Mesenchymal stem cell like (MSCl) cells generated from human embryonic stem cells support pluripotent cell growth

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1. Introduction

Mesenchymal stem (stromal) cells (MSCs), originally isolated from bone marrow [1], provide a supportive microenvironment for hematopoietic stem cells. Moreover, MSCs show multipotent stem cell characteristics [2] and special immunological features [3]. MSCs can also be obtained from various tissues including peripheral blood, umbilical cord, placenta, vein wall, muscle, adipose and connective tissues [4]. MSCs can be differentiated into canonical mesodermal tissues, e.g. bone, fat and cartilage, but may also retain a wider differentiation capacity [5]. MSCs play a role in tissue repair due to their differentiation potential and immunosuppressive activities [6], as well as by trophic effects mediated by growth factors and cytokines they produce [7].

These features initiated the use of MSCs in various cell-based therapies and by now more than 50 clinical trials related to this cell type have been reported (see http://www.clinicaltrials.gov) [8]. However, there are several limitations of large scale, reproducible, and well characterized production of human MSCs. The aspiration of MSCs from donors is an invasive procedure and several reports have demonstrated donor- and tissue-dependent variability of human bone-marrow-derived MSCs [4,9–13]. Isolation and enrichment of tissue-derived MSCs usually results in heterogeneous cell populations, and a long term ex vivo expansion of human MSCs has been shown to reduce replicative capacity, impair differentiation potential, alter gene expression profiles, and lead to karyotype instability [9,10].

In contrast to MSCs, pluripotent human embryonic stem cell (hESCs) lines have the capacity of unlimited growth and self-renewal [14] and can differentiate into all cell types of the human body [15]. There are numerous studies investigating the differentiation of hESCs, especially to yield the clinically most relevant cell types, including cardiomyocytes, hematopoietic cells, neuronal or pancreatic cells (for a recent review see [16]).

Recent publications have also reported the derivation of MSC-like cells from hESC lines [17–20], by using various protocols and producing MSCs with different characteristics. In the present study we have established a method to derive MSC-like cells from hESCs with well defined gene and protein expression patterns and immunological features, examined their differentiation potential, supportive role for culturing hESCs, and produced genetically modified MSC cell clones stably expressing fluorescent marker proteins. This work may significantly help our understanding of human MSC characteristics and the use of these cells in biotechnology applications.
2. Materials and methods

2.1. HuES, MSCI cells, HFF-1 and bmMSC cultures

The human embryonic stem cell lines (HUES9 and HUES1 originally provided by Dr. Douglas Melton, Harvard University) were cultured on mitotically inactivated mouse embryonic fibroblasts (MEF) [21]. Spontaneous differentiation of the hESCs were performed via embryoid body (EB) formation as described previously [21]. The cells were trypsinized at day 80 and were further cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) on a gelatinized 10-cm plate. The cells were trypsinized when confluent and split to 1:3 ratio. After two passages most of the cultured cells presented fibroblast-like morphology. The hESC-derived fibroblast-like cells were designated as hESC-derived mesenchymal stem cell like (MSCL) cells. All MSCI cells used in this study were of polyclonal origin.

HFF-1 cells were obtained from ATCC and were cultured according to the manufacturer’s instructions (http://www.stemcells.atcc.org). Collection of bone marrow samples from patients with hematological diseases was approved by the Regional and Institutional ethical review board of the Medical and Health Science Center of the University of Debrecen (protocol No.: UD-MHSC REC/IEC 2754-2008). Bone marrow samples were harvested from the iliac crest under medical examination. bmMSC isolated and cultured as described earlier [22]. The cells were used for phenotypic and functional analysis after P5.

2.2. Characterization of MSCI, HFF-1 and bmMSC cells

Cell surface expression of MSCI marker proteins on the surface of MSCI, HFF-1 and bmMSC cells was analyzed by three-color flow cytometry using FITC-, APC- or PE-conjugated monoclonal antibodies (mAb) with isotype-matched control mAbs; specific against hematopoietic-, MSC related-, endothelial markers, cell adhesion molecules and integrins as well (see Supplementary Table 1 for full list). Fluorescence intensities were measured by FACS Calibur flow cytometer and the data were analyzed by using the WinMDI software (Joseph Trotter, La Jolla, CA). Results were expressed as means of positive cells (% ± SD). Pluripotency markers of MSCI were tested by immunostaining (Oct4, SSEA4, PODXL) and by real time PCR (Nanog, ABCG2) performed as described previously [21]. The differentiation potential of MSCI was performed by using the Gibco’s StemPro® Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kits according to the manufacturer’s guide.

2.3. Lentiviral transduction of MSCI cells

For viral-based gene delivery, a third generation lentiviral vector system was used, as described in [23]. Determination of virus titers and the transduction procedures were performed as described previously [24]. The MSCI-2 cells were transduced by a MOI of 2–5, with an eGFP encoding lentiviral vector and further handling was the same as for the parental cell line.

2.4. Mitogen-induced cell proliferation and cytokine secretion

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). Mitogen-activated T lymphocyte proliferation was induced by concanavalin A (ConA) or phytohaemagglutinin (PHA) used at a final concentration of 10 μg/ml (Amersham Pharmacia Biotech) and 1 μg/ml (Sigma–Aldrich), respectively added to 1 × 10⁶ PBMCs. MSCs were added to 1 × 10⁶ PBMCs at 10³ and 10⁴ cell numbers and co-cultured for 3 days. On day three proliferation was detected by the BrDU colorimetric assay directly in the culture plate according to the manufacturer’s instructions (Roche). IL-6, IL-10 and IFN-γ cytokine secretion was quantitated by enzyme linked immunosorbent (ELISA) assay (OptEIA, BD Pharmingen) following the supplier’s instruction. All experiments were performed in triplicates.

2.5. Statistical analysis

Each experiment was performed at least three times and each sample was tested in triplicates. Data are expressed as mean ± SD. Statistically significant differences were determined by two-way ANOVA or paired student-t tests. *p < 0.05, **p < 0.01, ***p < 0.001.

3. Results and discussion

3.1. Generation of HUES9-derived MSCI cells – application as feeder cells

Pluripotent HUES9 cells were spontaneously differentiated via EB formation (6 days), and then the differentiation process was

![Fig. 1. Pluripotency markers of HUES9 cells cultured on different feeder layers, HUES9 cells were grown on MEF (A) or MSCI-2-GFP (B) feeder cells for two days in eight-well chambers for confocal microscopy. Co-culture of HUES and feeder cells were fixed and stained with the antibodies recognizing the following proteins: Oct4, SSEA4, PODXL (red). Antibodies specific for undifferentiated cell markers stained only the HUES9 clumps, while the MEF or MSCI-2-GFP cells did not show any staining with these markers. Nuclei were counterstained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
completed by further cell culturing on adherent surface in the presence of 10% FBS for 80 days. The differentiated fibroblast-like cells were separated from the other cell types by short trypsinization. After 2–3 passages on adherent surface the cells achieved uniform, fibroblast like morphology, and these cells (termed MSC1 cells) could be propagated at least for 15 passages without morphological or kariotypical changes (Supplementary Fig. 1). By using the same protocol, more than five polyclonal MSC1 cultures were independently generated from the HUES9 cell line.

In the following experiments we tested the capacity of the MSC1 cells as autogenic feeder cells for maintaining pluripotency of hESCs. When undifferentiated HUES1 or HUES9 cells were passaged to mitomycin treated MSC1 cultures, each MSC1 cell line supported the growth of these pluripotent hESCs without any change in the expression of pluripotency markers or the rate of cell growth, as compared to those seen with cells growing on MEF (Supplementary Fig. 1). By using the same protocol, more than five polyclonal MSC1 cultures were independently generated from the HUES9 cell line.

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Fig. 2. Differentiation of MSC1-2, HFF1 and bmMSC cells. Adipogenic differentiation is demonstrated by intracellular lipid vacuoles stained in red by OilRed O (red) and with nuclei counterstained with DAPI (blue) (A). Osteogenic differentiation of MSCs is demonstrated by the formation of calcium-hydroxyapatite-positive areas stained in red by Alizarin Red (B). High density cultures showed the development of chondrogenic phenotype when cultured in micromass; pink extracellular matrix staining marks proteoglycans stained with toluidine blue (C). Magnification: 200x. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MSCs should fit the functional and phenotypic criteria defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [28]. We first tested whether the MSC1 cells could be differentiated toward the canonical mesodermal (osteoblastic, chondrogenic, adipogenic) directions. We found that all four established MSC1 cell lines exhibited the potential to differentiate to at least one direction, but one of them (MSC1-2) showed robust differentiation potential toward all three directions (Supplementary Fig. 3). To complete a more detailed characterization we next compared the differentiation capacity of MSC1 cells with the commercially available human foreskin fibroblast cells (HFF-1) widely used as a feeder cell line for hESC cultures, and that of MSCs isolated from human bone marrow. All these MSCs of different origin could be differentiated in vitro to adipogenic, osteogenic and chondrogenic directions. Followed by a three-week adipogenic induction period, a large number of

MSCI-2 and bmMSC cells showed oil red positive staining, characteristic for adipocytes (Fig. 2A), while only a small number of HFF-1 cells became oil red positive. Similarly, when differentiation was induced by osteogenic induction medium for 2–3 weeks, MSCI-2 and bmMSC cells showed dense calcium deposits stained with alizarin red, while the HFF-1 cells exhibited weak osteogenic potential (Fig. 2B). After 3 weeks of differentiation sections made from chondrogenic mass cultures showed metachromasy upon toluidin-blue staining indicating chondrocyte formation from all three cell types (Fig. 2C). Based on these data we suggest that MSCI-2 cells generated from HUES9 cells retained their multilineage differentiation potential, similar to that found in bmMSCs [4,29], whereas HFF-1 cells possessed reduced differentiation capacity.

3.2.1. Cell surface markers of MSCI cells

In the first set of experiments summarized in Fig. 3 we compared the phenotypic characteristics of three different HUES9-derived polyclonal MSCI cell lines: MSCI-2, MSCI-3 and MSCI-4, which could be cultured for a long period of time, for the expression of the key cell surface molecules of MSCs. As shown by flow
In order to elicit their immunosuppressive function, MSCs cells when ConA but not PHA was used for stimulation (Fig. 4B). Proliferation of lymphocytes could also be suppressed by HFF-1 whereas the PHA-induced mitogenic response was significant in PBL efficiently reduced ConA-induced lymphocyte proliferation, the mitogenic response of lymphocytes in a dose-dependent manner when using bmMSCs as suppressors (Fig. 4C).

Whereas the PHA-induced mitogenic response was significant in HLA-DR. The unique property of HFF-1 cells was their high cell surface expression of CD14, which was undetectable in bmMSC or MSCl cells. The unique property of HFF-1 cells was their high cell surface expression of CD14, which was undetectable in bmMSC or MSCl-2 cells (Table 1). Thus in line with previous publications[17,20,30], the detailed phenotypic analysis of HUES9-derived MSCl-2 and adult bmMSCs cells revealed close phenotypic similarity.

### Expression of common phenotypic markers by MSCs of various origins.

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<th>MSCl-2</th>
<th>HFF-1</th>
<th>bmMSC</th>
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<tr>
<td>CD44</td>
<td>96.97 ± 2.81</td>
<td>93.23 ± 9.44</td>
<td>89.69 ± 10.04</td>
</tr>
<tr>
<td>CD105/Endoglin</td>
<td>89.61 ± 3.95</td>
<td>95.73 ± 6.19</td>
<td>86.08 ± 8.85</td>
</tr>
<tr>
<td>CD73/NTSE</td>
<td>99.51 ± 0.25</td>
<td>99.21 ± 0.22</td>
<td>93.57 ± 6.44</td>
</tr>
<tr>
<td>CD90/Thy-1</td>
<td>96.24 ± 2.41</td>
<td>87.38 ± 7.87</td>
<td>89.78 ± 7.67</td>
</tr>
<tr>
<td>CD24</td>
<td>0.00</td>
<td>1.24 ± 2.49</td>
<td>0.00</td>
</tr>
<tr>
<td>CD14</td>
<td>0.00 ± 1.88</td>
<td>85.27 ± 10.47</td>
<td>0.09 ± 0.19</td>
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<tr>
<td>CD54</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>CD117/c-kit</td>
<td>0.00</td>
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<td>CD133/Prominin 1</td>
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<td>HLA-DR</td>
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In the next set of experiments we sought to measure the concentration of interleukins (IL)-6, IL-10 and IFNγ cytokines in the supernatant of mitogen-activated PBMCs co-cultured with MSCl-2, HFF-1 and bmMSC cells (Supplementary Fig. 4). Our results revealed that bmMSCs induced significant increases in IL-6 (Supplementary Fig. 4C), IFNγ (Supplementary Fig. 4F) and IL-10 (Supplementary Fig. 4I) cytokine levels, whereas MSCl-2 cells were unable to do so even though they efficiently inhibited T lymphocyte proliferation (Fig. 4A, D, and G). The presence of HFF-1 cells induced a slight increase in IL-6 levels (Supplementary Fig. 4B), but the concentration of secreted IFNγ and IL-10 did not change (Supplementary Fig. 4E and H). These results indicate that the production of the functionally relevant cytokines is not an absolute requirement for exerting the immunosuppressive effects of MSCs, and the MSCl-2 cells may use different means to inhibit T-lymphocyte associated effector functions.

Although the detailed immunological characterization of HUES9-derived MSCl-2 needs further investigations, our present results indicate a strong immunosuppressive effect by the HUES9-derived MSCl-2 cells. Our current experiments reinforce previous observations [20,30,45] demonstrating that MSCs derived from human embryonic stem cells have the capacity to suppress peripheral blood T lymphocyte proliferation and also extend them by demonstrating the lack of MSCs-induced enhancement of cytokine secretion upon their immunosuppressive action.

As a summary, we have prepared and characterized human MSCl cells obtained from the human embryonic stem cell line, HUES9. In previous studies various protocols have been applied to obtain MSCs from hESC, including co-culture with mouse OP9 cells [46], embryoid body differentiation [47], spontaneous differentiation in monolayer [20], selection of cell populations by FACS [17], using Rho-associated kinase inhibitors [30], overexpression of HOX84 gene [18] and mechanical separation [19]. In the present study we have developed a novel, simplified method to obtain phenotypically and functionally homogeneous hESC-derived MSC populations. Our derivation protocol required neither the use of xenogenic feeder cell, nor selection (manual or FACS), nor chemical inducers. These MSCl cells showed long term stability and could be maintained in culture over 30 passages without detectable phenotypic or functional changes. Cell surface markers, multipotent differentiation potential and immunosuppressive effects of MSCl-2 proved to be similar to bmMSCs but was different from that of HFF-1. Our data suggest that MSCl-2 cells may be successfully applied as a model system for studying human MSC features and may be used in biotechnology applications.

### 4. Disclosures

The authors indicate no potential conflicts of interest.
Fig. 4. Effect of MSCl-2, HFF-1 and bmMSC cells on mitogenic lymphocyte stimulation. Mitogenic stimulation of PBMCs were measured in the presence of the indicated number of HUES9-derived MSCl-2 (A), HFF-1 fibroblast (B) or human adultBMSC (C). Stimulation of lymphocytes was induced by ConA or PHA and the cells were co-cultured with the indicated numbers of MSCs. Mean ± SD of relative cell growth, Biochem. Biophys. Res. Commun. (2011), doi:10.1016/j.bbrc.2011.09.089

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Appendix A. Supplementary material


References


References (continued)


