Stem Cell Rev and Rep (2009) 5:72–80
DOI 10.1007/s12015-009-9054-4

Separation of SSEA-4 and TRA-1–60 Labelled Undifferentiated Human Embryonic Stem Cells from A Heterogeneous Cell Population Using Magnetic-Activated Cell Sorting (MACS) and Fluorescence-Activated Cell Sorting (FACS)

Chui Yee Fong · Gary S. L. Peh · Kalamegam Gauthaman · Ariff Bongso

Published online: 31 January 2009

Abstract A major concern in human embryonic stem cell (hESC)-derived cell replacement therapy is the risk of tumorigenesis from undifferentiated hESCs residing in the population of hESC-derived cells. Separation of these undifferentiated hESCs from the differentiated derivatives using cell sorting methods may be a plausible approach in overcoming this problem. We therefore explored magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) to separate labelled undifferentiated hESCs from a heterogeneous population of hESCs and hepatocellular carcinoma cells (HepG2) deliberately mixed respectively at different ratios (10:90, 20:80, 30:70, 40:60 and 50:50) to mimic a standard *in vitro* differentiation protocol, instead of using a hESC-differentiated cell population, so that we could be sure of the actual number of cells separated. HES-3 and HES-4 cells were labelled in separate experiments for the stem cell markers SSEA-4 and TRA-1–60 using primary antibodies. Anti-PE magnetic microbeads that recognize the PE-conjugated SSEA-4 labelled hESCs was added to the heterogeneous cell mixture and passed through the MACS column. The cells that passed through the column ('flow-through' fraction) and those retained ('labelled' fraction') were subsequently analysed using FACS. The maximum efficacy of hESCs retention using MACS was 81.0±2.9% (HES-3) and 83.6±4.2% (HES-4). Using FACS, all the undifferentiated hESCs labelled with the two cell-surface markers could be removed by selective gating. Both hESCs and HepG2 cells in the 'flow-through' fraction following MACS separation were viable in culture whereas by FACS separation only the HepG2 cells were viable. FACS efficiently helps to eliminate the undifferentiated hESCs based on their cell-surface antigens expressed.

Keywords Fluorescence activated cell sorting · Magnetic activated cell sorting · Human embryonic stem cells · Hepatocellular carcinoma cells

C. Y. Fong · G. S. L. Peh · K. Gauthaman · A. Bongso (✉)
Department of Obstetrics and Gynaecology,
Yong Loo Lin School of Medicine,
National University of Singapore,
5 Lower Kent Ridge Road,
Singapore 119074, Singapore
e-mail: obgbongs@nus.edu.sg

Introduction

The isolation of human embryonic stem cell (hESC)-like cells from the inner cell mass of human blastocysts [4] followed by the derivation of a hESC lines [22, 29] has made hESC-derived tissue transplantation therapy a future possibility for the treatment of a variety of incurable diseases. In fact, successful engraftment of hESC-derived neurons, pancreatic islets and cardiomyocytes have been recently reported in Parkinsonian [32], diabetic [27] and ischaemic [13] animal models. However, hESC-derived tissue transplantation is fraught with major hurdles which need to be overcome before this science can enter human clinical trials. These problems include (1) inadequate cell numbers for treatment (2) immunorejection and (3) tumorigenesis. Generally, pluripotent cells of human or mouse origin induce teratomas in normal and immunosuppressed mice. Thus far, hESCs derived from surplus IVF embryos,

induced pluripotent stem cells (iPSCs) and human embryonal carcinoma cells (hEC) have been confirmed to induce teratoma formation in mice [1].

How teratomas are actually formed *in vivo* in mice when hESC-derived tissues are transplanted have not been properly studied to help understand what would actually happen when such tissues are transplanted in the human. Mouse embryonic stem cell (mESC)-derived insulin producing islets [9], mESC-derived cardiomyocytes [5] and mESC-derived neurons [8] produced teratomas when injected into immunosuppressed mice. Undifferentiated mESCs consistently formed teratomas when injected into normal and infarcted hearts of nude mice [18]. However, injection of hESC-derived cardiomyocytes [13] and osteocytes [3] into ischemic hearts and malformed bone respectively did not yield teratomas within one month of injection. Injection of hESC-derived neurons into the brain of Parkinsonian rats did not yield teratomas after 12 weeks [2] and in a more recent study, hESC-derived neurons injected into the brain of immunosuppressed fetal mice did not generate teratomas after 8 weeks [32].

Certain organs appear to favour teratoma formation while others do not. For example the rate of teratoma formation with hESCs in immunodeficient mice was site-dependent with a 25–100% incidence when injected subcutaneously, 60% intratesticularly, 12.5% intramuscularly and 100% when injected under the kidney capsule [20]. In another study, the pancreas appeared to be partially site-privileged while the thymus and lung had the highest incidence of teratoma formation [26].

It is also not clear as to how many undifferentiated hESCs actually induce a teratoma *in vivo*. Lawrenz et al. [15] carried out studies in the mouse where they injected defined numbers of mESCs together with normal lung cells (MRC-5) under the kidney capsule of nude mice and separately with matrigel subcutaneously. They reported that as few as 2 mESCs induced teratoma formation. Different results were obtained with hESCs when Shih et al. [26] injected hESCs into immunosuppressed mice. In all mice studied by them, up to 5,000 hESCs were required to produce teratomas while up to 50 hESCs did not produce teratomas at all in any of the animals studied. Thus, at the present moment in time it is not clear as to what the minimum number of hESCs are that will induce a teratoma at least 8 weeks after injection which is the usual cut-off time for any teratoma formation in the conventional teratoma assay in SCID mice used for hESCs. It is also not clear in all the above studies whether actual single cells in suspension were injected or whether the cells aggregated into clusters at the time of injection. It is also possible that the longer hESCs are encouraged to differentiate *in vitro*, the lesser the risk of teratoma formation from any left-over undifferentiated hESCs.

Various strategies have been suggested to eliminate rogue undifferentiated hESCs residing in differentiated hESC-derivatives. These include the selective induction of apoptosis of the hESCs in the heterogeneous cell population, the development of antibodies that specifically target undifferentiated hESCs, the separation of the undifferentiated hESCs from the differentiated cell population prior to transplantation using cell sorting methods alone or in combination with density gradient separation, deliberate extended differentiation of hESC-derivatives to allow any undifferentiated hESCs to differentiate into an undesirable cell type. If one approach alone is not successful, it is possible that combinations of two or more of the above approaches may be required. In this paper we report the preliminary results of an attempt at separation of undifferentiated hESCs from a mixed heterogeneous cell population containing hESCs and somatic cells using surface antigen labelled hESCs with magnetic activated cell sorting (MACS) and fluorescent activated cell sorting (FACS).

Materials and Methods

Cell Culture

HepG2 cells were used instead of normal somatic cells in this study because (1) they were freely available in our laboratory, (2) their large nuclear size and phenotype helps to distinguish them more easily from undifferentiated hESCs and any left-over differentiated hESCs from culture and (3) they closely resemble normal hESC-derived liver cells.

Ethical approval for the use of hESC lines (HES-3, 46XX and HES-4, 46XY); ES Cell International, Singapore) and the commercially available human hepatocarcinoma cell line, HepG2 (American Type Culture Collection, Rockville, MD, USA) was obtained from the Institutional Review Board, National University of Singapore. The hESC lines were maintained in DMEM/F12 medium containing 20% knock-out serum-replacement, 2 mM L-glutamine, penicillin (50IU), streptomycin (50 μg/ml), 1× nonessential amino acids, and 0.1 mM β-mercaptoethanol (Invitrogen Life Technologies, CA, USA) cultured on mitomycin-C-treated mouse embryonic fibroblasts (MEFs; $1.8\times10^4/cm^2$) and passaged weekly using Type IV collagenase (Invitrogen) at a 1:4 ratio. Individual colonies were mechanically cut with sharp hypodermic needles into small pieces and transferred to fresh mitomycin-C-treated MEFs (MMC) at each passage. HepG2 was maintained in DMEM containing 10% fetal bovine serum (Biochrom), and propagated as a monolayer culture. Cells were maintained at 37°C in a humidified incubator of 5%CO_2/95% air.

Dissociation, Labelling and Mixing of Cells

HES-3, HES-4 and HepG2 cells were dissociated using TrypLE Express (Invitrogen) for 5 min at room temperature. Subsequently, the cells were spun down at ×1,000 rcf for 5 min and resuspended in 10 mL wash buffer (PBS^{-} containing 4 mM Hepes buffer and 5% normal goat serum; 0.22 μm filtered). Dissociated cells were filtered through a 40 μm cell strainer cap (Becton Dickinson, BD) to eliminate cell clumps before cell counts were performed on the single cell suspension for both HES-3, HES-4 and HepG2.

The cells were labelled with two different primary antibodies: mouse IgM α-TRA-1–60 (10 μg/ml; Chemicon), and Phycoerythrin (PE) conjugated mouse IgG_3 α-SSEA4 (0.6 μg/ml; eBioscience) and their respective secondary antibodies: goat α-mouse IgM fluorescein isothiocyanate (FITC) conjugate (6.5 μg/ml; Sigma) and magnetic α-PE microbeads (Miltenyi Biotech) for 15 min at room temperature. The magnetic microbeads recognise SSEA-4 positive hESCs, which aid in their retention upon passing through the MACS column, and were used at a concentration of 20 μl for 10^7 cells as per the manufacturer's protocol. Based on the initial cell counts, labelled hESCs were then mixed with HepG2 at the ratios of 10%, 20%, 30%, 40% and 50%.

Magnetic Cell Sorting and Flow Cytometry

For MACS, cell mixtures were washed with MACS buffer (PBS containing 2 mM EDTA and 0.5% BSA). The washed cells were re-suspended and applied to the MS column of MiniMACS magnetic separation kit (Miltenyi Biotech). The cell mixture was then passed through the magnetic column followed by a series of three washes (500 μl each). The total effluent was collected as the 'flow-through' fraction. The MS column was then removed from the magnet and by the means of a plunger, 1 ml of MACS buffer was used to flush the microbead labelled cells out from the column which were initially retained by the magnetic field. The effluent was collected as the 'labelled' fraction. To investigate the purity of the magnetically separated cells, both the 'flow-through' and 'labelled' fractions, together with the unsorted cell mixture (the original cell mixture that were not subjected to the MS column) were subsequently analysed using fluorescence activated cell sorting (FACS).

The FACS analyses of the MACS separated cells were performed using a FACS-Altra instrument (Beckman), whereas cell sorting were performed using a FACS-Vantage SE (BD). For each experiment, the background fluorescence was measured using both unlabelled cell mixture and cell mixture labelled with the appropriate concentration of isotype-matched controls: α-mouse IgM (10 μg/ml; Caltag) and PE-conjugated α-mouse IgG_3 (0.6 μg/ml; eBioscience). These set the gating parameters between the positive and negative cell population. Data obtained were evaluated using winMDIv2.8 program (http://facs.scripps.edu/software.html) and expressed as Mean ± standard deviation.

Results

Efficacy of MACS to Separate hESCs from HepG2 Cells

FACS analysis of the starting cell populations showed that 77.2±8.4% of HES3 cells were SSEA-4 positive (Fig. 1A) and 71.6±7.5% of HES-3 cells were TRA-1–60 positive (Fig. 1B). HES-4 showed 88.8±1.6% for SSEA-4 expression (Fig. 1C) and 77.7±5.6% for TRA-1–60 expression (Fig. 1D). HepG2 cells showed artifactual staining of 1.2±0.7% and 2.1±1.2% for the SSEA-4 (Fig. 1E) and TRA-1–60 (Fig. 1F) groups respectively.

When MACS was used, the retentions of SSEA-4 positive HES3 and HES4 cells (PE tagged magnetic microbead bound to SSEA-4) varied between the different mixed ratios. The efficacy of MACS to deplete SSEA-4 positive HES3 and HES4 cells from the heterogeneous mixture of hESCs and HepG2 cells was calculated using the formula:

$$\mathbf{E}(\text{efficacy}) = 1 - \frac{\%\text{SSEA}-4 \text{ positive cells detected in the 'flow}-\text{through' fraction}}{\%\text{SSEA}-4 \text{ positive cells in the unsorted starting population}}$$

As such, the retention of the SSEA-4 positive cells on the magnetic column were 81.0%, 79.7%, 75.8%, 74.8% and 64.6% for HES-3 and 83.6%, 82.7%, 82.7%, 78.4%, 75.1% for HES-4 when mixed with HepG2 in ratios of 10:90, 20:80, 30:70, 40:60 and 50:50 respectively (Fig. 2A). It appears that the efficacy of separation decreased as the percentage of hESC ratios increased. The 'unlabelled' flow-through fraction showed that the percentage of SSEA-4 positive HES-3 cells decreased linearly from 18.1±3.5% to 2.7±0.5% as the ratios of HES-3 to HepG2 cells in the heterogeneous population decreased from 50% to 10% (Fig. 2B; Table 1). Similarly, TRA-1–60 labelled HES-3 cells also decreased linearly from 21.7±4.3% to 4.0±2.0% as the ratios of HES-3 cells decreased from 50% to 10% (Table 1). The overall expression of TRA-1–60 positive cells was greater than then SSEA-4 positive HES-3 cells. Similar observations were seen in studies using the second hESC line (HES-4; Table 1).

To understand why the microbead-labelled hESCs were not efficiently retained by the MACS column, we separately analysed the two hESC lines HES-3 and HES-4 as three different fractions viz., the 'unsorted', 'flow-through' and 'labelled' by FACS following MACS separation. The levels

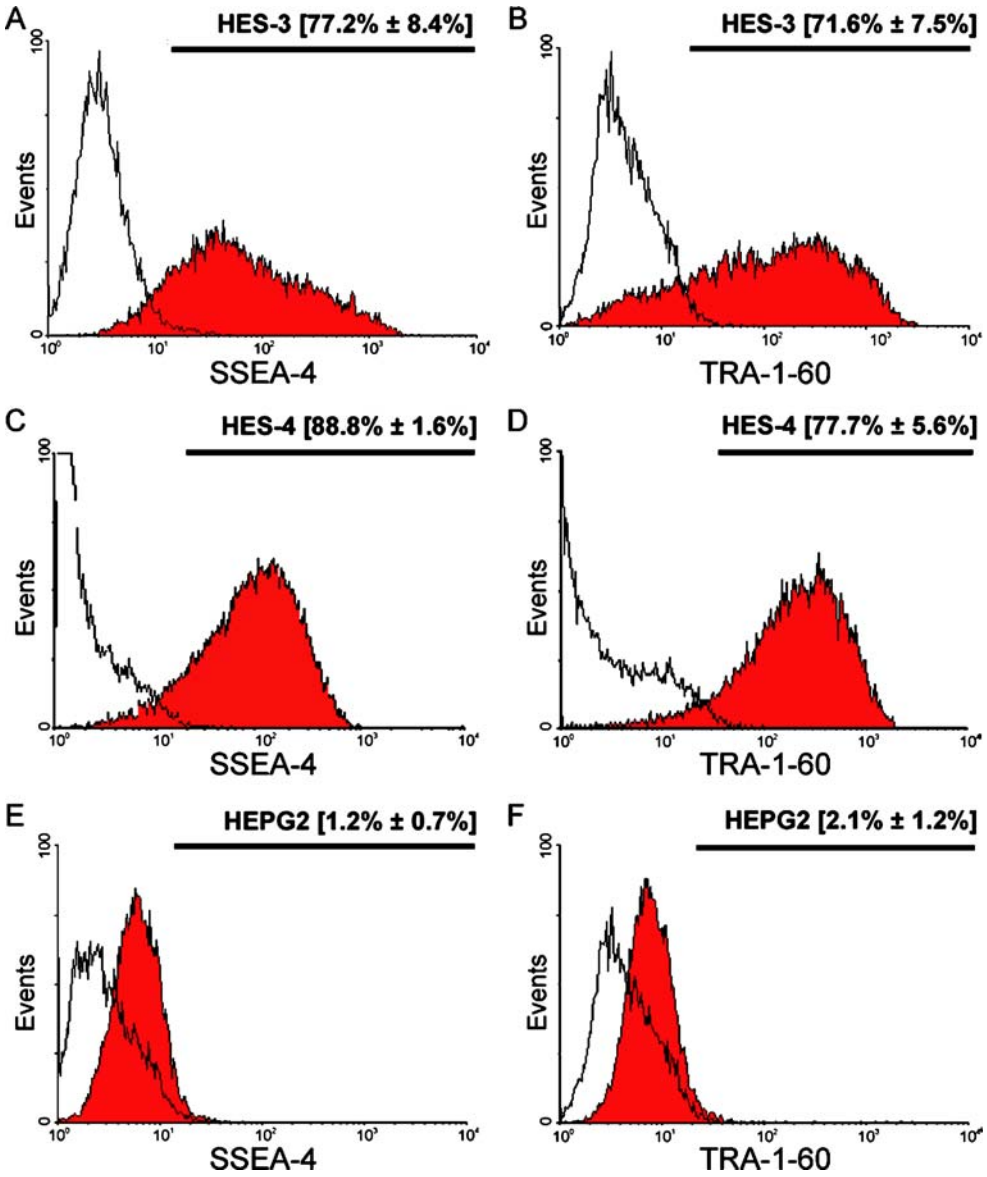

Fig. 1 Representative flow cytometric plots of human embryonic stem cell (hESCs) HES-3 labelled with antibodies for **A** SSEA-4, and **B** TRA-1–60; and HES-4 for **C** SSEA-4, and **D** TRA-1–60, each with their respective isotype-matched negative controls (*clear plot with black lines*). The hepatocellular carcinoma cells (HepG2) used in this study did not express **E** SSEA-4 or **F** TRA-1–60. All values are expressed as Mean ± SD

of background fluorescence for SSEA-4 and TRA-1–60 were determined using the appropriate isotope-matched controls (Fig. 3A). Based on the above control, four subpopulations of hESCs were observed: SSEA-4 positive / TRA-1–60 positive (63.9±1.1% for HES-3 and 71.7±4.2% for HES-4); SSEA-4 positive /TRA-1–60 negative (21.8± 1.9% for HES-3 and 17.0±5.3% for HES-4); SSEA-4 negative /TRA-1–60 positive (6.7±3.1% for HES-3 and 6.0±0.2% for HES-4); and SSEA-4 negative /TRA-1–60 negative (7.5±0.7% for HES-3 and 5.3±1.3% for HES-4; Fig. 3B). The purity of the SSEA-4 positive hESCs isolated using MACS ('labelled' fraction) were over 97%. However, it was evident from our data that 72.8±1.7% (HES-3) and 78.3±5.9% (HES-4) of these SSEA-4 positive hESCs were also TRA-1–60 positive; whereas 25.8±1.3% (HES-3) and 19.3±6.7% (HES-4) were not (Fig. 3C). For the flow-through fraction obtained, 46.3±2.2% of the SSEA-4 positive HES-3 cells were Tra-1–60 positive while 18.8± 3.1% was Tra-1–60 negative. Similarly, for HES-4, 48.1± 3.9% of the SSEA-4 positive cells were Tra-1–60 positive while 10.6±4.6% were Tra-1–60 negative (Fig. 3D). Interestingly, in comparison to the SSEA-4 positive hESCs isolated using MACS, these SSEA-4 positive hESCs had lower levels of fluorescence.

Discussion

Complete differentiation of pluripotent embryonic stem cells (ESCs) into a particular cell type is not possible and almost always at least a few ESCs would escape differentiation and such rogue cells pose a great risk of teratoma

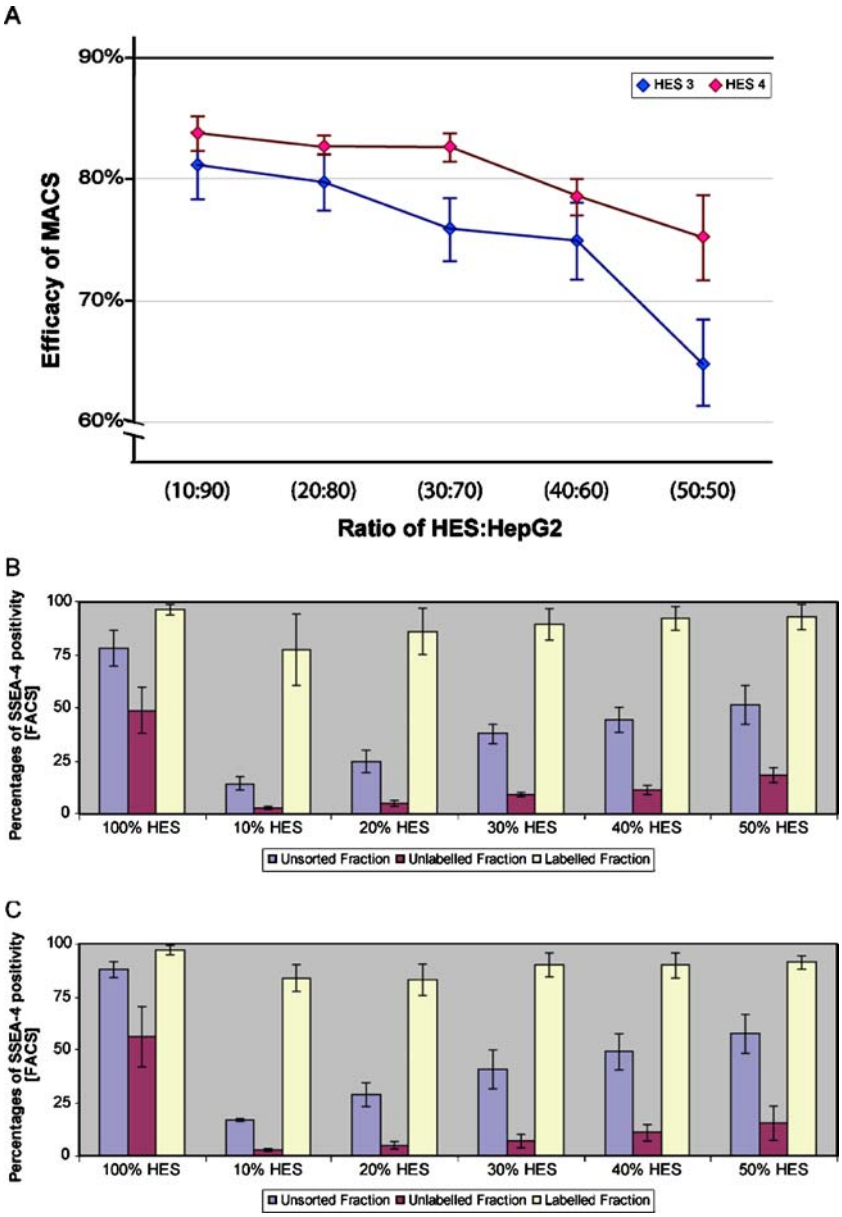

Fig. 2 The heterogeneous populations of hESCs and HepG2 were mixed respectively at different ratios (10:90, 20:80, 30:70, 40:60 and 50:50) and separated using magnetic activated cell separation (MACS). **A** Graph showing the efficacy of MACS in separating HES-3 and HES-4 from HepG2 using the cell-surface marker SSEA-4. **B** Histogram showing the percentages of SSEA-4 positive hESCs found in the unsorted, unlabelled ('flow-through' fraction) and labelled fractions of the various HES-3:HepG2 mix ratios and **C** the various HES-4: HepG2 mix ratios following MACS as analysed by FACS

formation in cell transplantation. Human embryonic stem cells express a specific set of markers which include surface marker antigens such as the SSEA, and TRA series. All these markers are commonly used to monitor differentiation during embryogenesis in mammalian embryos and embryonic stem cells [24, 11, 12]. Conventionally, SSEA-4 positiveness and SSEA-1 negativeness are used as a reliable characterization test for human and non-human primate embryonic stem cells. The most commonly used methods for cell sorting are magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) as the total time required to process the cells, the range of cell samples sizes that can be processed, and the relative cost of the two methods are comparable to each other. However, as observed by us and others, each of these has its own limitation ranging from cell recovery and survival in terms of cell viability post-sorting, and their capacity to separate the labelled cells in terms of the efficacy of the separation protocol [10, 23]. Nevertheless, adaptation of MACS and FACS protocol has been shown to contribute to the successful sorting and purification of other specific cell types following the directed differentiation of hESCs [19, 21].

Recently, Shibata et al. [25] used SSEA-4 as a marker together with FACS for the negative selection of undifferentiated cell fractions from among cynomolgus embryonic stem cell (cyESC)-derived progenitor cells. When their differentiated tissues were purged of SSEA-4 labelled undifferentiated ESCs and then transplanted, tumours were no longer detected in their monkeys. They thus concluded that purging SSEA-4 labelled pluripotent cells with MACS

Table 1 Expression levels of both SSEA-4 and TRA-1–60 hESCs

Ratio of heterogeneous cell mixture	Flow-through fractions			
	HES-3		HES-4	
	SSEA4 [%]	TRA-1–60 [%]	SSEA4 [%]	TRA-1–60 [%]
hESC 100%	48.8±11.1	57.1±4.7	55.0±10.9	65.0±8.9
hESC:HepG2 (10:90)	2.7±0.5	4.0±2.0	2.6±0.5	3.4±0.5
hESC:HepG2 (20:80)	5.1±1.5	7.1±3.8	4.9±1.1	5.3±2.1
hESC:HepG2 (30:70)	9.1±1.1	12.1±4.5	6.6±2.9	8.8±3.5
hESC:HepG2 (40:60)	11.1±2.2	14.0±5.9	9.4±4.7	12.7±5.4
hESC:HepG2 (50:50)	18.1±3.5	21.7±4.3	14.9±7.4	19.3±8.6

The heterogeneous population of human embryonic stem cells (HES-3 and HES-4) and hepatocellular carcinoma cells (HepG2) were mixed respectively at different ratios (10:90, 20:80, 30:70, 40:60 and 50:50) and separated using magnetic activated cell sorting (MACS). The cells in the various 'flow-through' fractions following MACS were re-analysed by fluorescence activated cell sorting (FACS) for the expression of both SSEA-4 and TRA-1–60 positivity. The values are expressed as Mean ± SD

was an efficient separation method and that the same protocol would be a promising approach of producing clinical progenitor cell preparations using human ESCs [25].

In order to eliminate these undifferentiated cells, we explored in the present study two different strategies namely MACS and FACS to evaluate the separation of undifferentiated hESCs from a mixed population of normal human embryonic stem cells (HES-3 and HES-4) and hepatocellular carcinoma cells (HepG2). Rather than use a hESC-derived cell population for this study we chose to deliberately mix known cell numbers in different ratios to closely mimic a standard *in vitro* differentiation protocol so that we would know the actual numbers of undifferentiated cells in the population and more reliable cell sorting percentages could be obtained. Additionally, in our study we used two well characterised monoclonal antibodies, namely SSEA-4 and TRA-1–60, to detect hESC surface antigens as a basis for elimination of hESCs using either MACS or FACS technology. Using MACS, although there

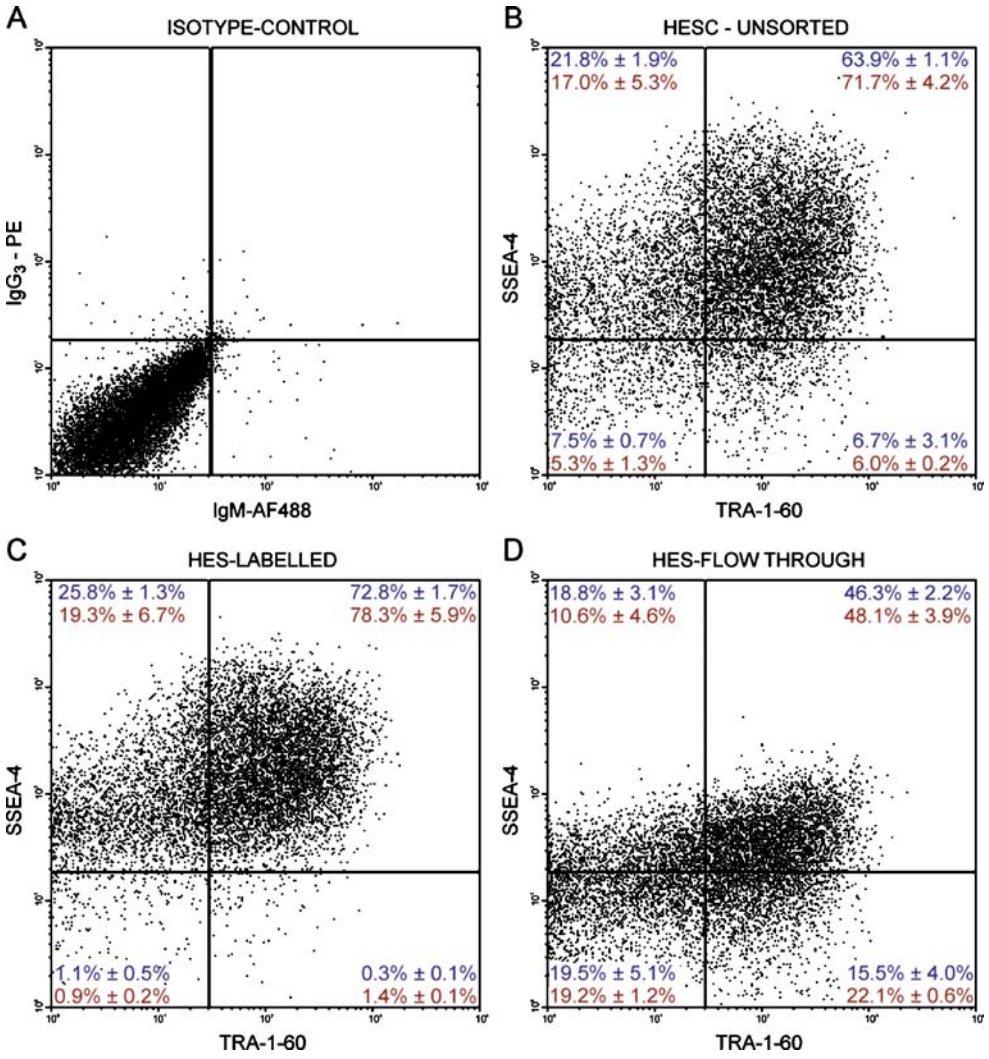

Fig. 3 Representative fluorescence activated cell sorting (FACS) expression profiles of **A** 100% hESCs labelled with isotype-match negative controls, **B** the unsorted hESCs expressing SSEA-4 and TRA-1–60, **C** the expression of SSEA-4 and TRA-1–60 of cells retained in the magnetized column following MACS, and **D** the expression levels of SSEA-4 and TRA-1–60 of hESCs within the 'flow-through' fraction. Separate data for HES-3 (*blue*) and HES-4 (*red*) are annotated in each of the representative expression profiles. The values are expressed as Mean ± SD

was no complete elimination of undifferentiated hESCs, more than 80% elimination was achieved when the differentiated cell type mixed was 10%. The inability to eliminate undifferentiated hESCs completely is probably due to the existence of various sub-populations of hESCs as well as the sensitivity of the MACS technology. This was confirmed when the 'flow-through' fraction of each mix ratio was further subjected to FACS analysis. Generally, the percentages of SSEA-4 positive hESCs that escaped the MACS separation procedure as analysed by FACS ranged between 18.9% to 35.4% for HES-3 and 16.4% to 24.9% for HES-4 for the various ratios of hESCs and HepG2 cell mixtures studied (Table 1). In our pilot studies when 100% hESCs were initially run through the MACS column we were surprised to find over 60% of SSEA-4 positive hESCs escaped the MACS column. However, based on subsequent FACS analysis we noticed that hESCs retention by the MACS column was clearly limited to those hESCs expressing high-levels of SSEA-4. The majority of the hESCs that escaped the separation protocols using MACS had low expression of SSEA-4 (SSEA-4^{LOW}) relative to the overall expression profile of SSEA-4 positive hESCs. These results highlight the critical limitation of MACS for hESC separation. Our findings are consistent with that of Geens and co-workers who also reported that depletion of malignant mouse testicular tumour cells was insufficient by using MACS [10]. Additionally it has been postulated that within a colony of undifferentiated hESCs, subpopulations of hESCs exist that are positive for different surface marker antigens and with serial passaging the same hESCs change their surface marker characteristics (M. Pera, personal communication). Therefore due consideration must be applied to interpret the efficacy when hESC-labelled sorting is done with either MACS or FACS.

Moreover, in our 'flow-through' fractions, out of the 65.1% and 58.7% of the hESCs which were identified to be SSEA-4^{LOW}, as much as 46.3±2.2% and 48.1±3.9% were highly expressing the TRA-1–60 cell surface antigen (TRA-1–60^{HIGH}) for HES-3 and HES-4 respectively, further confirming the existence of subpopulations within hESCs. Although, the identification of hESCs displaying such phenotypes is unclear, Laslett and co-workers showed that lineage commitment of hESCs is dynamic whereby quantitative expression levels of different cell-surface markers represents subpopulations of hESCs expressing varying levels of both pluripotency and lineage specific genes [14]. Although Shibata et al. [25] suggested the elimination of pluripotent cells with a single cell-surface marker SSEA-4 is efficient in producing clinically relevant progenitor cell preparation for transplantation of primate ESC-derivatives in an allogeneic setting, our present study clearly showed that use of a single cell-surface marker may not be sufficient (6.7±3.1% for HES-3 and 6.0±0.2% for HES-4 of SSEA-4 negative hESCs were TRA-1–60 positive) to have complete depletion of hESCs. Therefore great emphasis and more studies may have to be carried out on the use of multiple antibodies detecting different epitopes expressed by hESCs. Moreover, monoclonal antibodies can alter cellular homeostasis and this fact needs consideration while using antibodies to enrich the necessary cell population for cell based therapies [16, 28].

In conclusion, our results demonstrate that MACS is not efficient in complete depletion of hESCs from a heterogeneous population of cells. This limitation is compounded by the expression intensity of specific cell-surface antigens expressed by the hESCs. On the other hand, complete elimination of the undesirable hESCs may be possible using FACS by negative selection. However, the viability of cells (hESCs or their derivatives) may be compromised with FACS unlike MACS. Considering cell viability retention following MACS separation, it may be worthwhile to study further the efficiency of MACS following the use of multiple magnetically labelled antibodies for the different cell surface antigens.

Other approaches for the separation of rogue hESCs residing in hESC-derived cell populations such as the use of apoptotic agents that selectively destroy the rogue cells, the use of density gradients and development of antibodies that specifically target the rogue hESCs may be feasible but have not as yet been properly worked out. Recently, Choo et al. [6] used a cytotoxic antibody recognizing podocalyin-like protein-1 to select against undifferentiated hESCs. Complete elimination of rogue hESCs may not be brought about by a single approach but perhaps a combination of two or more approaches followed by extended in vitro differentiation of any left-over rogue hESCs.

Also, improvements to current FACS methodologies for sorting hESC-derivatives [19]; the adaptation to hESCs of recently developed methods for the capture and enrichment of haematopoietic and bone marrow stem and progenitor cells [17, 31]; incorporation of technical advances such as the usage of Rho-associated kinase inhibitor [30]; subsequent co-culture of the sorted cells with an equivalent cell population [7]; may contribute to eliminating the problem of tumorigenesis induced by hESCs.

Until such time as studies have conclusively confirmed the minimum numbers of hESCs required to that generate teratomas in animal models, it is mandatory that the above laboratory approaches at hESC separation be researched so as to make hESC-derived cell therapy a safe clinical application.

Acknowledgements The authors acknowledge the grant support provided by the National University of Singapore (R-174-000-089-133) and National Medical Research Council, Singapore (R-174-000-103-213). The technical assistance of Miss Manasi and Mr Arjunan Subramanian is greatly appreciated.

References

1. Aleckovic, M., & Simon, C. (2008). Is teratoma formation in stem cell research a characterization tool or a window to developmental biology? *Reproductive Biomedicine Online*, *17*, 270–280.
2. Ben-Hur, T., Idelson, M., Khaner, H., Pera, M., Reinhartz, E., Itzik, A., et al. (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. *Stem Cells*, *22*, 1246–1255. doi:10.1634/stemcells.2004-0094.
3. Bielby, R. C., Boccaccini, A. R., Polak, J. M., & Buttery, L. D. (2004). In vitro differentiation and in vivo mineralization of osteogenic cells derived from human embryonic stem cells. *Tissue Engineering*, *10*, 1518–1525.
4. Bongso, A., Fong, C. Y., Ng, S. C., & Ratnam, S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Human Reproduction*, *9*, 2110–2117.
5. Cao, F., Lin, S., Xie, X., Ray, P., Patel, M., Zhang, X., et al. (2006). In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery. *Circulation*, *113*, 1005–1014. doi:10.1161/CIRCULATIONAHA.105.588954.
6. Choo, A. B., Tan, H. L., Ang, S. N., Fong, W. J., Chin, A., Lo, J., Zheng, L., et al. (2008). Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyin-like protein-1. *Stem Cells*, *26*, 1454–1463. doi:10.1634/stemcells.2007-0576.
7. Elkabetz, Y., Panagiotakos, G., Al Shamy, G., Socci, N. D., Tabar, V., & Studer, L. (2008). Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. *Genes & Development*, *22*, 152–165. doi:10.1101/gad.1616208.
8. Erd, F., Buhrle, C., Blunk, J., Hoehn, M., Xia, Y., Fleischmann, B., et al. (2003). Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *Journal of Cerebral Blood Flow and Metabolism*, *23*, 780–785. doi:10.1097/01.WCB.0000071886.63724.FB.
9. Fujikawa, T., Oh, S. H., Pi, L., Hatch, H. M., Shupe, T., & Petersen, B. E. (2005). Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *American Journal of Pathology*, *166*, 1781–1791.
10. Geens, M., Van de Velde, H., De Block, G., Goossens, E., Van Steirteghem, A., & Tournaye, H. (2006). The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. *Human Reproduction*, *22*, 733–742. doi:10.1093/humrep/del418.
11. Henderson, J. K., Draper, J. S., Baillie, H. S., Fishel, S., Thomson, J. A., Moore, H., et al. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells*, *20*, 329–337. doi:10.1634/stemcells.20-4-329.
12. Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P., Beighton, G., et al., International Stem Cell Initiative. (2007). Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nature Biotechnology*, *25*, 803–816. doi:10.1038/nbt1318.
13. Laflamme, M. A., Chen, K. Y., Naumova, A. V., Muskheli, V., Fugate, J. A., Dupras, S. K., et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology*, *25*, 1015–1024. doi:10.1038/nbt1327.
14. Laslett, A. L., Grimmond, S., Gardiner, B., Stamp, L., Lin, A., Hawes, S. M., et al. (2007). Transcriptional analysis of early lineage commitment in human embryonic stem cells. *BMC Evolutionary Biology*, *7*, 12. doi:10.1186/1471-213X-7-12.
15. Lawrenz, B., Schiller, H., Willbold, E., Ruediger, M., Muhs, A., & Esser, S. (2004). Highly sensitive biosafety model for stem-cell-derived grafts. *Cytotherapy*, *6*, 212–222. doi:10.1080/14653240410006031.
16. Ludwig, D. L., Pereira, D. S., Zhu, Z., Hicklin, D. J., & Bohlen, P. (2003). Monoclonal antibody therapeutics and apoptosis. *Oncogene*, *22*, 9097–9106. doi:10.1038/sj.onc.1207104.
17. Narasipura, S. D., Wojciechowski, J. C., Charles, N., Liesveld, J. L., & King, M. R. (2007). P-Selectin coated microtube for enrichment of CD34+ hematopoietic stem and progenitor cells from human bone marrow. *Clinical Chemistry*, *54*, 77–85. doi:10.1373/clinchem.2007.089896.
18. Nussbaum, J., Minami, E., Laflamme, M. A., Virag, J. A., Ware, C. B., Masino, A., et al. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB Journal*, *21*, 1345–1357. doi:10.1096/fj.06-6769com.
19. Peh, G. S., Lang, R., Pera, M., & Hawes, S. (2008). CD133 expression by neural progenitors derived from human embryonic stem cells and its use for their prospective isolation. *Stem Cells Dev.* doi:10.1089/scd.2008.0124.
20. Prokhorova, T. A., Harkness, L. M., Frandsen, U., Ditzel, N., Burns, J. S., Schroeder, H. D., et al. (2008). Teratoma Formation by Human Embryonic Stem Cells is site-dependent and enhanced by the presence of Matrigel. *Stem Cells Dev.* doi:10.1089/scd.2007.0266.
21. Pruszak, J., Sonntag, K. C., Aung, M. H., Sanchez-Pernaute, R., & Isacson, O. (2007). Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. *Stem Cells*, *25*, 2257–2268. doi:10.1634/stemcells.2006-0744.
22. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., & Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nature Biotechnology*, *18*, 399–404. doi:10.1038/74447.
23. Servida, F., Soligo, D., Caneva, L., Bertolini, F., de Harven, E., Campiglio, S., et al. (1996). Functional and morphological characterization of immunomagnetically selected CD34+ hematopoietic progenitor cells. *Stem Cells*, *14*, 430–438.
24. Shevinsky, L. H., Knowles, B. B., Damjanov, I., & Solter, D. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell*, *30*, 697–705. doi:10.1016/0092-8674(82)90274-4.
25. Shibata, H., Ageyama, N., Tanaka, Y., Kishi, Y., Sasaki, K., Nakamura, S., et al. (2006). Improved safety of hematopoietic transplantation with monkey embryonic stem cells in the allogeneic setting. *Stem Cells*, *24*, 1450–1457. doi:10.1634/stemcells.2005-0391.
26. Shih, C. C., Forman, S. J., Chu, P., & Slovak, M. (2007). Human embryonic stem cells are prone to generate primitive, undifferentiated tumors in engrafted human fetal tissues in severe combined immunodeficient mice. *Stem Cells Development*, *16*, 893–902. doi:10.1089/scd.2007.0070.
27. Shim, J. H., Kim, S. E., Woo, D. H., Kim, S. K., Oh, C. H., McKay, R., et al. (2007). Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. *Diabetologia*, *50*, 1228–1238. doi:10.1007/s00125-007-0634-z.
28. Steff, A. M., Fortin, M., Philippoussis, F., Lesage, S., Arguin, C., Johnson, P., et al. (2003). A cell death pathway induced by antibody-mediated cross-linking of CD45 on lymphocytes. *Critical Reviews in Immunology*, *23*, 421–440. doi:10.1615/CritRevImmunol.v23.i56.40.
29. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, *282*, 1145–1147. doi:10.1126/science.282.5391.1145.

30. Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology*, *25*, 681–686. doi:10.1038/nbt1310.
31. Wojciechowski, J. C., Narasipura, S. D., Charles, N., Mickelsen, D., Rana, K., Blair, M. L., et al. (2008). Capture and enrichment of CD34-positive haematopoietic stem and progenitor cells from blood circulating using P-selectin in an implantable device. *British Journal of Haematology*, *140*, 673–681. doi:10.1111/j.1365-2141.2007.06967.x.
32. Yang, D., Zhang, Z. J., Oldenburg, M., Ayala, M., & Zhang, S. C. (2008). Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats. *Stem Cells*, *26*, 55–63. doi:10.1634/stemcells.2007-0494.