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| Original Article |

Selective Depletion of SSEA-3- and TRA-1-60-Positive Undifferentiated Human Embryonic Stem Cells by Magnetic Activated Cell Sorter (MACS)

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Abstract : The capacity of indefinite self-renewal and pluripotency make human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) an attractive source for potential regenerative medicine. However, teratoma formation is one of the major obstacles for implementing hESC-based therapeutics in a clinical setting. While this tumorigenic capacity is known to be lost upon lineage differentiation *in vitro*, there is a potential risk that any residual undifferentiated hESCs or progenitor cells may form tumors upon transplantation. To ensure the safety of hESCs in clinical application, we thus explored the magnetic activated cell sorter (MACS) as a tool for separating undifferentiated hESCs from a mixed population of hESCs and human blood mononuclear cells. Labeled with single or combinations of two fluorsecein-labeled monoclonal antibodies (SSEA-3 and TRA-1-60) and subsequently stained with anti-FITC and/or anti-PE magnetic microbeads, cells were subjected to MACS separation. While a reduction of hESCs by depletion with a single marker was observed, there was still a significant fraction of residual hESCs in the flow-through fraction. However, two consecutive MACS separations upon simultaneous staining of two different hESC markers, SSEA-3 and TRA-1-60, completely depleted hESCs, as validated by flow cytometer, real-time PCR and immunofluoresence microscopic analyses. The maximum efficacy of hESC removal using this protocol was higher than 99.9%. No teratoma formation was observed in hESC-depleted cell injection to NOD/SCID mice. These results indicate that the current MACS protocol with two antibodies can efficiently eliminate undifferentiated cells from differentiated cells and greatly alleviate concerns about tumor formation by hESC-derived cellular therapeutics.

Key words: *MACS, selective ablation, monoclonal antibody, embryonic stem cells, teratoma*

1. Introduction

Embryonic stem cells (ESCs) possess the ability to self-renew indefinitely and to differentiate into almost all known tissue cell types.[1] These characteristics have attracted great attention for their potential use in regenerative medicine. Clinical use of these cells in tissue and cell therapy requires not only specific pathogen-free, GMP-grade but also tumor-free terminally differentiated cells or progenitor cells of interest. Despite significant progress in controlling efficient differentiation of ESCs to a specific lineage, the cellular products are at most a mixture of differentiated and undifferentiated cells. Teratoma forming assay has been widely utilized as evidence by the pluripotency (i.e., developmental potential to three germ layers *in vivo*) of hESCs and induced pluripotent stem cells (iPSCs).[2] While differentiated cells derived from ESCs are not known to be tumorigenic, it is of a great concern that the contaminating undifferentiated cells residing within the population of cellular therapeutics can give rise to teratoma *in vivo* upon ectopic transplantation. Although benign, teratoma or other forms of abnormal cellular outgrowth (tumor) pose a significant health risk and can lead to loss of function of grafted cells and tissues. Indeed, a recent report showed that hESCs engrafted into human fetal tissues developed malignant tumors in SCID mice.[3] The presence of these potentially tumorigenic undifferentiated ESCs in cellular therapeutics for regenerative medicine hampers their clinical application.

While murine ESCs (mESC) as well as mESC-derived differentiated cells (insulin-producing cells, cardiomyocytes, neurons) produced teratomas upon injection into immunocompromised mice,[2] the incidence of teratoma formation

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in immunocompromised animal models by hESC-derived differentiated cells was relatively low compared to murine cells.[3] Lee *et al*.[4] suggested that the number of engrafted hESCs is a critical factor for teratoma formation, showing a minimum requirement of $1\times10^4 \sim 1\times10^5$ hESCs for teratoma development in the myocardium and in skeletal muscle, respectively. However, van der Bogt *et al*.[5] reported that subcutaneous injection of ~100 hESCs can induce teratoma formation, suggesting that contamination of hESC-derived therapeutic cells with undifferentiated cells may cause tumor formation. The discrepancies between mESCs and hESCs in terms of teratogenic potential could be due to species-barriers (microenvironment differences from human cells to murine tissues) or to differences in the de novo capacity of two ESCs in teratoma formation. Other variables including route of injection (subcutaneous, intratesticular, intramuscular and under kidney capsule), composite of injected cells (single cell suspension, aggregates and/or coinjection with Matrigel) and duration, all of which may greatly influence the outcome of tumorigenesis of the injected cells.[4,6] Since the absence of teratoma formation in hESC- or iPSC-derived differentiated cells in immunocompromised mice may also be due to immune rejection, these experimental models do not guarantee their safety in human application.

Due to these variables and uncertainties, clinical application of hESC-derived differentiated cells would require a firm guarantee that they are free from the risk of tumorigenesis. Various approaches have been introduced to avoid this problem.[7] First, generation of genetically engineered ESCs can ensure elimination of undifferentiated ESCs from a cellular product. ESCs equipped with suicide genes under the control of the promoter of stemness genes render undifferentiated cells sensitive to gancyclovir.[8] Alternatively, genetic modification of hESCs with a1,3-galactosyltransferase under hTERT promoter make the undifferentiated cells highly apoptotic to normal human serum.[9] While promising in removing undifferentiated cells, the introduction of xenogene(s) limits their clinical translation, as genetic manipulation raises another regulatory concern. Second, selective induction of apoptosis of the undifferentiated cells in the cellular mixture has been introduced.[10,11] While attractive, this approach requires further confirmation of lack of activity to differentiated cells, as some of these molecules may participate in vital processes of other cell types. In addition, intermediate stage (progenitor) therapeutic cells may be affected by the chemicals. Third, generation of pure differentiated cells from hESCs is an ideal, as differentiated cells lose their ability to form teratoma and possess extremely limited differentiation capacity *in vivo*. Brederlau *et al*. reported that the duration of *in vitro* differentiation of hESCs to differentiated cells was inversely related to the incidence of teratoma in rats, implying that long-term *in vitro* differentiation reduces the content of undifferentiated cells among differentiated cells.[12] However, current methods of differentiation are not able to yield differentiated cells without contaminating undifferentiated hESCs. As a small fraction of residual undifferentiated hESCs among the differentiated cells can generate teratoma in clinical settings, better, more efficient differentiation protocols for hESCs need to be further explored. Fourth, positive selection (enrichment) of differentiated cells or negative selection (depletion) of undifferentiated cells from heterogeneous cellular mixtures by antibody can be employed. Developed, explored and refined in the fields of immunology and hematology for isolation of specific cell types by antibody,[13] these strategies may complement the aforementioned strategies including efficient differentiation protocol. Currently, three major methods for cell isolation by monoclonal antibodies are available: (1) fluorescence-activating cell sorters (FACS), (2) magnetic-activated cell sorter (MACS) and (3) complement-mediated lysis. Of these, MACS provide fast and efficient segregation of undesired cells with great ease. Notably, if the efficiency of a single antibody is low, it is possible that combination(s) of two or more of antibodies can be applied for better resolution. Among the candidates, antigens of globo-series glycolipids (SSEA-3, SSEA-4) and keratin sulfate-related antigens (TRA-1-60 and TRA-1-81) are known to be highly expressed in hESCs and down-regulated upon differentiation.[14] Depletion of undifferentiated SSEA-4-positive cells ESCs prevented the formation of teratoma in ESC-derived hematopoietic precursor cells in allogeneic settings.[15] Recently, Choo *et al*.[16] reported that one monoclonal antibody to specific epitope of PODXL induces selective apoptosis to undifferentiated hESC. Still, the drawback to these approaches is that certain markers can be expressed in differentiated cells of specific lineage, as in the case of SSEA-4 in neural lineage (neural precursor cells),[17] thus limiting their utilization.

In the present study, we examined the efficiency of hESC ablation among differentiated cells by four well-known hESC-specific monoclonal antibodies with MACS to ensure the safety of differentiated cellular therapeutics derived from hESCs. To do this, hESC depletion by single and combinations of antibodies to known hESC makers were examined. To determine whether this strategy prevented tumor formation, we transplanted the sorted cells to immunocompromised mice and demonstrated that a combination of SSEA-3 and TRA-1-60 completely ablated undifferentiated hESCs among the differentiated cells.

2. Materials and Methods

2.1 Human Embryonic Stem Cells and Cell Culture

The work with human ESCs was approved by the Korean Food and Drug Administration as well as the Institutional Review Board of Yonsei University College of Medicine (IRB No. 4-2010-0277). The human embryonic stem cell lines, SNU-hES3 (P30–36) and CHA-hES3 (P88-93), were provided by Seoul National University Hospital (Seoul, Korea) and CHA Hospital (Seoul, Korea), respectively. The cells were routinely cultured in DMEM/F12 supplemented with 20% (v/v) Knockout Serum Replacements, penicillin (100 IU/ml) and streptomycin (100 ug/ml), 0.1 mM nonessential amino acids (all from Invitrogen, Carlsbad, CA, USA), 0.1 mM mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), and 4 ng/ml basic FGF (Invitrogen) as previously described.[18] SNU-hES3 and CHA-hES3 cell lines were grown on the layer of mitotically arrested STO (ATCC, Manassas, USA) feeder cells and mouse embryonic fibroblasts (MEFs), respectively. The media were changed daily and hESC colonies were transferred to a fresh feeder layer every 5-7 days by mechanical passaging.[18]

2.2 Neural Differentiation of hESCs

To prepare embryoid bodies (EBs), hES cell colonies were detached from feeder cells by being incubated with 2 mg/ml of type IV collagenase (Invitrogen) for 30 min and transferring the colonies to Petri dishes containing normal hESC culture medium without bFGF (EB medium). For spontaneous differentiation, EBs were cultured for seven days with medium change every two days. Neural precursor cells and spherical neural masses (SNM) were prepared as described previously.[19] Seven days after EB formation, EBs were cultured in neural precursor cell (NPC) selection media (DMEM/F12 with 1× N2 supplement (Invitrogen) containing bFGF (20 ng/ml) for five days, and the resulting NPCs were expanded for another four days in a modified EB medium containing a 0.5% N2 supplement without serum or serum replacement. For SNM formation, neural rosettes and neural tube-like structures that were observed during neural expansion culture were mechanically isolated and cultured onto bacterial culture dishes containing the NP expansion medium. SNMs were mechanically fragmented into 4~6 pieces and expanded for 7–10 days for passaging.

2.3 Preparation of Single Cell Suspension for Sorting and Flow Cytometry

Single cell suspension of hESCs was prepared as described previously,[20] with some modifications. For detaching hESC cells from feeder layer, ROCK inhibitor (10 μM Y-27632, Sigma)[21] or thiazovivin (0.5 μM, Stemgent, San Diego, CA, USA)[22] was added prior to enzyme treatment. Upon treatment with 1.5 mg/ml of collagenase type IV (Warthington, NJ, USA), clumps of hESCs were collected and further treated with TryPLE Express (Invitrogen). Cell separation was performed using MACS according to the manufacturer's instruction (Miltenyi Biotech, Auburn, CA, USA). Dissociated cells were passed through a 70-μm cell strainer (BD Pharmingen, San Diego, CA, USA) and stained using PE-conjugated anti-SSEA-3, FITC-conjugated anti-SSEA-4 and FITC-conjugated anti-TRA-1-60 or a combination of PE-conjugated anti-SSEA-3 and FITC-conjugated anti-TRA-1-60 (all from BD Pharmingen) at 4°C in PBS/EDTA/0.1% BSA containing 100 units/ml DNase I (Sigma) for 15 min. Cells were washed in PBS containing 5 mM EDTA, resuspended in PBS containing 5 mM EDTA and BSA and stained with either anti-PE-, anti-FITC-microbeads or a combination of both for an additional 15 min at 4 C. After washing, stained hESCs were sorted with two consecutive MACS columns (LS or LC column) as shown in Fig 1. After collection, pre-stained cells and cells of positive and negative fractions were analyzed by flow cytometer, real time-PCR or immunofluorescence microscope for cells stained with fluorochrome-conjugated antibodies to assess purity.

Cell surface antigens were labeled through incubating with fluorescein-conjugated primary antibodies for 15 min in the dark at 4°C. The stained cells were analyzed by Cytomics™ Flow

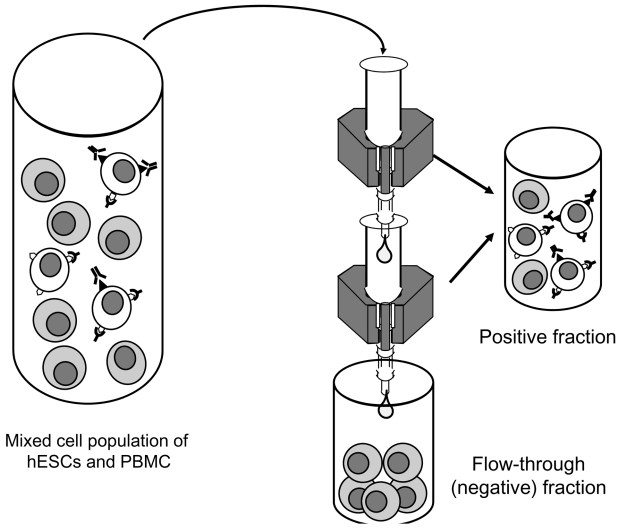

Figure 1. Schematic of serial MACS for the depletion of undifferentiated hESCs. After staining with two different antibodies to specific surface markers, the cells were then sorted into 2 consecutive MACS columns (LS column) to deplete unwanted (antibody-bound) cells from the mixture of heterogenous cells.

Cytometer (Beckman Coulter, Fullerton, CA, USA). Twenty thousand events were registered per sample and cell analysis was performed using the appropriate scatter gates to avoid cellular debris and aggregates. Data were processed using WinMDI 2.8 software (http://facs.scripps.edu/help/html/read1ptl.htm). Efficiency of hESC depletion was calculated using the following equation: Efficiency (%) = 1 – (% hESC cells in the flow-through fraction/% hESC in starting population).

2.4 Formation of Tumors in SCID Mice and Histological Analysis

All animal procedures were approved under the guidelines of the Health Sciences Animal Policy and Welfare Committee of the Yonsei University College of Medicine (IACUC #2010-0193). Single cell suspension of hESC-depleted (negative fraction of SSEA-3 and TRA-1-60 MACS-sorting) or hESC-enriched fraction cells were washed once in PBS, then resuspended in PBS at 2×10^6 cells/ml. A 100 μl aliquot was implanted beneath the testicular capsule of young (8-week) female NOD/SCID mice (Orent Bio, Seoul, Korea). Teratoma growth was determined weekly by palpation, and the mice were sacrificed (cervical dislocation) 12 weeks after implantation. The tissue was fixed, and sections were stained with Hematoxylin & Eosin. Immunohistochemistry was performed on formalin-fixed/paraffin-embedded tissues. The sections were deparaffinized in xylene and hydrated through sequential ethanol gradient. Antigen retrieval was carried out by heating the sections in citrate-EDTA buffer (10 mM citric acid, 2 mM EDTA, 0.05% Tween-20, pH 6.2) or Tris-EDTA buffer (10 mM Trizma-base, 1 mM EDTA, 0.05% Tween-20, pH 9). The sections were blocked for 30 minutes in 5% serum. Human nuclear antigen (HuNu, Chemicon, Temecula, CA, USA) staining was performed using an HRP-conjugated secondary antibody followed by incubation with diaminobenzidine solution (Vector Laboratories, Burlingame, CA, USA).

2.5 Quantitative RT-PCR (qRT-PCR) Analyses

Total RNA was extracted using a Easy-Spin® total RNA purification kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions and then 1 μg of the total RNAs were reverse transcribed with Power cDNA synthesis kit (iNtRON). qRT-PCR was performed using SYBR Premix Ex Taq TM (Takara Bio Inc, Shiga, Japan) and the reaction was carried out using the My-iQ or CFX96 Real-Time System (Bio-Rad, Hercules, USA) under the following conditions; (step 1) 1 min at 95°C; (step 2) 40 cycles of 20 s at 95°C, 20 s at 63°C, and 20 s at 72°C; (step 3) final extension for 1 min at 72°C. The expression values (Ct values) of specific marker genes were collected and normalized according to those of β-actin, and then the normalized expression levels of the markers were compared between MACS-sorted cells according to the ΔΔCt method.[23] All of the data was confirmed by at least three independent experiments. The primer sequences were as follows: Oct4-F 5'-tgg gct cga gaa gga tgt g-3" Oct4-R 5'-gca tag tcg ctg ctt gat cg-3'; β-actin-F 5'-gct ctt ttc cag cct tcc tt-3'; β-actin-R 5'-ctt ctg cat cct gtc agc aa-3'. For statistical analysis, ΔΔCt values of samples were subjected to a one-tailed t-test. Significant differences between groups were defined as $p < 0.01$.

3. Results

3.1 Marker Changes During *In Vitro* Neural Differentiation of hESCs

Undifferentiated hESCs are typically characterized by positive staining for pluripotent stem cell markers, such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, Oct4 and Nanog. We examined the expression profiles of the surface markers during the differentiation of hESCs toward neural lineage cells. While surface expression of SSEA-3 and TRA-1-60 was dramatically down-regulated in neural precursor cells (from 98.3±5.2 to 8.3 ±4.6 for SSEA-3 and from 83.7±9.1 to 1.6±1.2 for TRA-1-60, respectively), TRA-1-81 was expressed in substantial amount in neural precursor cells (Fig 2).

Stage specific embryonic antigen 4 (SSEA-4) was continuously expressed on neural rosettes and spherical neural masses (SNM), albeit at a reduced level. This result implies that (1) ESC-derived neural progenitor and precursor cells resemble native human embryonic brain-derived neural progenitor cells as the latter expresses SSEA-4[17] and (2) SSEA-4 is not a suitable marker for undifferentiated ESC ablation in neural lineage cells. Thus, our data indicate that cells of SNM are highly related to typical neural progenitor cells of the normal human brain.

3.2 Depletion of Undifferentiated hESCs by a Single Marker

The data from flow cytometric analyses of key stem cell markers suggest that these markers can be used for hESC depletion from heterogeneous population of differentiated cells. To do this, we examined whether each marker can deplete significant portions of hESCs among differentiated cells by MACS. For separation and subsequent transplantation, we deliberately generated a mixture of hESCs and normal human peripheral blood mononuclear cells (PBMCs) at a ratio of 1:4 ~1:5. Thus, the starting cell population contained 20~25% hESC-marker positive cells (Fig 3). PBMC cells displayed little

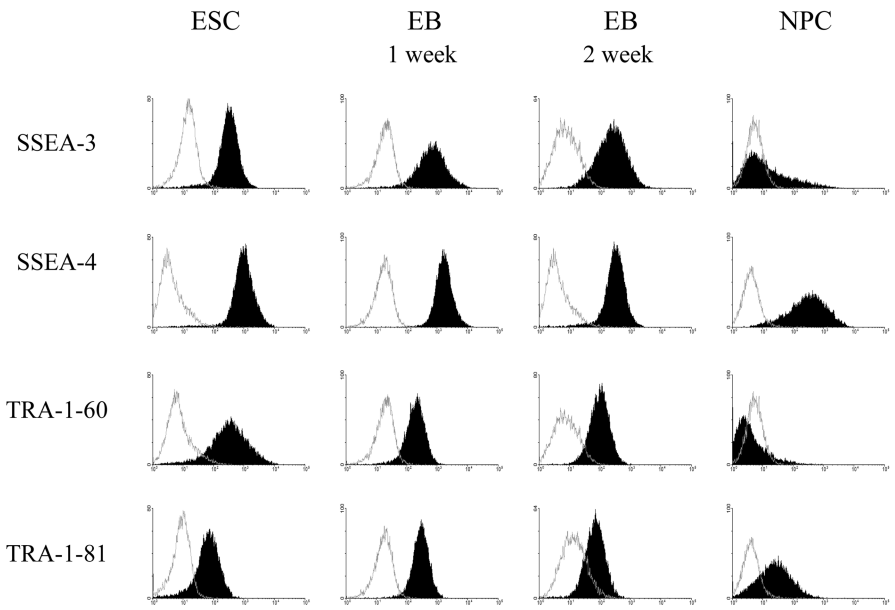

Figure 2. Flow cytometric analysis of hESCs, EBs and neural precursor cells. Representative histograms of the human ESC line (SNU-hES3) expressing SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. SSEA-4 and TRA-1-81 are substantially expressed on neural precursor cells. Isotype control is shown as an open histogram.

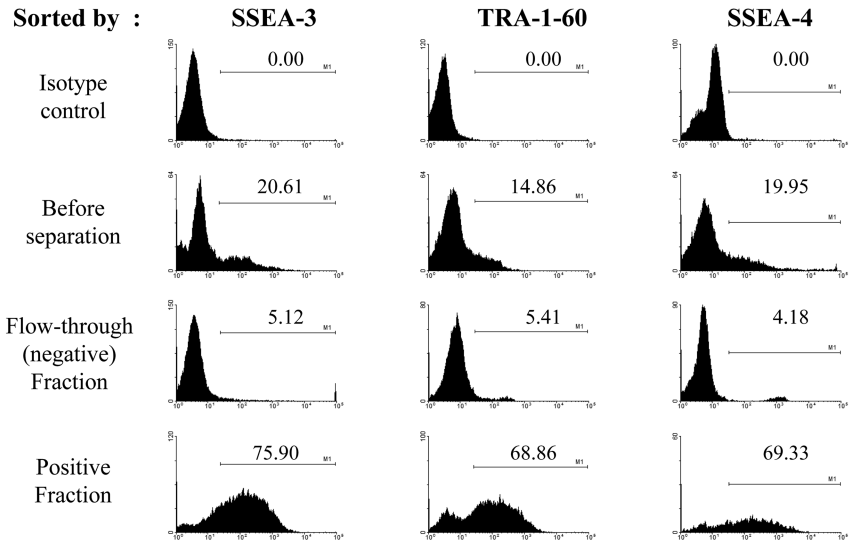

Figure 3. Immunomagnetic selection of hESCs from a mixture of hESCs and normal human PBMCs. Prior to separation, hESCs and normal human PBMCs were deliberately mixed with a ratio of 1:4 ~1:5. After staining with fluorochrome-conjugated antibodies against SSEA-3, TRA-1-60 or TRA-1-81 cells and subsequently with microbead-conjugated anti-PE- or anti-FITC antibodies, cells were subjected to MACS separation. Depletion of marker expressing cells in the flow-through fraction still contained substantial amounts of undifferentiated marker-positive cells.

positivity to these hESC makers. Since SSEA-4 was continuously expressed on differentiated cells derived from hESCs, we did not perform further analysis of SSEA-4. When a monoclonal antibody to a single marker was utilized, depletion of hESCs among flow-through (negative) fraction was incomplete. Routinely, 3-5% of negative fractions contained marker-positive cells (hESCs). Retention of marker-positive cells in the MACS column were 60~80%. This is in line with one previous report,[24] in which a single antibody yielded 4-7% contaminated hESCs on the negative fraction.

3.3 Depletion of Undifferentiated hESCs by Dual Markers

Since these pluripotency markers are not completely shared by all hESCs, we attempted to utilize two monoclonal antibodies to completely deplete hESCs expressing even a single antigen. In addition, we assembled two MACS column top to bottom to deplete further the antibody-stained cells from the flow-through fraction of the first column (Fig 1). Upon retrieval from the MACS column, the purity of flow-through reached an almost 100% hESC-free cell population (Fig 4A). Combination of SSEA-3 and TRA-1-60 antibodies yielded less than 0.1% hESCs. Two serial MACS purifications significantly reduced the number of eluted cells by 50%. Increases in marker-negative cell numbers in the positive fraction were due to high retention during the two consecutive column separation, as compared to the single quick MACS separation for single marker-base sorting. In addition, blood monocytes in the PBMC served as a main contaminating cell type in the positive fraction as they express FcR and captured monoclonal antibodies to hESCs in a nonspecific manner, despite the presence of a high concentration of FcR blocking reagents.

The absence of contaminating undifferentiated hESCs (SSEA-3- and/or TRA-1-60-positive cells) in the negative fraction was further confirmed by detection of immunofluorescent cells under microscopic observation (Fig 4B). In contrast, fluorescent cells were frequently detected on the positive fraction. In addition, qRT-PCR further confirmed the absence of Oct-4+ cells in the negative fraction (Fig 4C).

3.4 Teratoma Formation

Flow-through (negative) fraction failed to generate teratoma in NOD/SCID mice for at least 12 weeks. The viability of both positive and negative fractions was routinely above 80%. These data suggest that purified single cell suspensions are highly fragile or unable to gain access to the vascular network of host tissue to survival. Undifferentiated hESCs as aggregates corresponding to 1×10^5 cells formed teratoma in NOD/SCID mice (Fig 5), positive fractions containing single cell suspension of hESCs failed to produce teratoma in the immunodeficient mice after 12 weeks. We further tested whether the transplanted hESCs remained 12 weeks after transplantation by immunohistochemical staining of anti-

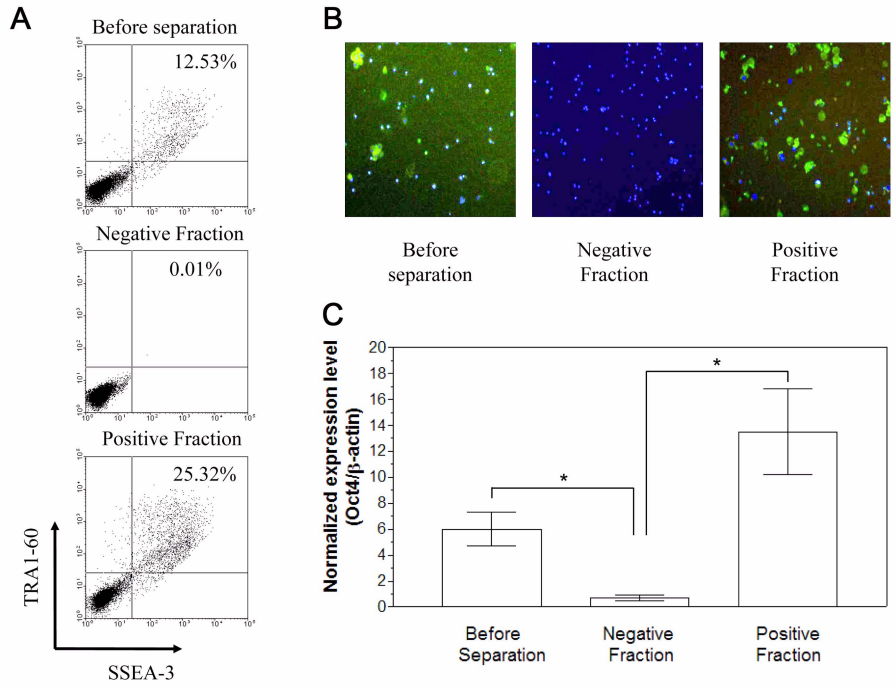

Figure 4. Complete depletion of hESCs by serial MACS with two hESC surface markers. Representative flow cytometric data of SSEA-3 and TRA-1-60 expression in (A) the unsorted mixed population of hESCs and PBMCs, the cells retained in the column (positive fraction), and the flow-through fraction are shown. The values are ratios of single positive or double positive cells to SSEA-3 or TRA-1-60. (B) No SSEA-3$^+$ could be detected in the "flow-through" fraction in contrast with frequent fluorescent cells in the positive fraction and unsorted cells. Immunofluorescence of SSEA-3-stained cells (green) is shown with DAPI-stained nuclei (shown in blue). (C) qRT-PCR of Oct4 confirmed the absence of hESCs in the "flow-through" fraction. The relative Oct4 gene expression of each cell fraction was determined by real-time PCR. Oct4 gene expression was normalized to β-actin. *p <0.01.

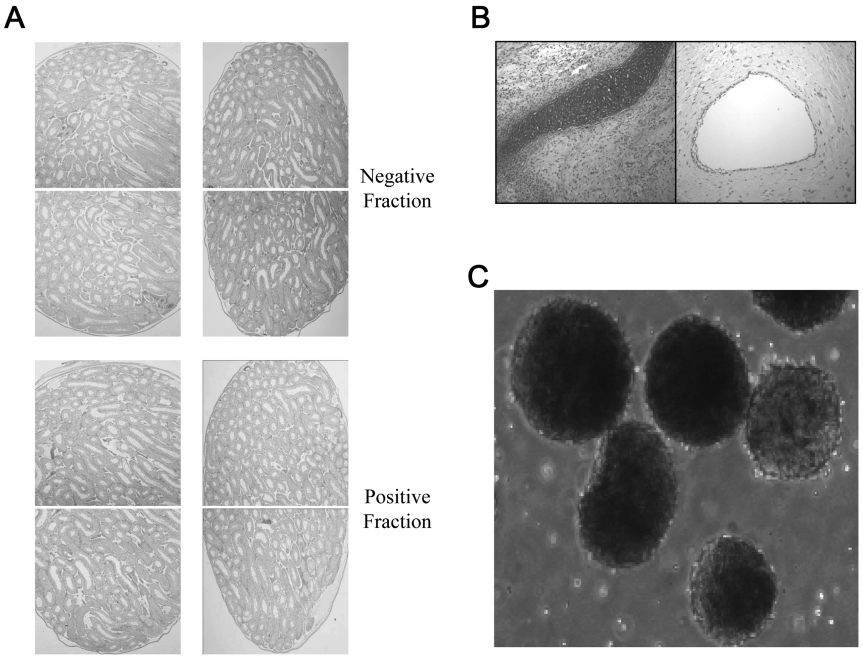

Figure 5. Histological analysis of testis tissue upon intratesticular transplantation reveals no teratoma formation. (A) Photomicrographs of NOD/SCID mouse testis tissue obtained 12 weeks postimplantation of 2×10^5 cells of hESC-depleted (negative fraction) or hESC-enriched (positive) fraction into the testicular capsule of the host mice. None of the transplanted testes showed evidence of tumor or teratoma formation on histological evaluation upon H&E staining. (B) Formation of teratomas after transplanted hESC aggregates into the testes of NOD/SCID mice. Histological analysis of the teratoma reveal the presence of mesodermal and endodermal tissues defined by Alician blue stained cartilage (left) and gut epithelium-like structure (right), respectively. Magnification ×100. (C) EB aggregates were formed from SSEA-3^+TRA1-60^+ fraction of MACS isolation. Positive fraction cells from MACS column retained viability during in vitro manipulation.

human nuclear antigen. No hESC-derived cells were detected in the cells of testis tissue (data not shown). Normal mouse testis tissue sections served as an internal negative control. In order to assess the viability of hESCs upon dual MACS column separation, we induced the formation of an embryoid body from cells of positive fraction. As shown in Fig 5B, typical EB aggregates were formed, indicating that cells subjected to MACS separation retain viability during *in vitro* manipulation (i.e., cell harvesting, washing, staining, MACS separation and elution from column).

4. Discussion

At present, the generation of pure therapeutic cells from hESCs remains a challenge; thus, it is essential to develop a strategy to delete the residual undifferentiated cells with teratogenic potential. Here, we have presented our data showing that two consecutive MACS column separation with a combination of two monoclonal antibodies were able to achieve this goal. Conventional hESC markers (SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81) were examined for their efficiency of hESC depletion by MACS. For decades, MACS has provided the most selective approach for positive selection and negative depletion and had been successfully applied to basic and clinical research for purifying, ablating specific cell types of interest. The limiting factors of MACS separation are the presence of (1) selectable markers and (2) high affinity antibody to the markers. From the flow cytometric data, we gathered that SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 were good candidates for MACS selection and applied them to our current strategy.

While we provided evidence that a combination of ESC markers could completely deplete undifferentiated cells, certain ESC marker(s) may not be suitable for depleting undifferentiated cells depending on cell lineage. For example, Shibata *et al.*[15] demonstrated that removal of SSEA-4-positive cells in cynomolgus ESC-derived hematopoietic precursors prevented the formation of teratoma in allogeneic transplantation. However, this marker cannot be used in neural cell lineages as we found that ESC-derived neural precursor cells commonly expressed this marker, albeit at a lower level than ESC. This is in accordance with a previous report[17] showing that human brain neural progenitor cells expressed SSEA-4.

The two-consecutive MACS strategy provided that SSEA-3$^+$ and/or TRA1-60$^+$ cells of flow-through fraction contained less than 0.1% of cells from a mixture of 10-25% hESCs with differentiated cells (PBMC). Since the resolution of flow cytometric phenotyping routinely exceeds the level of 0.01%, we further examined the content of hESCs among the negative fraction by immunofluorescence microscope. Of more than 10,000 cells examined, no fluorescent cell (antibody-stained cell) was detected implying that the contamination did not exceed 0.01% of the total cells. In addition, no signal was detected by RT-PCR of the negative fraction, implying that this approach can yield almost complete ablation of undifferentiated hESCs among negatively selected cells. In addition, we failed to observe any teratoma formation in hESC-depleted (negative) fraction as well as in hESC-positive fraction for at least 12 weeks. Unlike in other reports[6,25] with hESC aggregates (equivalent to 10^6 hESCs), we injected single cell suspensions of 2×10^5 cells (i.e., containing less than 5×10^4 hESCs in the positive fraction of MACS separation) and observed no teratoma formation in the testis by the positive fraction. Typically, the rate of teratoma formation in the testis has been found to be 94% when 1×10^6 undifferentiated cells were injected.[26] The reason for this could be due to (1) the lack of tumorigenic potential in small numbers of hESCs, (2) suboptimal number of hESCs or (3) poor viability of single cell suspension after MACS as compared to the aggregate form normally used. This is in line with a previous report by Hentze *et al*.,[6] which stated that there was a linear correlative relationship between the number of cells and the time required to form teratoma by single cell-adapted hESC lines at passage below 30. Low efficiency of teratoma formation may also be due to the subculture method of hESC maintenance. Collagenase-passaged hESCs failed to generate teratoma when single cell suspension was prepared using trypsin digestion, unlike efficient teratoma formation by trypsin-adapted hESCs.[27] Given that teratoma formation must be prevented in clinical cases, this result with single cell suspension containing hESCs provides promise for a cell therapy strategy.

A recent publication by Schriebl *et al*.[28] with a mESC model proposed that MACS technology is theoretically insufficient to achieve purification of undifferentiated cell-free cellular therapeutics derived from ESCs. By their mathematical estimation, more than 30 steps of repetitive MACS separation is required to achieve less than 10^{-1} cells among 10^9 cells for therapy, which may not be feasible for viability and quantity of outcome. This model, however, reflected the safety issue too strictly by limiting contaminating cells to 0.0000001% of the total. Based on the differences in efficiency of teratoma formation between murine and human hESCs, the minimum required number of hESCs for teratoma formation has not been evaluated thoroughly and may not be in this range, but at least to the 2-4 order higher than this in reality. In addition, other variables, including the viability of hESCs *in vivo* as a single cell suspension, survival, immunological recognition and eventual formation of tumors in the local microenvironment will further increase the order by a factor of 2-3. Considering these parameters, 10^4~10^5 cells in 10^9 cells (i.e., 0.001~0.01%) would serve as a safe margin for their clinical application. In line with this, Shibata *et al*.[15] reported that the purging of SSEA-4-positive cells from cynomolgus ESC-derived hematopoietic progenitor cells prevented the formation of teratoma upon transplantation into the allogeneic monkey model. While flow cytometric depletion of hESCs would yield a purer population with little or no contaminating cells, only a few studies have succeeded with this technique due to its slow sorting speed, which affects the viability of sorted cells significantly.[15,29,30] Further refining of MACS separation by high avidity antibodies and protocols, on the other hand, may provide means to fast and efficient depletion or purification with greater survival.

In conclusion, our results demonstrated that two-column MACS separation is efficient in complete depletion of hESCs among a heterogeneous cell population. As the overall process of staining and separation do not exceed one hour, the viability of both positive and negative was acceptable as determined by trypan blue and EB forming assay. Thus, MACS strategy can yield a highly pure undifferentiated hESC-free, viable cell preparation suitable for their clinical application. Multi-step MACS with antibodies against two or more markers with high specificity and avidity may offer a better resolution for conferring cellular therapeutics with no tumorigenic potential. Utilization of other strategies for selective ablation and/or apoptosis by chemical or cytotoxic antibody may further enhance the efficiency of specific cell isolation and/or depletion.

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