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Derivation and Immunological Characterization of Mesenchymal Stromal Cells from Human Embryonic Stem Cells

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Abstract

Objective—We have previously shown the simultaneous generation of CD73+ mesenchymal stromal cells (MSCs) along with CD34+ hematopoietic cells from human embryonic stem cells (ESCs) when they are co-cultured with OP9 murine stromal cells. We investigated whether MSCs can be derived from human ESCs without co-culturing with OP9 cells, and if such cells exhibit immunological properties similar to MSCs derived from adult human bone marrow (BM).

Materials and Methods—Our starting populations were undifferentiated ESCs cultured on matrigel-coated plates without feeder cells. The differentiated fibroblast-looking cells were tested for expression of MSC markers and their potential for multi-lineage differentiation. We investigated surface expression of HLA molecules on these MSCs before and after treatment with interferon gamma (IFN- γ). We also tested the proliferative response of T-lymphocytes towards MSCs and the effects of MSCs in mixed lymphocyte reaction (MLR) assays.

Results—We derived populations of MSCs from human ESCs with morphology, cell surface marker characteristics, and differentiation potential similar to adult BM-derived MSCs. Similar to BM-derived MSCs, human ESC-derived MSCs express cell surface HLA class-I (HLA-ABC) but not HLA class-II (HLA-DR) molecules. However, stimulation with IFN- γ induced the expression of HLA-DR molecules. Human ESC-derived MSCs did not induce proliferation of T-lymphocytes when co-cultured with peripheral blood mononuclear cells. Furthermore, ESC-derived MSCs suppressed proliferation of responder T-lymphocytes in MLR assays.

Conclusions—MSCs can be derived from human ESCs without feeder cells. These human ESC-derived MSCs have cell surface markers, differentiation potentials, and immunological properties *in vitro* that are similar to adult BM-derived MSCs.

Introduction

Mesenchymal stromal cells (MSCs), originally isolated from adult bone marrow (BM), are multipotent cells that provide stromal micro-environmental support for hematopoietic stem cells (HSCs); as well as give rise to mesodermal tissues such as bone, fat and cartilage [1-4]. Over the last several years MSCs have been derived from a variety of other tissues of adult [5-7] or fetal origin [8,9]. Interestingly, these MSCs have also been shown to have a wider differentiation capability *in vitro* and *in vivo* than previously appreciated, at least in some

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experimental models, [10-12] and thus have attracted attention as very desirable cells for regenerative medicine. Furthermore, MSCs from BM and other tissues possess unique immunological characteristics that make them even more attractive for potential clinical applications [13-16].

More recently, MSCs have also been derived from human embryonic stem cells (ESCs) either through co-culturing with the OP9 murine bone marrow stromal cell line or directly from ESCs cultured without feeder cells [17-20]. Human ESCs could potentially provide an unlimited source of MSCs for clinical applications. However, there is little known about the immunological properties of human ESC-derived MSCs. In this manuscript, we report a potentially clinically applicable methodology to derive MSCs from human ESCs. Furthermore, we investigated the immunological characteristics of these cells *in vitro* in direct comparison to human BM-derived MSCs.

Material and Methods

hESC cultures

We used the WiCell human ESC cell lines H1 (WA01), H7 (WA07) and H9 (WA09), passages 25-35, that were originally maintained on murine embryonic fibroblast (MEF) cells. Prior to the differentiation experiments, we passaged these cells weekly for several times on matrigel-coated plates with daily change of media consisting of MEF conditioned media (CM) + basic fibroblast growth factor (bFGF, R&D Systems) to ensure the absence of MEF cells in the culture plates.

Derivation of human BM-derived MSCs

We isolated the human BM-derived MSC-1215 primary cell line by washing a filter discarded at the end of BM harvest from a normal healthy donor after obtaining informed consent approved by the University of Wisconsin Hospital and Clinics Regulatory Committee. Mononuclear cells (MNCs) were isolated by Ficoll Hypaque separation and plated in α -MEM media (Invitrogen) +10% fetal bovine serum (FBS, Hyclone Laboratories). After adherence of stromal cells to plastic plates, culture media was changed to remove non-adherent cells. Media changes were continued every 3-5 days until the culture plates were about 90% confluent. At that point, cells were trypsinized, harvested, and expanded in larger flasks. We also used BM-MSC-5066R cell line provided by the Tulane Center for Gene Therapy and cultured them using a similar methodology as another set of controls for our comparative studies.

Differentiation into osteogenic, adipogenic, and chondrogenic lineages

MSC differentiation into osteogenic, adipogenic and chondrogenic lineages and their detection by immunohistochemistry were done using established reported methodologies [3] and similar to what we have used previously [19]. Briefly, human ESC-derived MSCs were treated with osteogenic or adipogenic medium for 21 days with media changes every 3-4 days. Cell cultures were assayed for mineral content by the von Kossa method and for accumulation of lipid-rich vacuoles by Oil-O-Red staining. For chondrogenic differentiation of MSCs we used the cell culture mass methodology with chondrogenic media changes every 3-4 days for 28 days. Paraffin-embedded micro-mass sections were stained with Safranin-O stain for glycosaminoglycans. For all control experiments, we used human ESC-derived MSCs that were not induced to differentiate.

Fluorescent activated cell sorting analysis

For fluorescent activated cell sorting (FACS) analysis, single cell suspensions of collected cells were analyzed using a FACSCalibur flow cytometer with cell quest acquisition software (BD Biosciences) and FlowJo software (Tree Star). Only human specific monoclonal antibodies CD29-PE, CD31-PE, CD34-FITC, CD44-PE, CD45-PE, CD54-PE, CD73-PE, CD90-APC (BD Biosciences), CD105-APC (eBioscience), and SSEA-4-APC (R&D Systems) were used. Control staining with appropriate isotype matched monoclonal antibodies was included in all FACS experiments.

HLA-expression

Undifferentiated human ESC-derived MSCs, passages 3-6 after their derivation, and human BM-derived-MSCs (BM-MSC-1215 passages 3-6 after derivation, and BM-MSC-5066R passages 2-5 after thawing the original vial) were analyzed for the expression of cell surface bound HLA class-I (HLA ABC-FITC) and HLA class-II (HLA DR-PE) molecules (eBioscience), and the co-stimulatory molecules CD40-PE and CD80-PE (BD Biosciences). To assess the effects of interferon-gamma (IFN- γ) stimulation on expression of HLA molecules, 100 Units/ml of IFN- γ (R&D systems) was added to the culture medium and cells analyzed after 1, 3 and 5 days.

Labeling of cells with Carboxyfluorescein Diacetate Succinimidyl Ester

Peripheral blood (PB) MNCs were labeled with 1 μ M of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 15 minutes at 37°C in PBS with 0.1% bovine serum albumin. After washing the cells twice with PBS + 1% FBS, they were resuspended in RPMI-1640 media (Sigma) +10% FBS and incubated at room temperature for another 10 minutes.

Assessment of T-lymphocyte proliferation response to MSCs

To test the potential of MSCs to induce proliferation of T-lymphocytes 5×10^5 human ESC-derived MSCs or BM-derived MSCs were irradiated (100 Gy) or not irradiated and plated into 24-well plates containing 5×10^5 /ml of CFSE-labeled human PB-MNCs in RPMI-1640 +10% FBS. After 72 hours of co-culture, the cells were harvested and washed twice with PBS + 1% FBS. T-cells were labeled with CD3-APC (eBioscience) for 30 minutes at 4°C, washed, and resuspended in PBS+ 1% FBS. Propidium Iodide (PI) was added to the samples to exclude dead cells from the analysis. For positive controls we used third party pooled (3-5 donors) PB-MNCs, either with or without stimulation with 10 μ g/ml phytohemmagglutinin (PHA, Sigma) for 72 hours. Negative control experiments were done with single donor PB-MNCs. We analyzed at least 40,000 gated events with CellQuest software. Analysis of cell division was performed by flow cytometry using FlowJo software. All experiments were performed at least in triplicate. To study the effect of IFN- γ treatment on the potential of MSCs to induce T-lymphocyte response, MSCs were cultured with IFN- γ for 3 days prior to their use in T-lymphocyte proliferation experiments.

Mixed lymphocyte reactions assays

Mixed lymphocyte reaction (MLR) assays were performed in the following manner: 5×10^5 stimulator cells (allogeneic pooled PB-MNCs) were plated on 24-well plates containing 5×10^5 cells CFSE-labeled responder PB-MNCs in RPMI-1640 + 10% FBS. 5×10^5 effector cells (human ESC-derived MSCs or BM-derived MSCs) were also plated into the same wells. Control experiments included pooled allogeneic PB-MNCs used as stimulators with no MSCs added. To study if direct contact between MSCs and lymphocytes are required, we used the transwell method, in which MSCs were separated from lymphocytes by a 0.4 μ m size membrane (Corning) transwell culture plate. To test the effect of MSC-conditioned media

(CM), media were collected after 48-72 hours from 80-90% confluent ESC-derived MSC or BM-derived MSC cultures, filtered, and then added to MLR cultures in 24-well plates. For controls MSCs were omitted from transwells, and RPMI media used without CM. After 72 hours of MLR culture the cells were harvested and washed twice with PBS + 1% FBS; T-cells were labeled with CD3-APC for 30 minutes at 4°C, washed and resuspended in PBS + 1% FBS. PI staining was used to exclude dead cells, and analysis of cell division was performed as before.

Statistical analysis

To test the probability of statistically significant differences between paired samples the student t test for paired data was used. Differences were considered statistically significant if p value was less than 0.05.

Results

Generation of MSCs from human ESCs

Our starting populations of cells were human ESCs (H1, H7 and H9 cell lines) cultured on matrigel plates with MEF-CM + bFGF for several passages. Human ESCs cultured under these conditions retain their undifferentiated state. The differentiation of human ESCs towards MSCs was induced by increasing the interval of media culture changes to 3-5 days. Upon increasing the interval of media changes, a portion of the cells at the periphery of the ESC colonies differentiated toward spindle-shaped fibroblast-looking cells. After 9-10 days in this culture condition when between 40-50% of the cells in the culture were fibroblast-looking in appearance, the undifferentiated portions of ESC colonies were removed by physically scraping and suctioning. The remaining cells were then treated with collagenase and transferred into new matrigel-coated plates. These new cultures were continued with MEF-CM + bFGF culture media changes every 3-5 days. At this stage the proportion of fibroblast-looking cells continued to further increase. When the majority of the cells in the plates were comprised of fibroblast-looking cells, the remaining semi-differentiated ESC colonies were again removed physically by scraping and suctioning. At this point the remaining cells were trypsinized and transferred into gelatin-coated plates and cultured with α -MEM medium +10% FBS. Upon transfer, the majority of cells had a fibroblast-looking morphology and when confluent the cells were persistently more than 90% positive for CD73+, a cell surface marker of MSCs. After the next passage these cells were almost completely positive for CD73+ and negative for SSEA-4 (Figure-1); we labeled these cells passage-1. We were persistently able to derive MSCs (H1-MSC n=4, H7-MSC n=3, and H9-MSC n=7 times) by this methodology in about 4-6 weeks from the start of the differentiation protocol. Figure-1 graph-A shows a representative analysis of our starting population of human ESCs prior to inducing their differentiation, which are mostly positive for SSEA-4 but negative for CD73+ cells. During our differentiation protocol there is a progressive loss of SSEA-4 marker and acquisition of CD73 marker by cells in the culture (graphs-B and C). At the end of the differentiation process, our human ESC-derived MSCs do not express SSEA-4 anymore and are highly positive for CD73 marker (graph-D).

Human ESC-derived MSCs show spindle-shaped fibroblast-looking morphology. Representative photomicrographs of human ESC-derived MSCs from each cell line (H1-MSCs, H7-MSCs and H9-MSCs) are shown in the upper panel of Figure-2. MSCs derived by this method were karyotypically normal when tested at passage 4 or 5 (CellLine Genetics, Madison, WI) (Figure-2: middle panel). Further passages of human ESC-derived MSCs were done whenever the culture plates became near-confluent. We were also able to freeze, thaw, and subsequently passage these MSCs. We could passage the H1 and H9 ESC-derived MSCs up to about a total of 18-21. At that time the cells started to show signs of senescence, or slowed growth, similar to what has been reported by Olivier et al [17]. However, we were unable to

grow the H7-MSCs past passages 8-10 in three attempts. RT-PCR assays done on RNA samples from human ESC-derived MSCs for expression of HPRT gene mouse-specific sequence ruled out the presence of any remnant MEF cells (data not shown).

Differentiation potential of human ESC-derived MSCs

Undifferentiated human ESC-derived MSCs (H1-MSCs, H7-MSCs and H9-MSCs passages 4-8) were subjected to osteogenic, adipogenic and chondrogenic inducing culture conditions as previously described [3,19]. Human ESC-derived MSCs could differentiate into osteocytes, adipocytes and chondrocytes (Figure-2: lower panel is a representative example of the differentiation potential of H9-MSCs).

Cell surface marker characteristics of human ESCs-derived MSCs

The human ESC-derived MSCs exhibit similar cell surface marker characteristics when compared to BM-derived MSCs derived in our laboratory (BM-MSC-1215) as well as the BM-MSC-5066R cells. Figure-3 shows that our ESC-derived MSCs are positive for CD29, CD44, CD54, CD73, CD90, and CD105 but negative for CD34, CD45 and the endothelial marker CD31. Table-B of Figure-3 shows the mean values $\pm$ standard errors of the percentages of MSCs that were tested for each cell surface antigen expression by flow cytometry.

Expression of HLA and co-stimulatory molecules

Like human BM-derived MSCs (BM-MSC-1215 and BM-MSC-5066R), human ESC-derived MSCs express HLA-ABC molecules. On the other hand, human ESC-derived MSCs or BM-derived MSCs do not express HLA-DR at their steady state. Similarly, neither BM-derived MSCs nor hESC-derived MSCs express co-stimulatory molecules CD40 and CD80 (Figure-3). When IFN- γ was added to the culture medium, expression of HLA-ABC molecules did not change and remained high (Figure-4). However, after 1 day of treatment with IFN- γ , HLA-DR expression increased on H1-MSCs (mean value: 6.7%), H7-MSCs (7.7%), H9-MSCs (4.3%), BM-MSC-1215 (9.0%) and BM-MSC-5066R (31.7%). After 3 days of treatment, there was a further increase of HLA-DR expression on H1-MSCs (mean value: 49.5%), H7-MSCs (55.4%), H9MSCs (50%), BM-MSC-1215 (69.6%) and BM-MSC-5066R (91.2%). These levels of expression further increased after 5 days of treatment on BM-MSC-1215 (mean value 98.8%) and BM-MSC-5066R (94.3%) but did not show any further significant increase on human ESC-derived MSCs (H1-MSC 54.0%, H7-MSC 58.0%, and H9-MSC 48.9%). Figure-4 summarizes the data (mean value $\pm$ standard error) from at least three different sets of experiments. There were no significant changes in the expression of co-stimulatory molecules (CD40 and CD80) on the MSCs after treatment with IFN- γ (data not shown).

Immunogenicity of human ESC-derived MSCs

CFSE passively enters the cell cytoplasm, where it is rapidly hydrolyzed into a fluorescent hydrophilic metabolite unable to diffuse out and readily detectable by FACS. Thus, CFSE concentration decreases with each cell division, and provides a history of the proliferation of labeled cells. Figure-5-A is a representative experiment showing the gating of the cells to calculate the proliferation index (PI: the average number of cell divisions that a cell in the original population has undergone). To determine if MSCs elicit a T-cell proliferative response when co-cultured with PB-MNCs, responder cells were stained with CFSE. At the time of analysis, CFSE labeled cells were further stained with CD3 to specifically track cell division of the T-lymphocyte population within PB-MNCs. We found that human ESC-derived MSCs (H1-MSC PI=1.003, H7-MSC with PI=1.01, H9-MSC with PI=1.002) and BM-derived MSCs (BM-MSC-1215 PI=1.003 and BM-MSC-5066 PI=1.02) did not elicit the proliferation of T-cells (background PI= 1.0) (Figure-5-B). As expected, third party PB-MNCs that were stimulated or not stimulated with PHA were highly immunogenic to responder T-cells

(allogeneic PB-MNCs PI=4.24, PHA-treated allogeneic PB-MNCs PI= 4.5). Inhibition of T-cell proliferation were seen with both irradiated and nonirradiated MSCs as reported before [21].

We then investigated whether pre-treatment of MSCs with IFN- γ would elicit proliferation of T-lymphocytes. There was no proliferation of responder T-lymphocytes when co-cultured with IFN- γ pre-treated MSCs (H1-MSC PI=1.003, H7-MSC PI=1.01, H9-MSC PI=1.007, BM-MSC-1215 PI=1.003, and BM-MSC-5066R PI=1.008). Control IFN- γ treated pooled allogeneic PB-MNCs were able to stimulate the proliferation of T-lymphocytes (PI=4.78).

Effect of MSCs in MLR cultures

The effect of hESC-derived MSCs compared to BM-derived MSCs on proliferation of T-lymphocytes in MLR assays is summarized in Figure-6. Human ESC and BM-derived MSCs reduced proliferation of responder T-lymphocytes (all p values < 0.01) when the responder and stimulator cells were in direct contact with MSCs (effector cells). To investigate if direct contact is necessary to induce this immunosuppressive effect, we separated MSCs from the responder lymphocytes and stimulator PB-MNCs using a semi-permeable membrane. All human ESC-derived MSCs and BM-derived MSCs suppressed proliferation of T-lymphocytes (all p values < 0.05) compared to control experiments (no MSCs added). However, in direct head to head comparison with corresponding MSCs in contact cultures, MSCs separated by the semi-permeable membranes showed less immunosuppressive potency (P value did not reach statistical significance, < 0.05, for H9-MSCs and BM-MSC-5066R). To verify that secreted components play a role we also tested the effect of CM collected from MSCs. Again CM from all MSCs exerted an immunosuppressive effect compared to no CM (all P values < 0.05). This immunosuppressive effect was again less when compared to contact cultures (P value did not reach statistical significance for H9-MSCs).

Discussion

A panel of experts recently proposed three criteria to define MSCs, including adherence to plastic in standard culture conditions, expression of specific cell surface antigens and a lack of expression of certain other antigens, and finally their ability to differentiate into osteocytes, adipocytes and chondrocytes [22]. In the present study we have devised a practical methodology that allowed us to persistently isolate MSCs from three different human ESC lines (H1, H7, and H9) without the use of any feeder-cell supporting layers. We defined these cells as MSCs by the combination of their adherence to plastic in standard culture conditions, their cell surface immunophenotype, and their functional properties of differentiation into osteogenic, adipogenic, and chondrogenic lineages *in vitro*.

Although the generation of fibroblast-looking cells from human ESCs was originally reported a few years ago, those cells were not fully characterized as MSCs [23-25]. Barberi et al were the first to derive MSCs from human ESCs through co-culturing them with the OP9 murine BM stromal cell line for 40 days and characterize them according to accepted criteria for MSCs [20]. Recently, we reported the generation of MSCs from human ESCs through co-culturing with OP9 cells but in a shortened 2 weeks period [19]. Nevertheless, our intent in the current study was to move away from the use of murine OP9 feeder cells. Olivier et al. previously reported derivation of MSCs from human ESCs through a feeder free culture system [17]. However, that methodology was based on culturing MSCs from human ESCs that were grown into a thick multilayer epithelium. More recently, Lian et al. reported generating MSCs from H1 and H9 human ESCs by culturing ESC colonies on gelatinized plates in knockout DMEM and supplemented with 10% serum replacement, FGF2 and PDGF AB followed by cell sorting for CD105+ and CD24- cells by FACS [18], which is different from our method.

SSEA-4 is one of the markers expressed on undifferentiated human ESCs [26]. Although there are major differences in our derivation methodology we can not explain why our MSCs were uniformly negative for SSEA-4 expression in contrast to the study of Olivier et al study [17]. Interestingly, we only saw negligible amounts of cells that were double-positive for SSEA-4 and CD73 markers at any time point in our culture system (Figure-1). The expression of SSEA-4 on adult human BM-derived MSCs is also disputed between different groups. For example, Wagner et al [27] and Cheng et al [28] reported that MSCs derived from human BM do not express SSEA-4 marker. However, Gang et al reported that SSEA-4 are expressed on human BM-derived MSCs [29]. These differences might be due to differences in the derivation methodologies used by different groups; for example, Battula et al showed that culturing human placenta or BM-derived MSCs in serum-free bFGF-containing medium induces expression of SSEA-4 [30]. Shibata et al. have shown that purging SSEA-4 positive cells from hematopoietic cells differentiated from cynomolgus monkey ESCs prevented teratoma formation when cells were subsequently transplanted into fetal animals, compared to transplantation of un-purged cells that resulted in teratoma formation in all animals tested [31]. Thus, the lack of expression of SSEA-4 on our human ESC-derived MSCs, that otherwise possess all the other characteristics of adult BM-derived MSCs, makes our differentiation protocol of potential clinical value.

To our knowledge our report is the first on the *in vitro* immunological properties of human ESC-derived MSCs. Similar to adult BM-derived MSCs, our human ESC-derived MSCs express HLA-ABC but do not express HLA-DR or co-stimulatory molecules such as CD40 and CD80 [32]. Although treatment of human ESC-derived MSCs with IFN- γ could induce cell surface expression of HLA-DR, the expression of HLA-DR molecules on ESC-derived MSCs remained lower compared to BM-derived MSCs at day 3 and 5. It has also been shown that adult BM-derived MSCs do not elicit a proliferative response when co-cultured with allogeneic T-lymphocytes [16,33]. Similarly, our ESC-derived MSCs failed to induce proliferation of allogeneic T-lymphocytes when co-cultured with PB-MNCs. Thus, at least under these experimental conditions, human ESC-derived MSCs are not inherently immunogenic. Even after we cultured human ESC-MSCs with IFN- γ to induce surface expression of HLA-DR antigen they failed to induce proliferation of T-lymphocytes. Also, when we added human ESC-derived MSCs to MLR cultures they suppressed proliferation of responder T-lymphocytes. The suppressive effect of our ESC-derived MSCs persisted to a lesser degree, using semi-permeable membrane or MSC-CM. This suggests a role for secretory factors similar to the report by Di Nicola et al. [33].

The extent of MSC contribution to generation of other tissues, their ultimate clinical significance, and the potential mechanisms of their activities are matters of strong debate [34,35]. Nevertheless, interest in MSCs has already moved from *in vitro* and animal studies into actual clinical trials in patients [36]. Several phase I-II clinical trials have already shown the safety and efficacy of BM-derived MSCs for enhancing the engraftment of co-transplanted HSCs in patients with hematologic and non-hematologic malignancies [37,38]. BM-derived MSCs have also shown promising results for the treatment of mesenchymal diseases such as osteogenesis imperfecta [39] and congenital disorders such as metachromatic leukodystrophy [40]. BM-derived MSCs have shown to be safe in phase-I trials in myocardial infarction patients [41]. The immunological characteristics of BM-derived MSCs are another reason for the interest in the use of these cells for clinical applications [13-16]. Indeed, the use of third party ex vivo expanded HLA-mismatched MSCs in immune-dysregulation disorders such as graft versus host disease after allogeneic HSC transplantation and Crohn's disease have generated encouraging results, and are currently into phase-III trials [42-44].

Human ESC-derived MSCs could provide a novel and unlimited universal source of MSCs for a variety of potential clinical applications such as repair of mesodermal tissues, contribution

to repair of other tissues, and even potentially enhancement of engraftment of human ESC-derived HSCs. We have devised a reproducible and potentially clinically applicable method for deriving MSCs from human ESCs *in vitro* with morphologic, immunophenotypic, and *in vitro* functional and immunological characteristics very similar to adult BM-derived MSCs. These immunological properties of human ESC-derived MSCs could play a significant role in their eventual applications.

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Figure-1.

The majority of undifferentiated human embryonic stem cells (ESCs) (graph-A) are positive for the SSEA-4 marker but they do not express the CD73 marker, one of the markers expressed on mesenchymal stromal cells (MSCs). Progressively during the MSC differentiation process human ESCs lose their SSEA-4 marker and become positive for CD73 (graphs-B and C). Fluorescent activated cell sorting analysis of passage-1 human ESC-derived MSCs shows that they do not express the SSEA-4 marker, but are uniformly positive for the CD73 marker (graph-D).

Figure-2.

Upper panels show representative microscopic views of human embryonic stem cells (ESC)-derived mesenchymal stromal cells (MSCs) from H1, H7 and H9 cell lines. The middle panels show a normal karyotype for each human ESC-derived MSC line when tested at passage 4-5 after derivation. The lower panels show the differentiation of H9-ESC-derived MSCs into osteogenic (lower-left), adipogenic (lower-middle), and chondrogenic (lower-right) lineages. Von Kossa staining shows deposits of calcium crystals (lower left), Oil red O staining shows lipid vacuoles (lower middle), and Safranin O staining shows cartilage-specific glycosaminoglycans (lower right), respectively. All photomicrographs of MSCs or their differentiated progenies were taken with a Leica DFC320 digital camera on a Leica DM IRB microscope using C Plan 20×0.22 objective except the chondrogenic picture that was taken by N plan 10×0.12 objective (lower right).

Figure-3. Panel-A shows representative fluorescent activated cell sorting analysis of H1-MSCs, H7-MSCs, H9-MSCs, BM-MSC-1215, and BM-MSC-5066R for different cell surface markers. Table-B shows the mean value ($\pm$ standard error) of the expression of corresponding markers from at least three sets of experiments.

Figure-4.

Human embryonic stem cells (ESC)-derived mesenchymal stromal cells (MSCs) or bone marrow (BM)-derived MSCs express HLA-ABC but do not express HLA-DR. Treatment of human ESC-derived MSCs with IFN- γ induces the cell surface expression of HLA-DR but to a lesser degree compared to that seen from stimulated BM-derived MSCs.

Figure-5.

A: Upper panel shows one representative carboxyfluorescein diacetate succinimidyl ester (CFSE) assay showing the T-lymphocyte fraction of peripheral blood mononuclear cells (PB-MNCs) does not proliferate when they are cultured alone. Lower panels show T-lymphocytes labeled with CFSE dividing in response to third-party PB-MNCs. Proliferation index (PI) is calculated using FlowJo software.

B: Mean value for proliferation index ($\pm$ standard error based on at least three sets of experiments) of responder T-lymphocytes cultured alone, co-cultured with non-stimulated or PHA-stimulated PB-MNCs, and human embryonic stem cell-derived or bone marrow-derived mesenchymal stromal cells (MSCs) are shown alongside a representative CFSE assay. MSCs do not induce the proliferation of T-lymphocytes.

Figure-6.

Mesenchymal stromal cell (MSCs) cultured in direct contact or in the presence of a semi-permeable membrane in transwells, and also when their conditioned media (CM) were added to mixed lymphocyte reactions (MLRs) suppressed the proliferation of responder T-lymphocytes towards stimulator cells (pooled peripheral blood mononuclear cells). Negative controls included carboxyfluorescein diacetate succinimidyl ester (CFSE) stained T-lymphocytes alone and positive controls included MLRs performed in the absence of MSCs or CM. Culturing in direct contact was always more immunosuppressive compared to transwell culture (this difference did not reach statistical significance for H9-MSCs and BM-MSC-5066R) and compared to CM (this difference did not reach statistical significance for H9-MSCs).