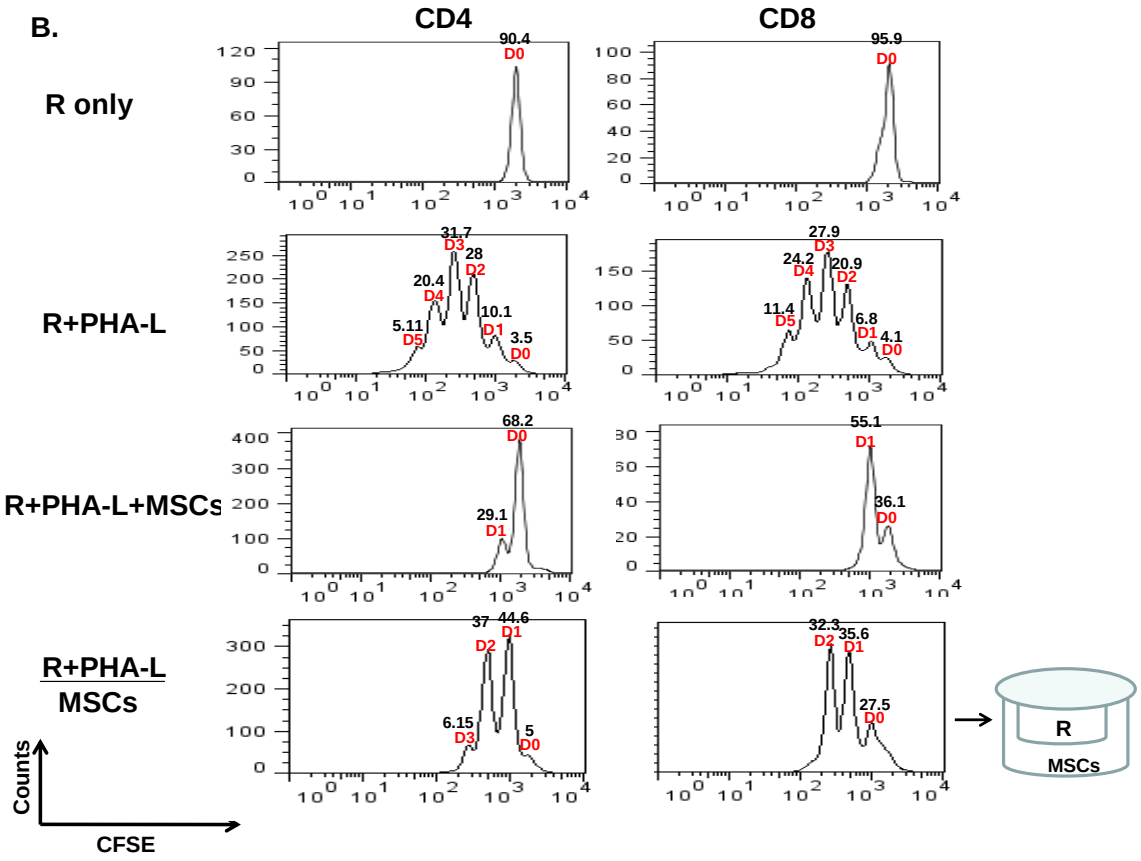

Figure 14: WJ-MSCs partially suppressed alloantigen-stimulated T cells responses in mutually separated condition. *(Continued)*

B. CFSE-labeled CB MNCs (R) were stimulated with PHA-L, and then subjected to the co-culture with WJ-MSCs in the presence or absence of transwell chambers. After 4 days $CD4^+$ or $CD8^+$ T cells divisions were analyzed by flow-cytometry. The data are representative of three independent experiments.

(R: CB-MNCs; MSCs: Auto WJ-MSCs;)

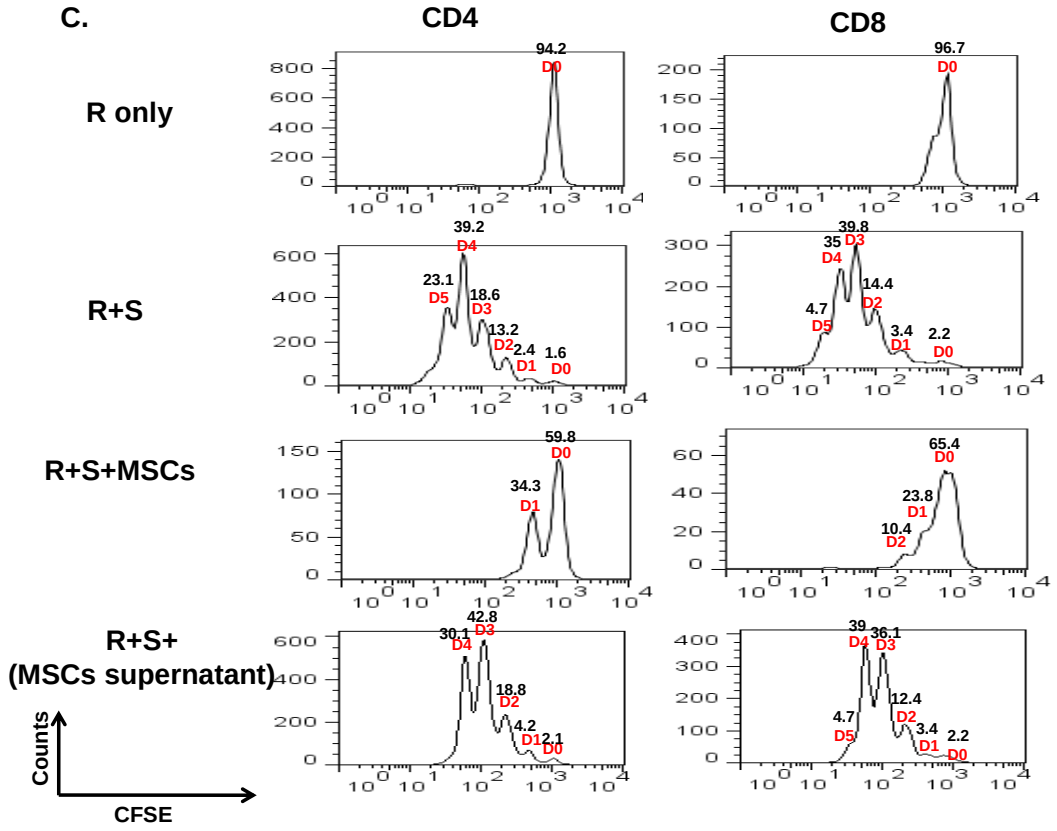

Figure 14: WJ-MSCs partially suppressed alloantigen-stimulated T cells responses in mutually separated condition. *(Continued)*

C. CFSE-labeled CB MNCs (R) were mixed with CB DCs (S), and then incubated by addition with the culture supernatant of WJ-MSCs. After 4 days, $CD4^+$ or $CD8^+$ T cells divisions were analyzed by flow-cytometry. The data are representative of three independent experiments.

(R: CB-MNCs; S: Allo CB-DCs; MSCs: 3rd WJ-MSCs;)

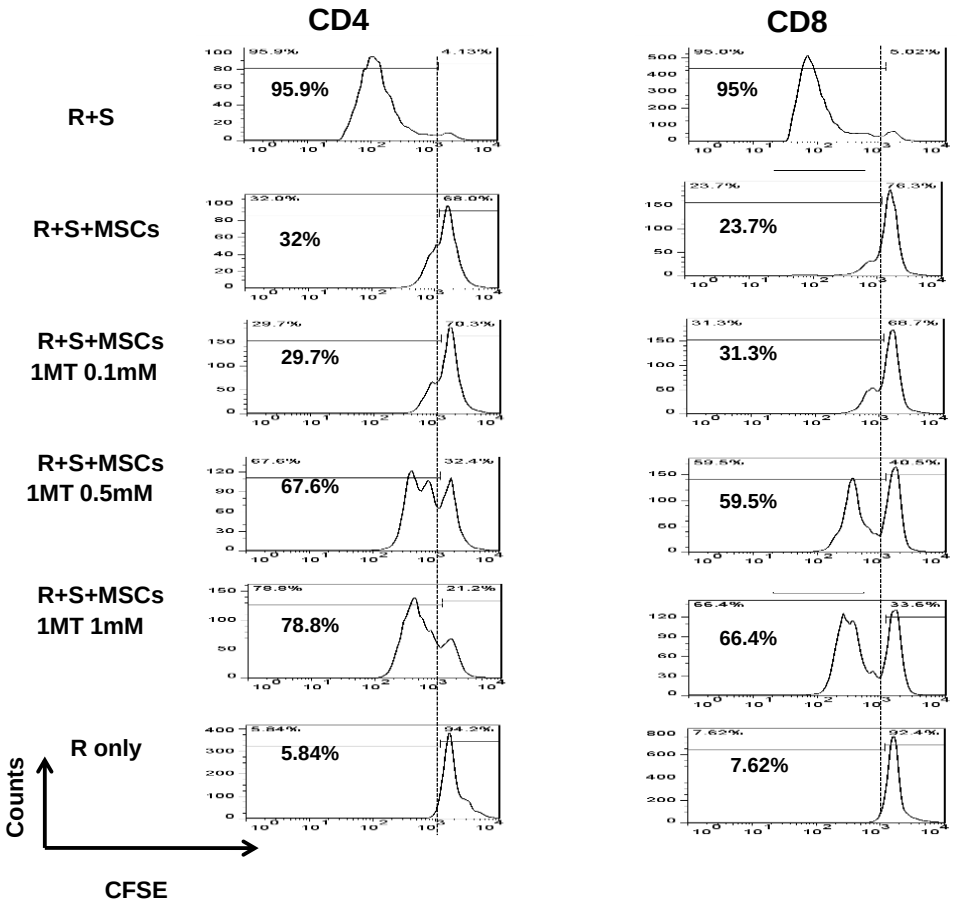

Figure 15: An IDO inhibitor, 1-MT, reversed WJ-MSCs-mediated inhibition of alloantigen-stimulated T cells responses in a dose-dependent manner.
CFSE-labeled PB MNCs (R) were mixed with CB DCs (S), and then subjected to the co-culture with WJ-MSCs in the presence of grading doses of an IDO inhibitor, 1-MT (0, 0.1, 0.5, and 1 mM). After 4 days, $CD4^+$ or $CD8^+$ T cells divisions were analyzed by flow-cytometry. The data are representative of three independent experiments.

(R: PB-MNCs; S: Allo CB-DCs; MSCs: 3rd WJ-MSCs;)

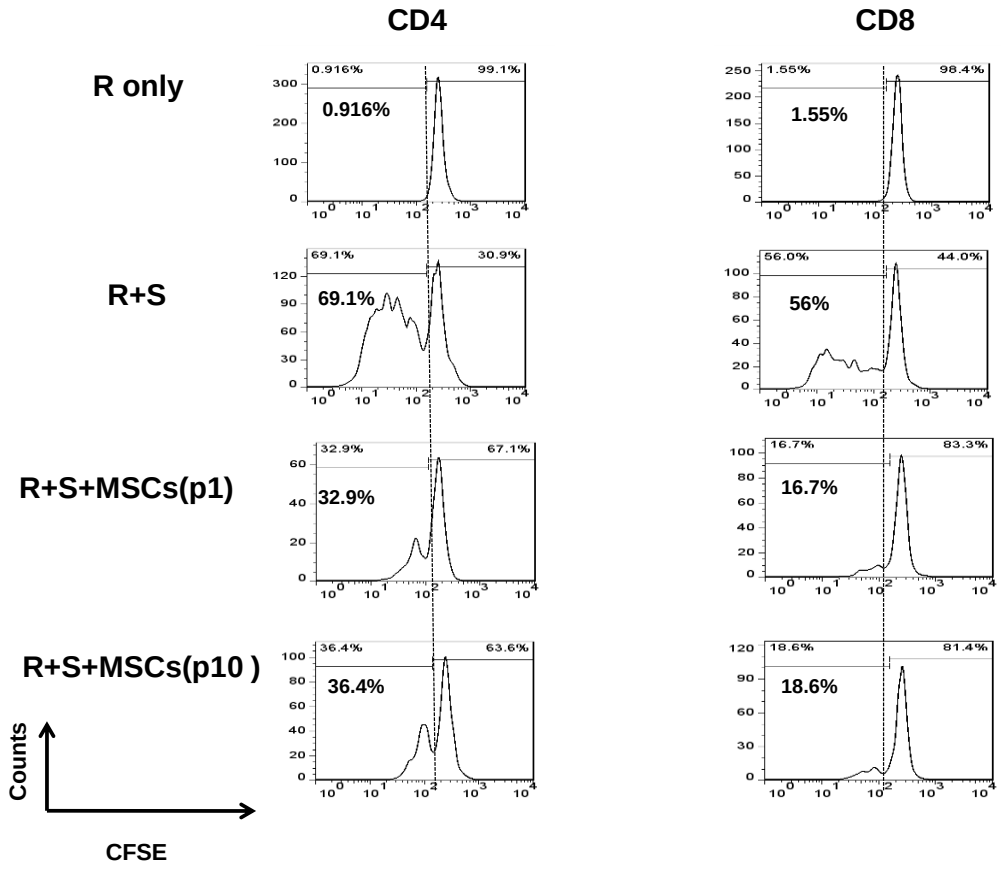

Figure16: Immunosuppressive activity of WJ-MSCs was not affected by the passage number.

CFSE-labeled CB MNCs (R) were mixed with CB DCs (S), and then subjected to the co-culture with WJ-MSCs of early (p1) or late passage (p10). After 4 days, $CD4^+$ or $CD8^+$ T cells divisions were analyzed by flow-cytometry. Respectively, the data from one of the two independent experiments was shown.

(R: CB-MNCs; S: Allo CB-DCs; MSCs: 3^{rd} WJ-MSCs;)

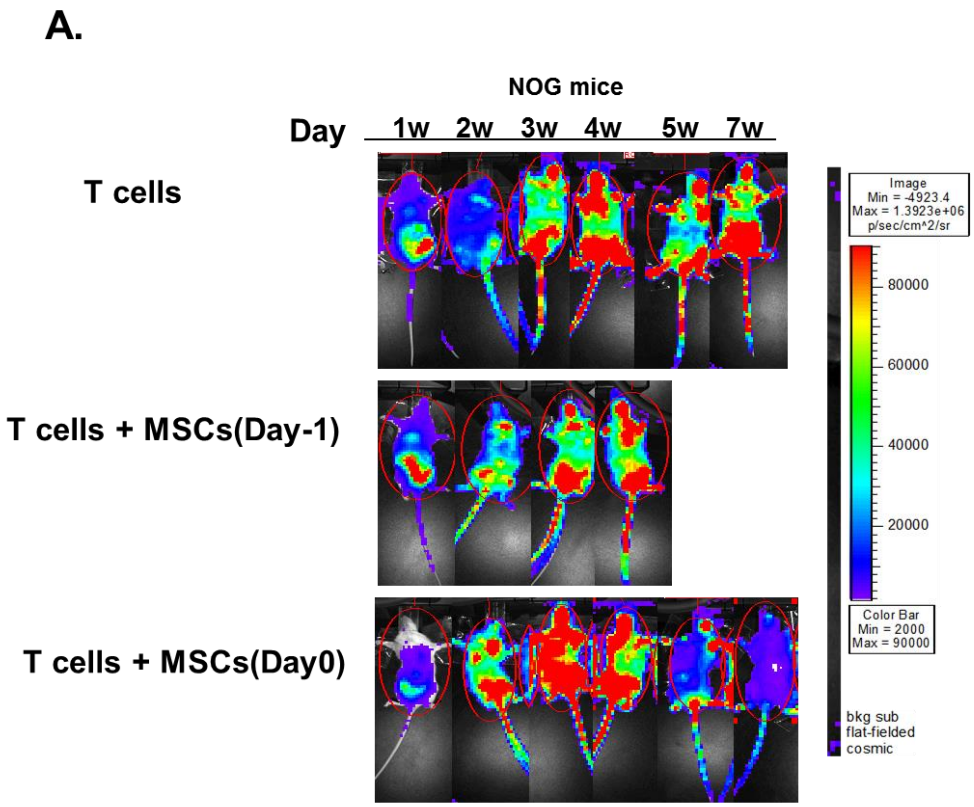

Figure17: Xenogenic GVHD mice treated with or without WJ-MSCs

A. Bioimaging analysis of xenogenic GVHD mice treated with or without WJ-MSCs. NOG mice injected with 2 x 10^7 luc-transduced T cells on Day 0 were divided into 3 groups; control group (n = 4):T cells, treatment group with $1x10^6$ WJ-MSCs on Day -1 (n = 3): T cells + MSCs (Day -1), and treatment group with $1x10^6$ WJ-MSCs from Day 0 (n = 3): T cells + MSCs (Day 0). These mice were weekly monitored by IVIS for T cell expansion. Whole-body images were obtained and analyzed with Living Image 2.50 software (Xenogen).

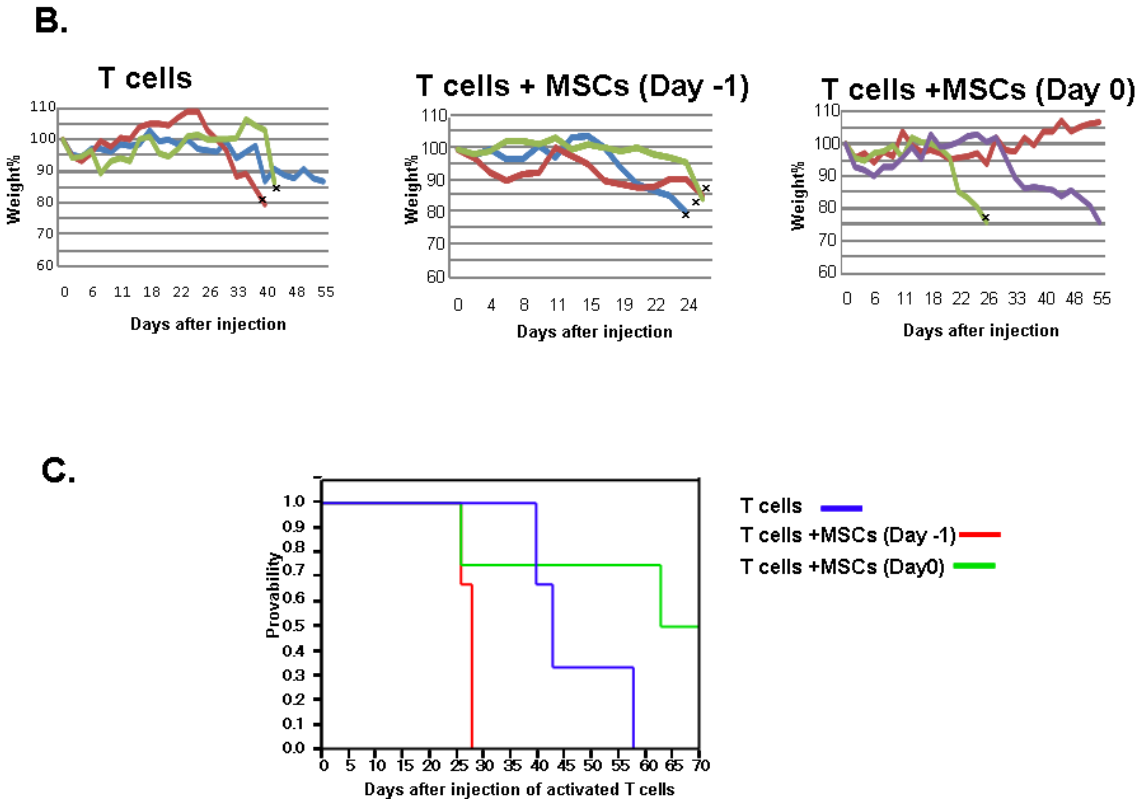

Figure17: Xenogenic GVHD mice treated with or without WJ-MSCs *(Continued)*
B. Body weight of NOG mice developing xenogenic GVHD was monitored at the indicated periods. **C.** Survival curve of xenogenic GVHD mice treated with or without WJ-MSCs

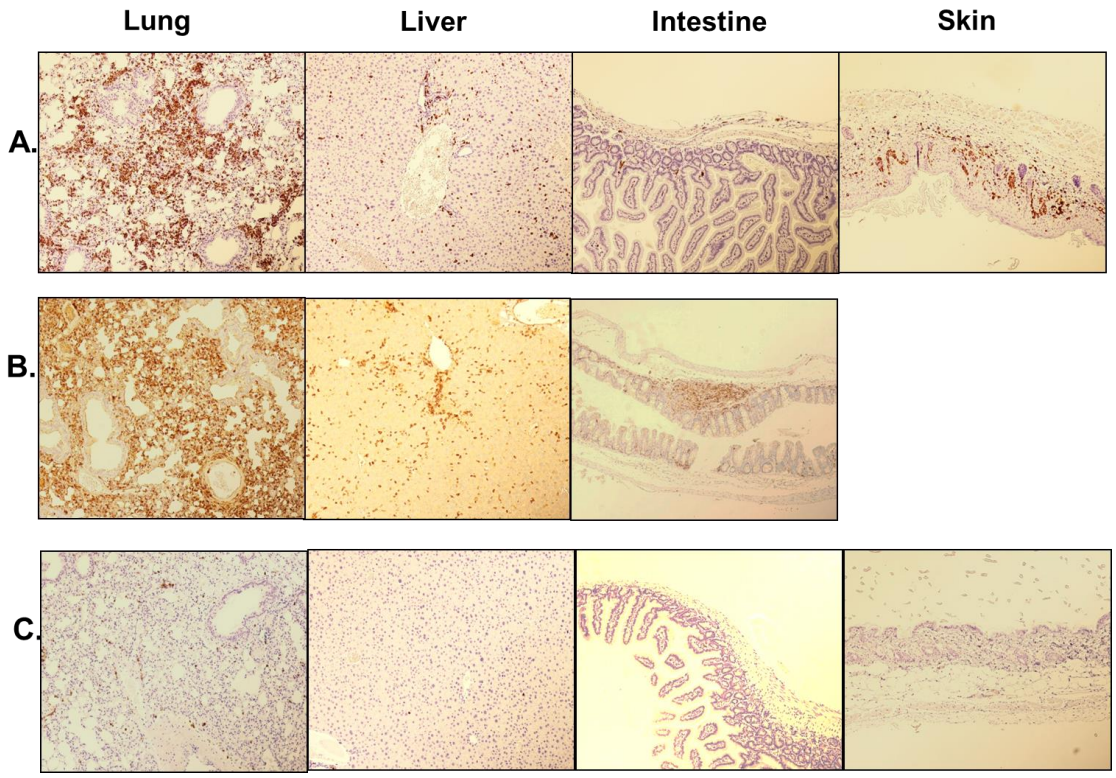

Figure18: Immunopathological analysis of tissues involved in xenogenic GVHD
Tissue specimens including lung, liver, intestine and skin were prepared and stained with anti-human CD45 mAb.
A. T cells group. **B.** T cells +MSCs (Day -1) group. **C.** T cells +MSCs (Day 0) group**.**

Table 1. Human primer sequences used for RT-PCR

Gene		Primer sequence	Product size
hOCT3/4	sense	5' GACAGGGGGAGGGGAGGAGCTAGG 3'	144bp
	Anti-sense	5' CTTCCCTCCAACCAGTTGCCCCAAAC 3'	
REX1	sense	5' CAGATCCTAAACAGCTCGCAGAAT 3'	306bp
	Anti-sense	5' GCGTACGCAAATTAAAGTCCAGA 3'	
NANOG	sense	5' CAGCCCCGATTCTTCCACCAGTCCC 3'	391bp
	Anti-sense	5' CGGAAGATTCCCAGTCGGGTTCACC 3'	
hSOX2	sense	5' GGGAAATGGGAGGGGTGCAAAAGAGG 3'	151bp
	Anti-sense	5' TTGCGTGAGTGTGGATGGGATTGGTG 3'	
hKLF4	sense	5' ACGATCGTGGCCCCGGAAAAGGACC 3'	397bp
	Anti-sense	5' TGATTGTAGTGCTTTCTGGCTGGGCTCC 3'	
hGAPDH	sense	5' AACAGCCTCAAGATCATCAGC 3'	338bp
	Anti-sense	5' TTGGCAGGTTTTTCTAGACGG 3'	

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