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SSEA-5, an antibody defining a novel surface glycan on human pluripotent stem cells and its application to remove teratoma-forming cells as part of a surface antibody panel

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Abstract

A significant risk in the clinical application of human pluripotent stem cells (hPSCs), including human embryonic and induced pluripotent stem cells (hESCs and hiPSCs), is teratoma formation from residual undifferentiated cells. We have raised a monoclonal antibody (mAb) against hESCs, designated SSEA-5, which binds a novel antigen highly and specifically expressed on hPSCs--the H type-1 glycan. Separation of SSEA-5 high cells through fluorescence-activated cell sorting (FACS) drastically reduced teratoma formation potential. To ensure complete removal we identified additional markers exhibiting a large dynamic expression range during differentiation: CD9, CD30, CD50, CD90, and CD200. Immunohistochemistry (IHC) conducted on human fetal tissues and bioinformatics analysis of a microarray database revealed that concurrent expression of these markers is both common and specific to hPSCs. When applied to incompletely differentiated hESC cultures, immunodepletion with SSEA-5 and 2 additional markers completely removed teratoma formation potential.

Human pluripotent stem cells possess significant potential as a source for cell-based therapeutics. However, an important clinical risk is the possibility of teratoma formation from residual undifferentiated cells remaining among intended differentiated products^{1,2}. Earlier reports have focused on the retrospective removal of formed teratomas through methods including introduction of suicide genes and chemotherapy^{3,4}. However, major caveats of such methods include adverse side effects, drug resistance, and most importantly, their retrospective design. As a result, recent approaches have focused on the prospective

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Author contributions

C.T., J.V., M.D., and I.L.W. designed the experiments and wrote the manuscript. C.T., A.S.L., J.V., D.S., A.R.M., D.N., M.A.I., and M.D. performed the experiments and analyzed data. R.A., S.L.C., R.R.P., B.B., J.C.W. provided samples and reagents. All authors endorse the full content of this work.

Competing financial interests

None

removal of undifferentiated cells prior to transplantation. Choo et al. and others have made an important step in this direction by deriving a mAb capable of inducing cell death in pure cultures of undifferentiated hESCs^{5,6}. Although these studies represent important advances, they were not extended for the depletion of residual teratoma-initiating cells from heterogeneous differentiated cultures.

To create a universally applicable protocol to prospectively remove residual undifferentiated cells hPSC-derived products, we sought to identify a surface marker combination for FACS-based separation. We utilized two mAb sources including a mouse hybridoma library raised against undifferentiated hESCs⁷ and a library of commercially available mAbs recently showed to bind undifferentiated cells⁸. We used flow cytometry to identify hESC specific markers by analyzing mAb binding to undifferentiated hESCs and following 3-day differentiation in the presence of retinoic acid (RA) or bone morphogenetic protein 4 (BMP4). We found that one mAb, designated SSEA-5 (clone 8e11), from our hybridoma library brightly labeled undifferentiated cells. Differentiation resulted in a 2-3 orders of magnitude reduction in SSEA-5 binding signal, a reduction substantially greater than the established markers TRA-1-81, SSEA-3, and SSEA-4⁹ (Fig. 1a). We confirmed that SSEA-5 binds undifferentiated cells by comparing the transcription of pluripotency genes *POU5F1* (*OCT3/4*), *NANOG*, and *SOX2* in SSEA-5+ and SSEA-5-sorted fractions (Fig. 1b). In addition, we tested SSEA-5 specificity to PSCs *in vivo* by immunostaining human embryonic day 6 (E6) *in vitro* fertilization (IVF) derived blastocyst-stage embryos. We found that SSEA-5 brightly labeled the inner cell mass (ICM), from which hESCs are derived^{10,11}. This was most evident by intense and specific staining of both ICMs from monozygotic twin, a frequently occurrence during IVF¹² (Fig. 1c).

To test SSEA-5 binding to a range of differentiated cells, we performed immunohistochemistry staining (IHC) of 12-week-old hESC-derived teratomas that contain immature tissues representing the 3 germ layers. SSEA-5 was found to label only a subset of SSEA-4 and epithelial specific antigen (ESA) positive epithelial cells, comprising ~2% of total cells by flow cytometry (Fig. 1d). SSEA-5+ structures exhibited morphology reminiscent of primordial hPSCs, suggesting teratoma stem cells^{13,14}. To test this hypothesis, we dissociated hESC-derived teratomas to single cells followed by sorting and injection of 10⁵ SSEA-5+ and SSEA-5-cells under the kidney capsules of immunodeficient mice, a model previously shown to be conducive for teratoma formation¹⁵. To track tumor progression, we utilized a H9 hESC clone expressing a luciferase-GFP fusion protein and monitored luciferase signal^{16,17}. We found that the SSEA-5+ cells grew rapidly while the average signal from the SSEA-5-cells remained low ($P < 0.05$) (Fig. 1e). From 3 independent experiments, all 7 SSEA-5+ transplants formed large (>1cm in maximal dimension) teratomas while only 3 out of 11 SSEA-5-transplants gave rise to smaller growths (Table 1). IHC of a panel of 12 human tissues from 7-month-old fetuses revealed that SSEA-5 is not significantly expressed in any of the tested tissues (Fig. 1f). In addition, SSEA-5 did not bind *in vitro* differentiated hESC-derived hematopoietic CD34+CD43+ precursors¹⁸, but rather, labeled a distinct undifferentiated SSEA-5+CD34-CD43-population (Fig. 1g). Taken together, these experiments provide considerable evidence for the specificity of SSEA-5 to hPSCs and suggest its application to remove residual teratoma-initiating cells.

To determine the identity of the SSEA-5 antigen, we immunoprecipitated solubilized hESC membranes with SSEA-5 followed by SDS-page gel electrophoresis. Multiple bands were visualized at ~127 kDa and greater than 190 kDa, indicating that SSEA-5 is not a single protein antigen (Supplementary fig. 1a). Accordingly, mass spectrometry of isolated bands was unsuccessful in identifying a single peptide (data not shown). Since hPSCs express abundant carbohydrate antigens on their surface¹⁹, we next tested the ability of SSEA-5 to bind specific glycans by probing the surface of glycan arrays through the Consortium for

Functional Glycomics²⁰. SSEA-5 was found to specifically bind all 6 glycans with a terminal presentation of the motif: Fuc α 1-2Gal β 1-3GlcNAc β , also known as the H type-1 (H-1) antigen (Supplementary fig. 1b). This binding pattern was largely reproduced by a commercially available mAb (17-206 clone) targeting the H-1 antigen, albeit differences in binding preferences were noted. Importantly, SSEA-5 did not bind any glycan without the H-1 motif, including both H type-2 and globo-H motifs. Finally, glycans bound by commercial mAbs against H type-2 (H-2), SSEA-3, and SSEA-4 did not overlap with those bound by SSEA-5 (Supplementary fig. 1c).

The H-1 antigen is a primitive terminal glycan capable of O- and N-linkage to surface proteins. This glycan is modifiable to other glycans including Lewis and ABO blood group antigens²¹ (Supplementary fig. 2a). Analyzing glycan expression during hESC differentiation revealed a shift in the predominant terminal glycan backbone from type 1 (e.g. Lewis(a) and H-1) to type 2 (e.g. CD15/SSEA-1/Lewis(x) and H-2) at 3 days of RA treatment (Supplementary fig. 2a). This glycan shift was reproduced in hESC lines HES2²² (data not shown) and H7¹⁰, and in hiPSC line IPS(BL) (Supplemental Fig. 2b). These findings suggest that blood group antigens exhibiting a type-1 backbone (such as H-1) are specific to hPSCs, which are later replaced with antigens exhibiting a type-2 backbone.

We applied 2 functional *in vivo* assays to assess the utility of SSEA-5 to remove teratoma-forming cells from hESC-derived preparations. We first tested the capability of SSEA-5 to separate undifferentiated hESCs spiked at 1:100 ratio into “fully” differentiated cells produced through 2 weeks of RA treatment (illustrated in Fig. 2a). Viability-sorted spiked mixtures produced large (>1 cm in maximal dimension) teratomas in all 8 replicates within 7 weeks (Fig. 2b; Table 1). However, when spiked mixtures were depleted of SSEA-5+ cells (illustrated in Fig. 2a), we observed the formation of small tumors in only 3 out of 8 replicates (Table 1). Quantification of luciferase signal indicated that the viability-sorted spiked preparations formed substantially larger growths ($P < 0.05$) than the SSEA-5 depleted population starting at 3 weeks following injection (Fig. 2b).

Second, we tested the ability of SSEA-5 to remove residual teratoma-forming cells from partially differentiated cultures created by 3-day exposure to RA. From these heterogeneous cultures, SSEA-5 high and low populations were sorted and transplanted (illustrated in Fig. 2c). Notably, teratomas formed from both populations, yet those derived from the SSEA-5^{hi} cells were substantially larger ($P < 0.05$) at 4 ($P = 0.036$) and 6 ($P = 0.049$) weeks compared to SSEA-5^{low} cells (Fig. 2d). This difference, however, decreased and was not significant past 6 weeks ($P = 0.23$). Similar to teratomas formed from spiked undifferentiated cells, any SSEA-5^{low} growths exhibited tissues with 3 germ layers (Fig. 2d). Taken together, these functional assays demonstrate enrichment for hPSCs in the SSEA-5^{hi} versus SSEA-5^{low} populations; however, SSEA-5 is insufficient by itself to separate all residual pluripotent cells. We therefore sought to identify additional markers to be used in combination with SSEA-5 for ensuring complete teratoma-initiating cell removal.

Analysis of the commercially available mAbs revealed 5 additional trypsin-insensitive hPSC markers (Supplementary fig. 3a) exhibiting high dynamic expression ranges during hESC differentiation: CD9, CD30, CD50, CD90, and CD200 (Supplementary fig. 3b). Of note, some of these markers were previously shown to correlate with pluripotency¹⁹. Including SSEA-5, these antigens are hereafter collectively referred to as the pluripotency surface markers (PSMs). We found that the hESC lines H7¹⁰ and HES2²² and hiPSC lines iPS(IMR-90)²³, IPS(BL), and IPS(SH) (the latter two were prepared for this study) exhibited similar PSM patterns (Supplementary fig. 4). To confirm that PSMs concurrently label a single population of undifferentiated cells, we performed multicolor flow cytometry analysis, which showed that a single population co-expressing high levels of four PSMs

(CD9, CD50, CD90, and SSEA-5) decreased in proportion during differentiation from 52% to 6% at days 3 and 10 of RA treatment, respectively (Supplementary fig. 5).

We next performed a bioinformatics analysis to evaluate the specificity of PSM combinations for undifferentiated hPSCs. We stratified >27,000 human microarray samples, within which 120 samples represented pluripotent sources, including: hESCs, hiPSCs, and germ cell tumors (both seminomatous and nonseminomatous morphologies)^{24,25}. Stratification was based on CD9, CD30, CD90, and CD200 transcript levels. We were unable to include CD50, as available probes were insufficiently sensitive, and SSEA-5, as this marker is a glycan. Applying high thresholds for concomitant CD9, CD30, CD90, and CD200 expression (set to levels in hPSCs) revealed that >99% of the non-pluripotent tissues did not express high levels of all 4 PSMs, while almost all pluripotent sources did (Fig. 3a,b). This specificity was maintained with combinations of 3 PSMs, but declined with PSM pairs and furthermore with individual PSMs (Fig. 3b **bottom**). IHC of 7-month-old human fetal tissues revealed that approximately half of the analyzed organs exhibited tissue co-labeling of 3 or more PSMs, but labeled structures rarely overlapped (Supplementary fig. 6 and 7). Taken together, these results suggest that concurrent high expression of 3 PSMs is almost exclusive to hPSCs and rarely found in non-pluripotent tissues. However, we stress that stringent co-labeling analyses are required to conclude whether rare stem or progenitor populations expressing high concomitant PSM levels exist within these tissues.

To functionally test whether 3 PSMs are capable of distinguishing and thereby eliminating undifferentiated from differentiated hPSCs, we sorted heterogeneously differentiated cultures created by 3-day RA treatment with representative mAb combinations (illustrated in Fig. 3c). We found that the SSEA-5, CD9, CD90 triple high population formed large (>1cm in maximal dimension) teratomas with evidence of 3 germ layers, while the triple low population did not (Table 1). In agreement, luciferase imaging revealed significant size differences between triple high and low grafts ($P=0.007$ at week 9) (Fig. 3d). Importantly, all growths emerging from SSEA-5, CD9, CD90 triple low population exhibited histological evidence of only epithelium and mesenchyme, but lacked many features typical to teratomas, including evidence of bone, cartilage, or neural rosettes (Fig. 3e). Similarly, we found that SSEA-5, CD50, CD200 triple high cells formed large teratomas while the SSEA-5, CD50, CD200 triple low cells did not (Table 1).

As a comparison, we examined the ability of two classic hPSC markers, TRA-1-81 and SSEA-4, to remove teratoma-initiating cells by sorting and transplanting the top 15% double positive and negative populations. We found that both TRA-1-81, SSEA-4 double high and low populations formed teratomas with 3 germ layers (Table 1) without significantly different in luciferase signal (Fig. 3d).

We provide here proof-of-concept for immunodepletion using 3 surface marker combinations to remove residual teratoma-initiating cells from heterogeneously differentiated hESC cultures. As the centerpiece of the technique, we highlight a novel mAb created through hESC immunization. We chose the name SSEA-5, as similarly to SSEA-1, -3, and -4, this mAb binds an immature embryonic glycan specific for a stage of embryonic development. Depletion with SSEA-5 alone dramatically reduced the teratoma-initiation potential of partially differentiated cultures. However, complete removal was achieved only after combining SSEA-5 with 2 additional PSMs (SSEA-5, CD9, CD90 and SSEA-5, CD50, CD200) with resultant formation of relatively small grafts without evidence of 3 germ layers. The limited tissue repertoires exhibited by the triple low grafts indicate that these populations did not consist of pluripotent cells but rather committed developmental precursors. At this point, we are unaware of clinical hurdles that may be imposed by

embryonic precursors, but further analysis of grafted therapeutic products depleted of teratoma-initiating cells is required to fully assess potential clinical risks.

The 6 PSMs presented here are not meant as an exhaustive list. Identification of additional markers distinguishing pluripotent from differentiated cells would further assist depletion of teratoma-initiating cells. We hypothesize that immunodepletion using SSEA-5 alone was insufficient to remove the teratoma potential since some SSEA-5^{low} cells may have not concluded their exit from pluripotency and require detection with additional PSMs. It should be noted that our immunodepletion approach may require optimization of the PSM panel to avoid detection and hence removal of desired progenies in the case that they express PSM(s). It is our hope that SSEA-5 and the additional PSMs would be immediately applied to advance hPSC research and ensure the safety of patients undergoing clinical trials utilizing hPSC-derived therapeutics.

Methods

Cell culture

H9⁸, H7⁸, and HES2¹⁸ hESC lines were maintained on irradiated mouse embryonic fibroblast (MEF) feeder layer in DMEM/F12 media supplemented with 20% Knockout Serum Replacement, 1% MEM Non-essential Amino Acids, 1% GlutaMAXTM, 1% Penicillin-Streptomycin, 0.05 mM 2-Mercaptoethanol (all obtained from Invitrogen), and 8 ng/ml recombinant human FGF-basic (PeproTech). Cells were split at 1:3 to 1:5 ratio every 5 days using Collagenase Type IV (Invitrogen). IMR90-derived hiPSCs¹⁹ (clones #1 and #4) were cultured on human ESC qualified Matrigel (BD Biosciences) coated plates and maintained using mTeSR1 (Stem Cell Technologies).

To promote differentiation, trypsin dissociated cells were transferred to Matrigel-coated tissue culture dishes in the presence of differentiation media prepared as above except knockout serum was replaced with 20% fetal bovine serum (Hyclone) and FGF-basic was not added. BMP4 (RnD) was used at final concentration of 100 ng/ml and all trans-retinoic acid (Sigma) at 0.5 mM. Undifferentiated controls were prepared similarly except these cultures were exposed to MEF conditioned media to maintain undifferentiated cells.

To differentiate hESCs towards the hematopoietic lineage, cells were passaged onto the OP9 stromal cell in α MEM media (Invitrogen) supplemented with 10% defined fetal calf serum (ThermoScientific), 100 μ M monothiglycerol (Sigma), 50 μ g/ml ascorbic acid (Sigma), 1% GlutaMAXTM-I, 1% Penicillin-Streptomycin as described¹⁴ and differentiated for 8 days prior to analysis.

Hybridoma library derivation

Hybridomas specific to hESCs were generated via the decoy immunization method as previously described⁶. Briefly, human peripheral blood mononuclear cells (PBMCs) were injected as antigen decoy into the left footpad of Balb/c mice followed by immunization of the right footpad with undifferentiated H9 hESCs. B-cells were isolated from the right popliteal lymph node and fused to SP/2 mouse myeloma cells. Hybridomas were subjected to limited dilution into 96-well plates and propagated for 10 days. Hybridoma supernatants were collected and screened for binding to undifferentiated and differentiated hESCs. To ensure clonality, hybridoma subclones were raised from single cells that were sorted into individual culture wells.

Antibodies

All primary and secondary antibodies used in this study are listed in Supplementary table 1.

Flow cytometry and fluorescence activated cell sorting

Cultures of hESCs were washed in PBS and dissociated in 0.25% trypsin for sorting experiments or non-enzymatically in EDTA-containing cell dissociation buffer (both Invitrogen) for flow cytometry screens⁷. To dissociate ovarian cancers and hESC-derived teratomas, tumors were minced and then placed in a solution of Liberase Blendzymes 2 and 4 (Roche) in Media 199 (Invitrogen). The resultant slurry was then filtered through a 40 μ m nylon mesh and treated with LCK buffer to eliminate erythrocytes as described²².

All immunostaining and washing steps were conducted in FACS media (2% FBS in PBS). For flow cytometry analyses, 10^5 cells in 100 μ l FACS media were incubated with 0.5 μ g unconjugated primary antibodies (listed in Supplementary table 1). For sorting experiments, cells were labeled at a concentration of 10^7 cells/ml.

For FACS experiments, cells were re-suspended in FACS media containing propidium iodide (PI) or 4',6'-diamidino-2-phenylindole (DAPI). Live single cells were gated based on PI or DAPI exclusion, side scatter area, and forward scatter area/width. Flow cytometry analysis was performed on a LSR or LSR II machine and FACS was performed on FACS ARIA or ARIA II machines (all Becton Dickinson). Data analysis was performed using FlowJo (Tri Star).

Quantitative real time PCR

RNA was isolated from sorted cells using Trizol (Invitrogen) followed by complementary DNA synthesis using SuperScript III first strand synthesis kit (Invitrogen). Taqman assays (Applied Biosystems) used in this study include GAPDH-Hs99999905_m1, OCT4-Hs00742896_s1, NANOG-Hs02387400_g1, and SOX2-Hs01053049_s1. Real-time PCR amplification was performed using ABI 7900HT. Gene expression was calculated for each gene by comparative CT ($2^{-\Delta\Delta CT}$) normalized to *GAPDH* transcript levels.

Bioinformatics analysis

Gene expression levels from 27,114 human sample microarrays were obtained from the Gene Expression Omnibus (GEO) database consisting of the Affymetrix U133 Plus 2.0 platform and normalized using Robust Multi-array Average (RMA) as described previously²³. PSM probes exhibiting the best hybridization signal and expression dynamic range across all the arrays were selected, including, CD9 (201005_at), CD30 (206729_at), CD50 (204949_at), CD90 (213869_x_at), and CD200 (209583_s_at). Within this database we identified 120 samples (~0.006% of total) from undifferentiated pluripotent sources including 64 from hESCs, 24 from hiPSCs, and 22 from germ cell tumors. To estimate the percentage of arrays from non-cancerous or normal tissues in our database (found to be 45%), we annotated 200 randomly selected microarrays. The majority of remaining arrays was derived from cancer tissues. The threshold for "high" PSM encoding gene expression level was computed as the lowest levels exhibited by undifferentiated hESCs and hiPSCs. As our analysis resulted in the lack of significant CD50 mRNA, we excluded this marker from further use.

Kidney capsule injections

Approximately 10^5 sorted cells were injected under the kidney capsules of 8 week old male RAG γ -double knockout immunodeficient mice²⁴. To monitor cell growth *in vivo*, mice were injected with an aqueous solution of D-fire-fly luciferin (375 mg/kg body weight; Xenogen) and imaged using the Xenogen *In Vivo* Imaging System (IVIS). Luciferase signal was quantified via Living Image software (Xenogen). Twelve weeks following transplantation, resulting tissues were removed, fixed in 10% formalin, embedded in paraffin and stained with Hematoxylin and Eosin (HE). For immunohistochemistry, teratomas were fixed in 4%

paraformaldehyde, transfused in 30% sucrose, and embedded in optimum temperature cutting medium (OCT, Tissue-Tek). All animals were maintained in Stanford University laboratory animal facility in accordance with Stanford animal care and use committee and National Institutes of Health guidelines.

Immunohistochemistry

OCT embedded ovarian cancer or teratomas were fixed and rehydrated with graded ethanol and PBS, and stained with unconjugated mAbs or control mouse IgG, followed by secondary antibody detection with Alexa488 anti-mouse for ovarian cancer sections and phycoerythrin anti-mouse for teratoma sections. Nuclei were counterstained with DAPI. Confocal image series of teratoma sections were taken via a SP2A AOBS Laser Scanning Microscope (Leica). Fetal frozen tissue arrays (FFE302, Biomax.US) were fixed and stained as described above. Tissue array slides were scanned with a tissue array scanner system (Bacus Laboratories). An experienced pathologist scored PSM staining results on fetal tissue arrays. A low threshold was set for positive staining to minimize false negative scores. Any tissue was scored as positively stained if at >5 cells exhibited specific labeling. Fetal heart, urinary/kidney, and colon exhibited high background when stained with IgG and were not analyzed further. All embryos used in this study were supernumerary embryos donated to research by informed consent in accordance with established IRB protocols.

Human blastocyst immunofluorescence

Day 7 live human embryos were obtained from Stanford Fertility and Reproductive Medicine Center after approval by the Stanford Institutional Review Board (IRB); all samples were obtained with written informed consent from all participants involved in the study. Staining: embryos were fixed in 2% paraformaldehyde for 20 min, treated with 0.1% triton X-100 for 1 hr, and incubated with Alexa-647 conjugated SSEA-5. Imaging was performed with Zeiss LSM510 Meta inverted confocal microscope.

Statistics

Student t-tests were used to compare luciferase values. Differences were deemed significant at p-value <0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SSEA-5 mAb is specific for hPSCs. **(a)** Representative FACS plots demonstrating bright SSEA-5 labeling of pluripotent hESCs (undiff - blue), and its decline following 7-day treatment with fetal bovine serum (FBS - green), and FBS supplemented with RA (red) or BMP4 (grey). Compared to TRA-1-81, SSEA-3, and SSEA-4, SSEA-5 exhibited higher staining intensity on hESCs and greater reduction in expression following RA treatment. **(b)** Pluripotency genes *OCT4*, *NANOG*, and *SOX2* are enriched in the SSEA-5+ versus SSEA-5- populations sorted following 7-day RA treatment of hESCs. **(c)** Immunostaining of human blastocyst-stage embryos with SSEA-5 (red) overlaid on bright-field (BF) images revealed bright labeling of embryonic days 6 (E6) monozygotic twin inner cell masses (indicated by arrows). **(d)** SSEA-5 (red) labels a subset of SSEA-4+ and epithelial specific antigen (ESA)+ epithelial cells and consist of ~2% total cells (flow cytometry). **(e)** SSEA-5+ (purple) but not SSEA-5- (red) populations sorted from dissociated hESC-derived teratomas reproduced teratomas *in vivo*. (*) p-value < 0.05, and (**) p-value < 0.01. **(f)** 7-month-old human fetal tissues did not exhibit specific immunostaining with SSEA-5. Analysis at high magnification (http://tma.stanford.edu/cgi-bin/viewAvailableStains.pl?array_block_name=TA-326) revealed that signals observed in skin and CNS were unspecific. **(g)** SSEA-5 and the hematopoietic markers CD34 and CD43 labeled different cell populations in hESC cultures differentiated towards the hematopoietic lineage. DAPI (blue) was used to stain nuclei. Scale bars: 100 μ m.

Figure 3.

Depletion of cells concurrently expressing 3 PSMs eliminates teratoma-initiation potential. (a, b) Concurrent expression of 3 and 4 PSMs is highly specific for hPSCs: (a) Analysis of PSM expression levels among >27,000 human tissue microarrays (light blue) highlights a distinct cluster of 120 samples representing undifferentiated hESCs/hiPSCs (red) and germ cell tumors (blue). (b) Representative expression analysis of 3 PSMs (CD9, CD30, and CD200) within the microarray database reveals concurrent high levels in undifferentiated hESCs/hiPSCs (red) and germ cell tumors (blue) compared to all other samples (grey). Dashed box indicates those samples expressing PSMs at levels similar to hPSCs, and inset pie chart presents the proportions of tissue types within the dashed box. Inset bar graph presents the percentage of non-pluripotent samples (% of total in database) excluded from the PSM high co-expression cluster analyzed with representative combinations of 1-4 PSMs. (c) Gating strategy used to sort 3-day RA-treated hESCs for SSEA-5/CD9/CD90 triple high and low sorted populations and transplanted into immunodeficient mice. (d) Time series luciferase signal of implants derived from SSEA-5/CD9/CD90 triple high (purple) and low (red) sorted populations. The Tra-1-81/SSEA-4 double high (green) implants were found to exhibit high luciferase signals similar to Tra-1-81/SSEA-4 double low (blue) and SSEA-5/CD9/CD90 triple high populations. (*) P-value < 0.05, and (**) P-value < 0.01 compared to SSEA-5^{low}CD9^{low}CD90^{low}. (e) Representative Hematoxylin and Eosin sections of SSEA-5/CD9/CD90 triple high and low implants at 9 weeks demonstrate cartilaginous, epithelial, and neural rosettes structures in the triple high grafts, while the triple low grafts consisted only of mesenchyme and epithelium structures. Scale bar: 100 μ m.

Table 1

Summary of growths formed from hESC-derived sorted populations

Experiment	Conditions	Teratoma >1 cm	Teratoma <1 cm	No tumors
Spiking Experiment: differentiated spiked with undifferentiated hESCs (Schematic in Fig. 2a)	Viability	8/8	-	-
	SSEA-5 Depleted	1/8	2/8	5/8
	RA viable	-	-	5/5
Heterogeneous Culture Experiment: heterogeneously differentiated hESCs after 3 day RA treatment (Schematic in Fig. 2c)	Viability	7/7	-	-
	SSEA-5 high	7/7	-	-
	SSEA-5 low	6/7	1/7	-
	SSEA-5/CD9/CD90 high	7/7	-	-
	SSEA-5/CD9/CD90 low	-	2/6*	4/6
	SSEA-5/CD30/CD200 high	4/5	-	1/5
	SSEA-5/CD30/CD200 low	-	1/4*	3/4
	TRA-1-81/SSEA-4 high	6/6	-	-
	TRA-1-81/SSEA-4 low	6/6	-	-