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REGULAR ARTICLE

Development of a decoy immunization strategy to identify cell-surface molecules expressed on undifferentiated human embryonic stem cells

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Abstract Little is known about the cell-surface molecules that are related to the undifferentiated and pluripotent state of human embryonic stem cells (hESCs). Here, we generated a panel of murine monoclonal antibodies (MAb) against undifferentiated hESCs by a modification of a previously described decoy immunization strategy. H9 hESCs were differentiated in the presence of retinoic acid and used as a decoy immunogen. Twelve Balb/c mice were immunized in the right hind footpads with differentiated H9 cells and in the left hind footpads with undifferentiated H9 cells. After immunization, the left popliteal lymph node cells were collected and were fused with mouse myeloma cells. The fusion resulted in 79 hybridomas secreting MAbs that bound to the undifferentiated H9 cells as shown by flow cytometric analysis. Of these, 70 MAbs bound to the undifferentiated H9 cells, but only weakly or not at all to the differentiated H9 cells. We characterized 37 MAbs (32 IgGs, 5 IgMs) recognizing surface molecules that were down-regulated during embryoid body cell formation. One of the MAbs, L125-C2, was confirmed to immunoprecipitate CD9, previously known as a surface molecule on the undifferentiated hESCs. To investigate the relationship between the MAbs and hESC-specific antibodies, two representative MAbs, viz., L125-C2 and 291-D4, were selected and studied by multi-color flow cytometric analysis. This showed that more than 60% of L125-C2- and 291-D4-positive cells were also positive for the expression of hESC-specific surface molecules such as SSEA3, SSEA4, TRA-1–60, and TRA-1–81, indicating the close relationship between the two MAbs and the hESC-specific surface molecules. Our results suggest that the decoy immunization strategy is an efficient method for isolating a panel of MAbs against undifferentiated hESCs, and that the generated MAbs should be useful for studying the surface molecules on hESCs in the pluripotent and undifferentiated state.

Keywords Decoy immunization · Monoclonal antibodies · Cell-surface molecules · Undifferentiation · Embryonic stem cells · Human

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Introduction

Embryonic stem cells (ESCs) represent populations of pluripotent and undifferentiated cells that have unlimited self-renewal capacity, and that can be differentiated into a variety of specialized cells (Boiani and Scholer 2005). ESC fate is regulated by a combination of intrinsic and extrinsic signals, many of which are poorly understood. The transcription factors Oct4, Nanog, and Sox2 have been identified as crucial intrinsic regulators of pluripotency and self-renewal in pluripotent ESCs (Boiani and Scholer 2005; Boyer et al. 2006). The regulatory mechanisms that control the pluripotent and undifferentiated state of ESCs must deal with externally originating signals, because pluripotency and "undifferentiation" is a fundamental biological function in multicellular organisms, and ESCs are surrounded by a number of different cell types during development. The changes in gene expression that lead to development are generally initiated through a response to external cues, whereby transcription factors are most often the ultimate targets of such signals. Indeed, together with Oct4, Nanog, and Sox2, extracellular matrix (ECM) signaling based on cell membrane receptors and adhesion molecules involved in nucleus-directed signaling pathways appear to modulate the pluripotent and undifferentiated state of ESCs both in vivo and in vitro (Boiani and Scholer 2005). Therefore, investigations into the way that extrinsic signals specify intrinsic gene expression programs and stem cell identity is an important next step in stem cell research.

To define the pluripotent and undifferentiated state of human embryonic stem cells (hESCs), cell-surface molecules such as SSEA1, SSEA3, SSEA4, Tra-1–60, and Tra-1–81 have been generally used (Andrews et al. 1984; Kannagi et al. 1983; Laslett et al. 2003). The surface molecules, however, are associated with carbohydrate epitopes, and their function in the pluripotent and undifferentiated state of hESCs has been uncertain to date (Badcock et al. 1999; Brimble et al. 2007). Some surface molecules such as CD9, E-cadherin, and PODXL have also been sporadically identified on hESCs in the undifferentiated state, but their function in the pluripotent and undifferentiated state of hESCs is also unknown (Cai et al. 2005; Carpenter et al. 2004). Recently, two studies have shown that IGF1R and ERBB2 play an important role in the survival and self-renewal of pluripotent hESCs (Bendall et al. 2007; Wang et al. 2007). In order to improve the definition and to study the undifferentiated and pluripotent state of hESCs, we need to identify surface molecules on undifferentiated hESCs systemically. Thus, for the efficient generation of a panel of monoclonal antibodies (MAbs) that bind to undifferentiated hESCs, but not to differentiated hESCs, we have used retinoic acid (RA)-treated hESCs as a decoy immunogen and generated a panel of MAbs that specifically recognize the cell-surface antigens expressed on the undifferentiated hESCs by a modification of a previously described decoy immunization strategy (Yin et al. 1997).

Materials and methods

Cell culture

hESC lines (H1 and H9) were cultured according to the protocols provided by the Wicell Research Institute (http://www.wicell.org/index.php). Briefly, cells were cultured on a layer of irradiated CF1 mouse embryonic fibroblast (MEF, Charles River Laboratories, Wilmington, Mass.) feeder cells in DMEM/F12 medium (Invitrogen, Seoul, Korea), supplemented with 20% serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol, 1% non-essential amino acids, 1 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 4 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, N.J.). The hESC colonies were subcultured every 5 days by detaching the clumps with 1 mg/ml collagenase IV (Invitrogen). Differentiation of H9 cells was induced by incorporating all-*trans*-RA (Sigma-Aldrich, Seoul, Korea) at 10^{-5} M into the medium and culturing the cells for at least 20 days. Human embryoid body (EB) was prepared from H9 cell colonies as described previously (Chadwick et al. 2003). Mouse embryonic stem cell (mESC) line J1 was cultured on a irradiated CF1 MEF in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (FBS; Invitrogen), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 500 U/ml leukemia inhibitory factor (PeproTech; Li et al. 1992). Human peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Paque Plus method (GE Healthcare, Seoul, Korea). Lung carcinoma cell line A549 (CCL-185, ATCC, Manassas, Va.) was cultured in DMEM supplemented with 10% FBS and antibiotics.

Generation and purification of MAbs

H9 cells were cultured in the presence of RA for 20 days and prepared as differentiated H9 cells. The differentiated or undifferentiated H9 cells were incubated with collagenase IV (1 mg/ml), and the detached cells were triturated with a Pasteur pipette. Then, the triturated cells were injected into the hind footpads of 12 female Balb/c mice (KOATECH, Pyungtaek, Korea). Approximately 2×10^6 cells in 50 μl phosphate-buffered saline (PBS), pH 7.4, were immunized per footpad. To generate a panel of hybridomas producing antibodies that bound to the undifferentiated but not to the differentiated H9 cells, the

differentiated H9 cells were immunized into the right hind footpads of 6-week-old mice, on days −3, 0, 3, 6, 13, 17, and 20, whereas the undifferentiated H9 cells were immunized into the left hind footpads on days 0, 3, 6, 13, 17, and 20 as described previously (Yin et al. 1997). On day 21, the left popliteal lymph nodes were removed. A lymphocyte suspension (total 1.5×10^8 cells) from the left popliteal lymph nodes was fused to FO myeloma cells (ATCC), and then plated on 96-well plates in DMEM supplemented with 20% FBS (Invitrogen) and HAT component (Sigma-Aldrich), as described previously (Kohler and Milstein 1975). Hybridoma supernatants were screened on the undifferentiated or differentiated H9 cell preparations by flow cytometric analysis as described below. Isotype analysis of each antibody was carried out with the Mouse Immunoglobulin Isotyping Kit (BD Biosciences, Seoul, Korea), according to the supplier's protocol. MAbs were purified from the culture supernatants of hybridomas by Protein G-Sepharose column chromatography as previously described (Ryu et al. 1997).

Biotinylation of MAbs

Biotinylation of MAbs was performed according to the supplier's protocol with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, Ill.). Briefly, 1.63 mg NHS-LC-biotin was dissolved in 1 ml PBS (pH 8.0), and 50 μl (l46.4 nmol) of this solution was used to biotinylate 1 mg MAbs. After a 1-h incubation at room temperature, the reaction mixture was dialyzed at 4°C against 0.1 M $NaHC0_3$, pH 8.0, for 48 h.

Flow cytometry

Undifferentiated hESCs, RA-treated hESCs, and mESCs were treated with collagenase IV for 1 h in normal growth medium, treated with cell dissociation buffer (Invitrogen) for 20 min in a 37°C incubator, and filtered through a 40-μm cell strainer as previously described (Chadwick et al. 2003). The dissociated cells were immediately resuspended at approximately 2×10^5 cells per ml in PBA (1% bovine serum albumin [BSA], 0.02% NaN_3 in PBS) and incubated with anti-SSEA1 (R&D Systems, Minneapolis, Minn.), anti-SSEA3 (R&D Systems), anti-SSEA4 (R&D Systems), anti-TRA-1–60 (Millipore, Billerica, Mass.), anti-TRA-1–81 antibodies (Millipore), or murine MAbs for 30 min at 4°C. Then, the cells were further incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rat IgM, anti-mouse IgM, or anti-mouse IgG (BD Biosciences) depending on the isotype of the primary antibody. For multi-color flow cytometric analysis, cells were incubated with appropriate primary antibodies for 30 min at 4°C. Primary antibodies used here were anti-SSEA3, anti-SSEA4, anti-TRA-1–60, or anti-TRA-1–81. Then, the cells were further incubated with FITC-conjugated anti-rat IgM, anti-mouse IgG, or anti-mouse IgM (BD Biosciences) depending on the combination of primary antibodies. After being washed with PBA, the cells were incubated with biotin-conjugated 291-D4 or L125-C2 followed by streptavidin-phycoerythrin (PE). After further washes, propidium iodide (PI)-negative cells were analyzed for antibody binding by using FACSCalibur (BD Biosciences) and Cell Quest software (BD Biosciences).

Immunocytochemistry

H9 cells were cultured for 4 days on a cover-slip in a 12-well culture dish, washed with Ca^{2+}- and Mg^{2+}-PBS, and then fixed in 4% paraformaldehyde for 30 min at 4°C. The cells were blocked with blocking solution (10% horse serum, 0.1% BSA in Ca^{2+}- and Mg^{2+}-PBS) for 20 min. Then, the cells were incubated with diluted primary antibody overnight at 4°C followed by Texas-red-conjugated anti-mouse IgM or FITC-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, Calif.) at room temperature in the dark for 1 h. Between each step, cells were washed with Ca^{2+}- and Mg^{2+}-PBS. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole).

Cell-surface biotinylation, immunoprecipitation, and Western blotting

Cell-surface biotinylation of H9 cells was performed according to the supplier's protocol with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology). Biotin-labeled H9 cells were treated with lysis buffer (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 2 μg/ml aprotinin, 100 μg/ml phenylmethane sulfonyl-fluoride, and 5 μg/ml leupeptin) at 4°C for 20 min. Nuclei were removed by centrifugation, and the cell lysates were stored at -70°C before use. To remove the cellular proteins that nonspecifically bind to Protein G plus-Sepharose (Santa Cruz Biotechnology, Santa Cruz, Calif.), the cell lysate from approximately 1×10^7 cells was incubated with 20 μl Protein G plus-Sepharose at 4°C for 2 h, and the beads were recovered and extensively washed with lysis buffer to use as a negative control for the immunoprecipitation experiment. To immunoprecipitate the antigens recognized by the MAbs and anti-CD9 antibodies, the pre-cleared lysates were incubated with approximately 1 μg MAbs or anti-CD9 antibodies (sc-9148, Santa Cruz; CBL162, Chemicon) at 4°C overnight and further incubated with Protein G plus-Sepharose as described above. The beads were extensively washed with lysis buffer, and the bound proteins were eluted from the beads by heating at 100°C for 5 min. The pre-cleared lysate and eluted proteins were

fractionated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel under denaturing conditions and transferred to a nitrocellulose membrane for Western blotting. The membrane was blocked in 5% skim milk in PBST (PBS containing 0.1% Tween 20) at room temperature for 1 h. After two rinses with PBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated streptavidin (1:4,000; GE Healthcare) at room temperature for 1 h. Following extensive washing, the biotinylated proteins were visualized by enhanced chemiluminescence (ECL) detection reagent (GE Healthcare). For Western blot analysis, cell lysates were prepared as described above without biotinylation and subjected to Western blotting with anti-CD9 antibodies (Santa Cruz or BD Biosciences), followed by HRP-conjugated anti-rabbit or mouse IgG (Santa Cruz) at room temperature in the dark for 1 h. The immunoblots were visualized by using ECL detection reagent as described above.

Results and discussion

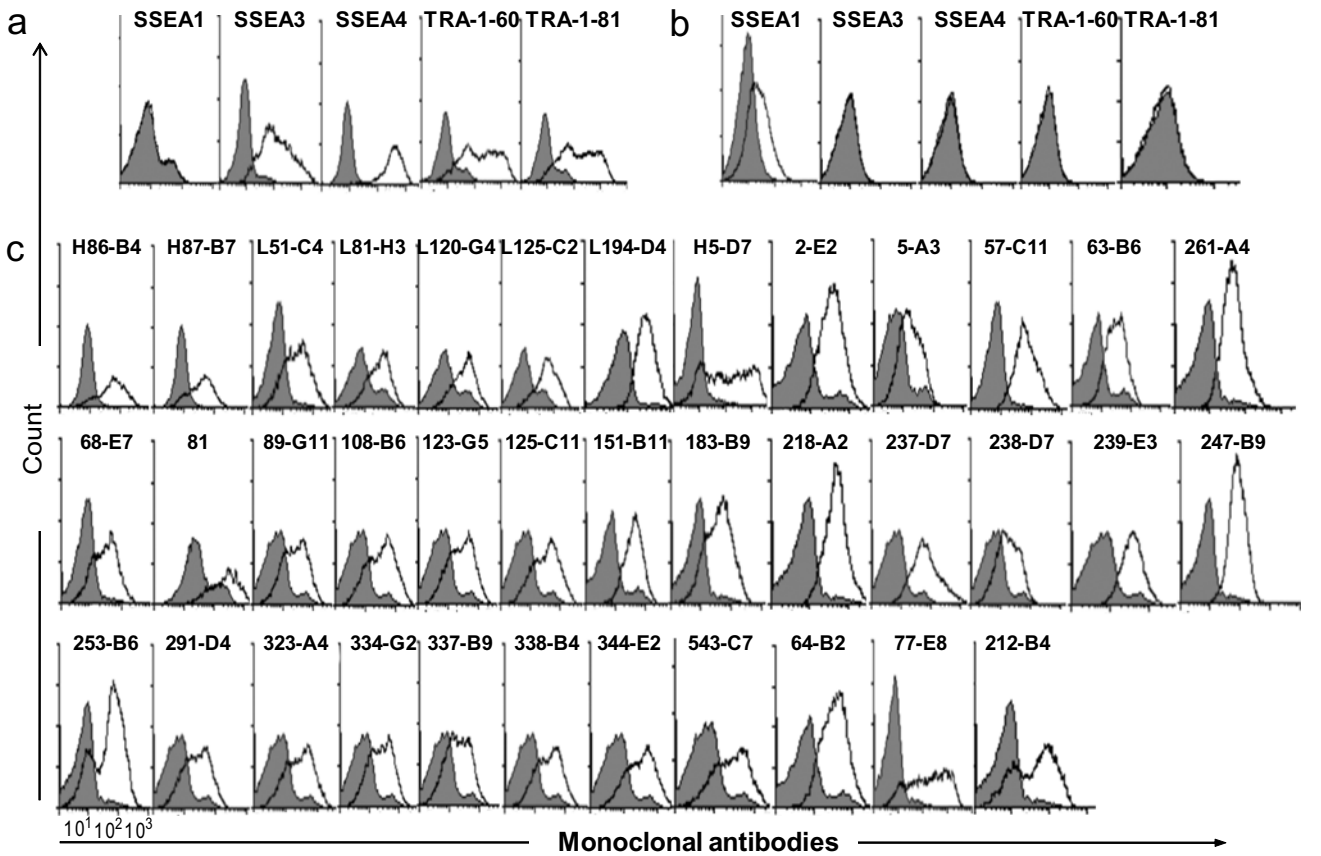
Generation of a panel of MAbs against undifferentiated H9 cells

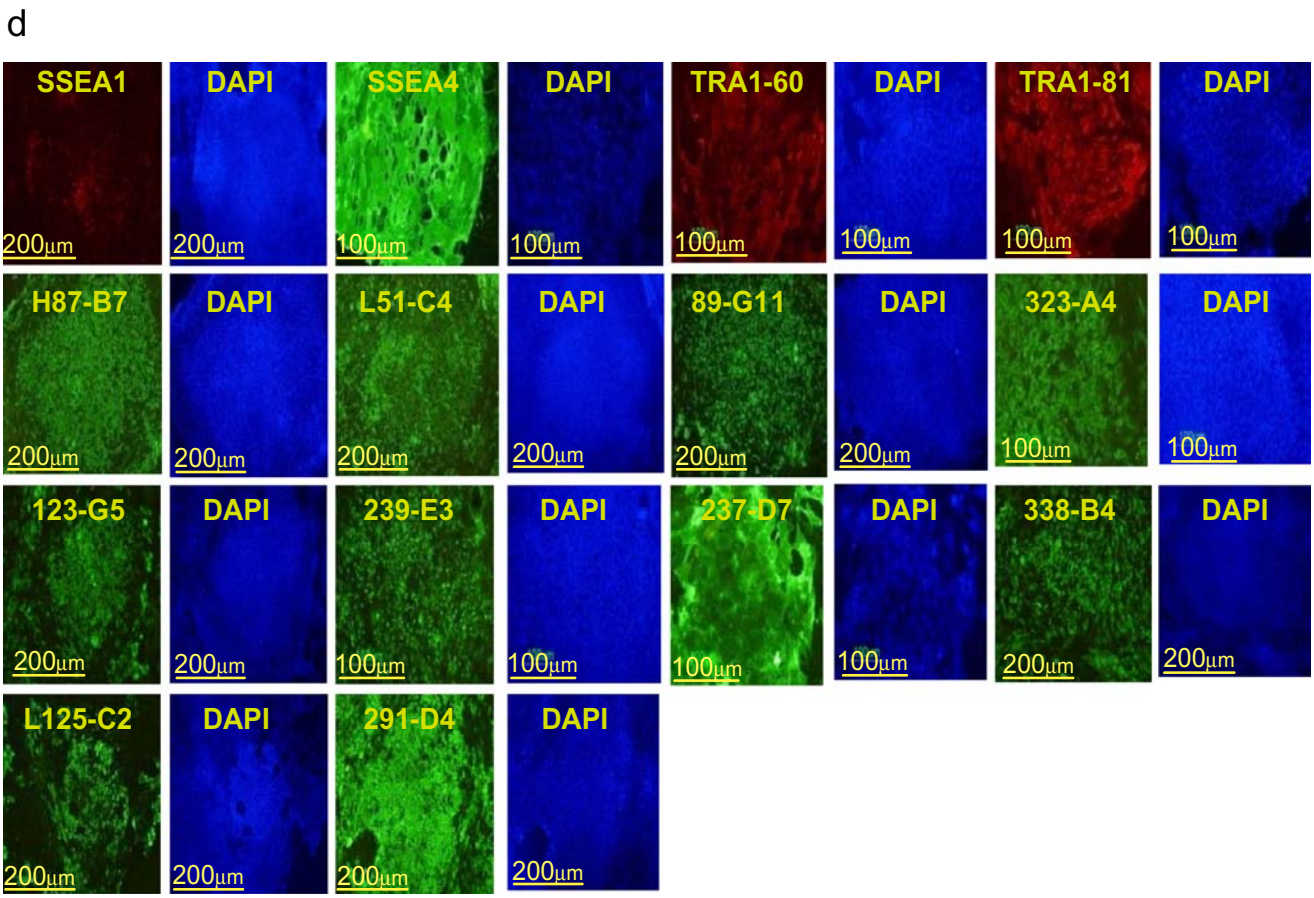
Undifferentiated H9 cells were cultured routinely as described above. Differentiated H9 cells were induced by incorporating RA into the medium and cultured for at least 20 days as described previously (Draper et al. 2002; Henderson et al. 2002). The undifferentiated state of H9 cells was confirmed in flow cytometric analysis by using anti-SSEA1, anti-SSEA3, anti-SSEA4, anti-TRA-1–60, and anti-TRA-1–81 antibodies as described previously (Fig. 1a; Chadwick et al. 2003; Son et al. 2005). After treatment of H9 cells with RA for 20 days, undifferentiated markers of hESCs such as SSEA3, SSEA4, TRA-1–60, and TRA-1–81 were drastically down-regulated, whereas differentiated marker SSEA1 was slightly up-regulated as expected (Fig. 1b; Draper et al. 2002). To generate a panel of hybridomas secreting MAbs that bound to the undifferentiated but not to the differentiated H9 cells, the differentiated H9 cells were immunized into the right hind footpads of Balb/c mice as a decoy immunogen, and then the undifferentiated H9 cells were immunized into the left hind footpads 3 days later as described previously (Yin et al. 1997). After seven injections, the lymphocytes from the left hind popliteal lymph nodes were fused to FO myeloma cells.

Out of a total of 480 hybridomas selected, 79 hybridomas secreted MAbs that bound to the undifferentiated H9 cells as shown by flow cytometric analysis. To determine whether the 79 clones were able to bind to the differentiated H9 cells, RA-treated differentiated H9 cells were also included in the flow cytometric analysis. Only nine clones (11.4%) were able to bind to both the undifferentiated and differentiated H9 cells, indicating that approximately 89% of the hybridoma clones exhibited a significant decrease in binding to the undifferentiated H9 cells upon differentiation. Of the 79 hybridoma clones, 37 clones that bound to the undifferentiated H9 cells, but weakly or not at all to the RA-differentiated H9 cells, were subcloned by limiting dilution (Table 1, Fig. 1). In the isotyping analysis of the subcloned MAbs, 32 MAbs were IgGs and κs, and 5 MAbs were IgMs and κs. Although some MAbs showed a similar binding pattern in the flow cytometric analysis, most of the MAbs exhibited different patterns of binding (Fig. 1c), suggesting that they recognized different surface molecules on the undifferentiated H9 cells. Binding was also confirmed by the immunocytochemical method (see Fig. 1d for a representative result).

To check whether the MAbs bound to other hESC lines, undifferentiated H1 cells were also included in the flow cytometric analysis (Table 1, data not shown). Of the 37 MAbs, 33 (89.2%) bound to the H1 cells, but four MAbs (10.8%; 81, 151-B11, 323-A4, and H5-D7) did not (Table 1). Therefore, the four molecules recognized by the four MAbs were not considered as general factors important for maintaining pluripotency. Based on previous reports (Abeyta et al. 2004; Skottman et al. 2005), the four molecules recognized by the H9-specific MAbs might reflect genetic variations between the H9 and H1 cells. The binding specificity of the MAbs to other cells was also investigated by flow cytometric analysis. The MAbs did not bind to human PBMCs, except for 2-E2, which showed partial binding capacities (Table 1). Interestingly, most of the MAbs did not bind to J1 mouse embryonic stem cells (mESCs) or to CF1 MEFs, except for 239 and 64-B2,

Fig. 1 Screening of MAbs against hESCs by flow cytometry and immunocytochemistry. **a** Undifferentiated H9 cells were stained with anti-SSEA-1, anti-SSEA-3, anti-SSEA-4, anti-TRA-1–60, or anti-TRA-1–81 followed by FITC-conjugated anti-mouse IgM, anti-rat IgM, anti-mouse IgG, anti-mouse IgM, or anti-mouse IgM, respectively. Non-viable cells were identified by using PI and excluded during analysis. The *unfilled* population indicates each antibody staining, whereas the *filled* population indicates FITC-conjugated secondary antibody staining without the primary antibody as a control. Each antibody is presented *top*. **b** Undifferentiated H9 cells were cultured in the presence of RA for 20 days and included as differentiated cells in the same flow cytometric analysis. **c** Undifferentiated H9 cells were stained with various MAbs followed by FITC-conjugated anti-mouse IgG or anti-mouse IgM. Each MAb is presented *top*. **d** Undifferentiated H9 cells were cultured on 0.1% gelatin-coated cover-slips plated with CF1 MEF cells. The cells were then stained with anti-SSEA1, anti-SSEA4, anti-TRA-1–60, anti-TRA-1–81, or various MAbs followed by FITC-conjugated anti-mouse IgG or Texas-red-conjugated anti-mouse IgM. Antibody staining is in *green* or *red*, whereas nuclear DAPI staining is in *blue*


a
SSEA1
SSEA3
SSEA4
TRA-1-60
TRA-1-81
b
SSEA1
SSEA3
SSEA4
TRA-1-60
TRA-1-81
c
H86-B4
H87-B7
L51-C4
L81-H3
L120-G4
L125-C2
L194-D4
H5-D7
2-E2
5-A3
57-C11
63-B6
261-A4
68-E7
81
89-G11
108-B6
123-G5
125-C11
151-B11
183-B9
218-A2
237-D7
238-D7
239-E3
247-B9
253-B6
291-D4
323-A4
334-G2
337-B9
338-B4
344-E2
543-C7
64-B2
77-E8
212-B4
Count
$10^1 10^2 10^3$
Monoclonal antibodies


d
SSEA1
DAPI
SSEA4
DAPI
TRA1-60
DAPI
TRA1-81
DAPI
H87-B7
DAPI
L51-C4
DAPI
89-G11
DAPI
323-A4
DAPI
123-G5
DAPI
239-E3
DAPI
237-D7
DAPI
338-B4
DAPI
L125-C2
DAPI
291-D4
DAPI
200μm
100μm

which partially bound to J1 mESCs and/or MEFs (Table 1). hESC and mESCs differ in growth conditions and cytokine requirements to maintain self-renewal and pluripotency in culture (Reubinoff et al. 2000; Thomson et al. 1998), although the overall strategies used to regulate self-renewal and pluripotency are similar between them. The above result is also reminiscent of the finding that human undifferentiated surface markers SSEA3, SSEA4, TRA-1–60, and TRA-1–81 are not expressed on the surface of mESCs (Andrews et al. 1984; Laslett et al. 2003) and suggests that many molecules on the surface of undifferentiated ESCs are not conserved between human and mouse.

From the above screening results, we considered that 33 MAbs could be useful for further studying surface molecules with respect to the undifferentiated state of hESCs. To compare the expression level of each molecule between the undifferentiated and differentiated hESCs, the antibody-binding percentages were measured on the undifferentiated and differentiated H9 cells by flow cytometric

Table 1 Characteristics of a panel of MAbs against human embryonic stem cells (*hESC*) H9 and H1, RA-treated H9 cells (*RA*), embryoid bodies (*EB*), mouse embryonic fibroblasts (*MEF*), and mouse embryonic stem cells (*mESC*). Binding was defined as: +++ strong, ++ medium, + weak, − absent (*ND* not determined)

Subclone	Isotype	Flow cytometric analysis						
		hESC lines		RA	EB	MEF	mESC	PBMC
		H9	H1					
H86-B4	IgG1,K	++	+	−	+	−	−	−
H87-B7	IgG1,K	++	++	−	+	−	−	−
L51-C4	IgG1,K	+++	+++	+	+	−	−	−
L81-H3	IgG1,K	++	++	−	−	−	−	−
L120-G4	IgG1,K	++	++	+	−	−	−	−
L125-C2	IgG1,K	+++	+++	−	+	−	−	−
L194-D4	IgG1,K	++	+++	+	+	−	−	−
2-E2	IgG1,K	+++	+++	+	++	−	−	+
5-A3	IgG1,K	++	+	−	−	−	−	−
57-C11	IgG1,K	+++	+++	+	++	−	−	−
63-B6	IgG1,K	++	+	+	+	−	−	−
68-E7	IgG1,K	++	++	+	+	−	−	−
81	IgG	++	−	−	−	ND	ND	−
89-G11	IgG1,K	+++	++	−	+	−	−	−
108-B6	IgG1,K	+++	++	−	+	−	−	−
123-G5	IgG1,K	+++	++	−	+	−	−	−
125-C11	IgG1,K	+++	+++	−	+	−	−	−
151-B11	IgG1,K	+++	−	+	ND	−	−	−
183-B9	IgG1,K	+++	+++	−	−	−	−	−
218-A2	IgG1,K	+++	+++	−	+++	−	−	−
237-D7	IgG3,K	+++	+	−	−	−	−	−
238-D7	IgG1,K	++	+	−	−	−	−	−
239-E3	IgG3,K	+++	++	−	+	+	+	−
247-B9	IgG1,K	+++	+++	+	+++	−	−	−
253-B6	IgG1,K	+++	+++	+	++	−	−	−
291-D4	IgG1,K	+++	++	−	+	−	−	−
323-A4	IgG1,K	+++	−	−	−	−	−	−
334-G2	IgG1,K	+++	+++	−	+	−	−	−
337-B9	IgG1,K	+++	++	−	−	−	−	−
338-B4	IgG1,K	+++	++	−	−	−	−	−
344-E2	IgG1,K	++	++	−	−	−	−	−
543-C7	IgG1,K	++	++	−	ND	−	ND	−
H5-D7	IgM,K	+++	−	−	−	−	−	−
64-B2	IgM,K	+++	+++	+	++	−	+	−
77-E8	IgM,K	+++	+++	−	ND	−	−	−
261-A4	IgM,K	+++	+++	+	+++	−	−	−
212-B4	IgM,K	+++	+++	−	−	−	−	−

analysis. The binding percentages of anti-SSEA-3, anti-SSEA-4, anti-TRA-1–60, and anti-TRA-1–81 to the RA-differentiated cells were markedly decreased (down to below 5%, Fig. 2a), whereas the percentages bound to the undifferentiated cells were around 90% (Fig. 2a). The results were similar to the previous findings observed with the hESC lines H7 and H17 (Draper et al. 2002; Henderson et al. 2002). The binding percentages of the 33 MAbs to the undifferentiated H9 cells were 60%–95%, but the percentages to the RA-differentiated H9 cells were drastically decreased, although the binding percentages for some MAbs such as 247-B9, 253-B6, and 261-A4 were mildly decreased (Fig. 2a–c). These results indicate that the 33 MAbs show decreased binding upon differentiation, but that the extent of the decrease differs.

hESCs can reproducibly differentiate in vitro into embryoid body (EB) cells comprising the three more differentiated embryonic germ layers (Chadwick et al. 2003; Itskovitz-Eldor et al. 2000). As the EB cells represent the earliest differentiated cells from hESCs in vitro, a comparison of the binding percentages of the 33 MAbs between EB cells and hESCs could explain how the surface

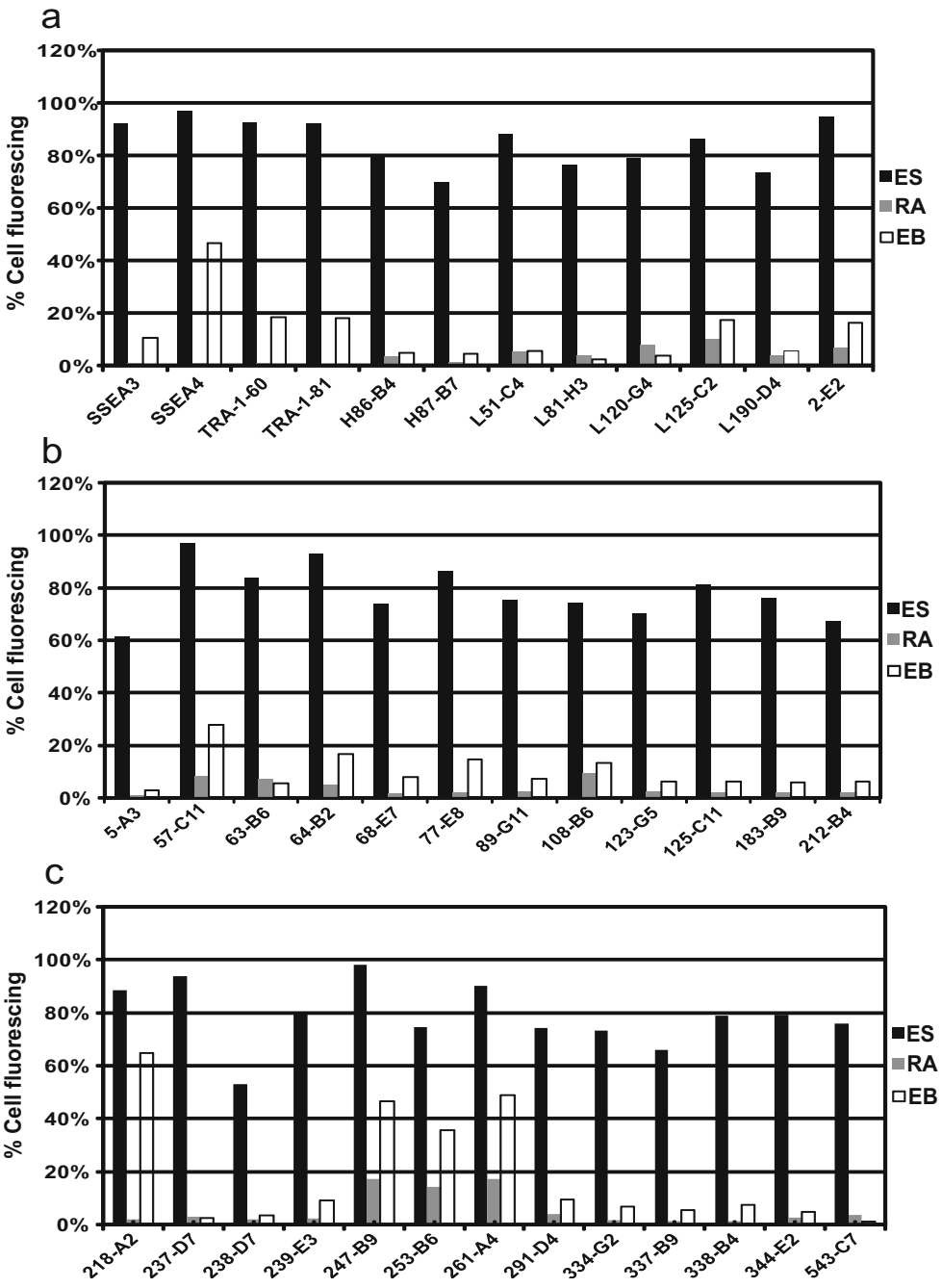


Fig. 2 Binding percentages of various MAbs to H9 cells, RA-treated H9 cells, and embryoid body cells. The binding percentages of anti-SSEA3 (**a**), anti-SSEA4 (**a**), anti-TRA-1–60 (**a**), anti-TRA-1–81(**a**), or various MAbs (**a**–**c**) to undifferentiated H9 cells (*ES*), RA-differentiated H9 cells (*RA*), or embryoid body cells (*EB*) were measured by flow cytometric analysis and calculated from control secondary antibody binding. RA treatment was carried out for 20 days, whereas EB formation was allowed to occur for 4 days. The binding percentages of all MAbs to ES, RA, and EB were measured at least twice. Averages of the measured percentages are presented

molecules recognized by the 33 MAbs are regulated in early hESC differentiation in vitro. Therefore, EB cells from the undifferentiated H9 cells were cultured for just 4 days in vitro as described previously (Chadwick et al. 2003; Son et al. 2005). The EB cells were triturated into single cells by means of cell dissociation buffer and a Pasteur pipette and were included in the flow cytometric analysis. The binding percentages of anti-SSEA3, anti-SSEA4, anti-TRA-1–60, and anti-TRA-1–81 to the EB cells were decreased as expected (Fig. 2a). The binding percentages of most of the MAbs were also decreased, although the degree of decrease was noticeably moderate for some molecules recognized by 218-A2, 247-B9, 253-B6, 261-A4, or 57-C11 (Fig. 2a–c). Taken together, most of the MAbs generated from the decoy immunization strategy recognize surface molecules that are expressed on the undifferentiated hESCs and that immediately decrease upon differentiation.

Identification of surface molecules on undifferentiated H9 cells by immunoprecipitation

To identify the cell-surface molecules recognized by the 33 MAbs, the surface proteins of the undifferentiated H9 cells were biotinylated and analyzed by immunoprecipitation and Western blotting as described above. A representative result with some IgG antibodies is shown in Fig. 3a. Six MAbs, viz., L125-C2, 63-B6, 218-A2, 247-B9, 291-D4, and 57-C11, immunoprecipitated around 24-, 140-, 90-, 35-, 26-, and 120-kDa proteins, respectively, from the undifferentiated H9 cells. Previously, studies had shown that surface proteins CD9 (24 kDa) and E-cadherin (120 kDa) were expressed on the undifferentiated hESCc and decreased upon differentiation (Cai et al. 2005; Carpenter et al. 2004). Therefore, the 24- and 120-kDa proteins immunoprecipitated by L125-C2 and 57-C11 were subjected to Western blotting by using commercially available CD9 and E-cadherin antibody, respectively. The 120-kDa protein was not detected with E-cadherin antibody (data not shown). However, the 24-kDa protein recognized by L125-C2 was detected with the polyclonal anti-CD9 antibody by Western blot analysis (Fig. 3b). To confirm whether the 24-kDa protein was indeed CD9, CD9 protein was immunoprecipitated from H9 and A549 cell extracts with MAb-CD9 and detected with L125-C2 by Western blot analysis. As shown in Fig. 3c, L125-C2 recognized 24-kDa proteins immunoprecipitated from both H9 and A549 cell extracts with MAb-CD9. This result thus confirmed that L125-C2 recognized CD9.

Close relationship between selected MAbs and hESC-specific MAbs

To examine the relationship between the MAbs and the known cell-surface molecules on the pluripotent and undifferentiated stage of hESCs, L125-C2 was subjected to multi-color flow cytometric analysis. To abolish cross-reactivity between the antibodies used, L125-C2 was biotinylated and detected with streptavidin-PE. Approximately 60%, 73%, 60%, and 60% of L125-C2-positive H9 cells were positive for the expression of hESC-specific surface molecules such as SSEA3, SSEA4, TRA-1–60, and TRA-1–81, respectively (data not shown). The results were similar to previous findings carried out with anti-CD9 antibody (Carpenter et al. 2004), although the percentage of CD9-positive cells amongst SSEA4-positive cells was

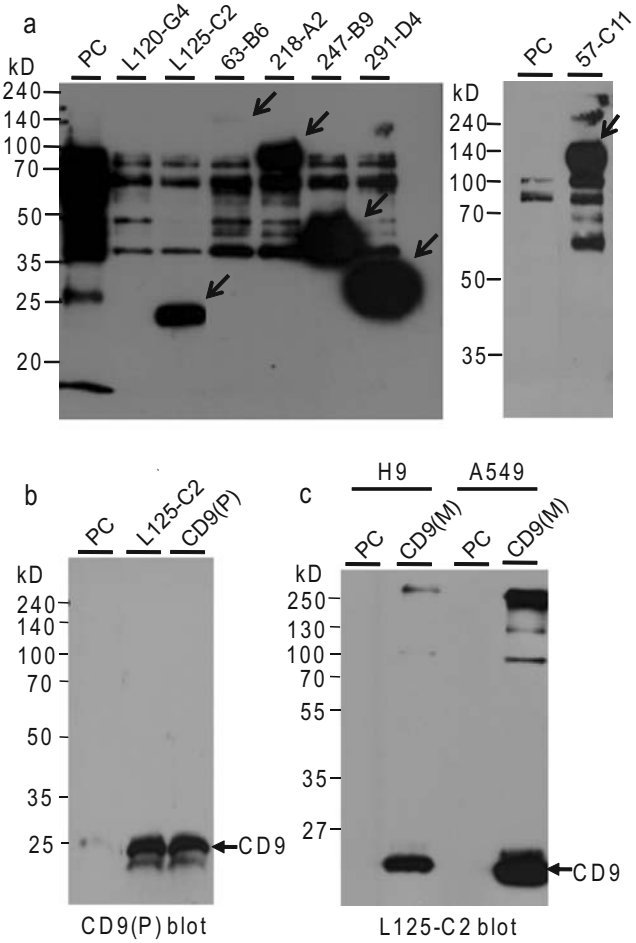


Fig. 3 Identification of cell-surface molecules with selected antibodies (*top*). Undifferentiated H9 cells were biotinylated, and the biotinylated cell extract was prepared by 1% NP-40 (*PC* precleared samples with Protein G plus-Sepharose beads alone). **a** The cell extracts were subjected to immuoprecipitation with the indicated MAbs and protein G-Sepharose beads and visualized (*arrows*). **b** The surface molecules immunoprecipitated with L125-C2 (*lane L125-C2*) or rabbit polyclonal anti-CD9 antibody (*lane CD9(P)*) were fractionated on an SDS-gel and subjected to Western blotting with the same anti-CD9 antibody. **c** Cell extracts from H9 and A549 cells were again subjected to immunoprecipitation with MAb-CD9 (*lane CD9 (M)*), and then the immunoprecipitated complexes were subjected to Western blotting with L125-C2 antibody. The recognized proteins (*arrow left*) were visualized by HRP-conjugated anti-rabbit or mouse immunoglobulin and an ECL detection kit

slightly lower under this experimental condition. Next, another MAb 291-D4 was also subjected to multi-color flow cytometric analysis. The purified 291-D4 antibody was biotinylated and used in the same method as that for L125-C2. Approximately 63%, 85%, 70%, and 68% of 291-D4-positive H9 cells were positive for the expression of SSEA3, SSEA4, TRA-1–60, and TRA-1–81, respectively (Fig. 4i–l). These results indicated the close relationship between the representative MAbs and the hESC-specific surface molecules. Taken together, our results suggest that the 33 MAbs generated by the decoy immunization strategy will be useful for investigating cell-surface molecules expressed on undifferentiated hESCs.

The identification and characterization of the cell-surface molecules that are involved in the pluripotent and undifferentiated state of hESCs are important steps for current stem cell research. In order to study such surface molecules, investigators have used microarray data gained from the total RNAs of undifferentiated hESCs. However, the generation of a panel of MAbs specific for the undifferentiated hESC represent a good alternative strategy, because MAbs are excellent tools for identifying and isolating the surface molecules, and the MAbs themselves can be conveniently used in further investigations. By employing RA-treated hESCs as a decoy immunogen, we have been able to generate 33 MAbs that bind to hESCs in the undifferentiated state and that show decreased binding upon differentiation (Fig. 2, Table 1). The result suggests that the decoy immunization protocol is another suitable way of finding surface molecules on the undifferentiated hESCs at the protein level. Furthermore, the MAbs generated by the decoy immunization protocol are mostly IgGs (60 IgGs, 10 IgMs; Table 1 and data not shown), whereas the MAbs obtained by simple intraperitoneal injection are mostly IgMs (9 IgGs, 16 IgM; Son et al. 2005). As IgG antibody is relatively easy to handle in immunopreciptation and purification experiments, the generation of IgG MAbs will be more convenient and useful in further investigations. Furthermore, 33 out of the 37 MAbs (89%) that we have generated here through our decoy immunization protocol are able to bind to another hESC line, viz., H1, suggesting that the 33 MAbs may also bind to other hESCs.

Concluding remarks

The decoy immunization protocol that we have developed in this study appears to be an efficient screening technique for identifying new cell-surface molecules that may have roles in cell signaling pathways in hESCs. The 33 MAbs that we have generated here should be useful

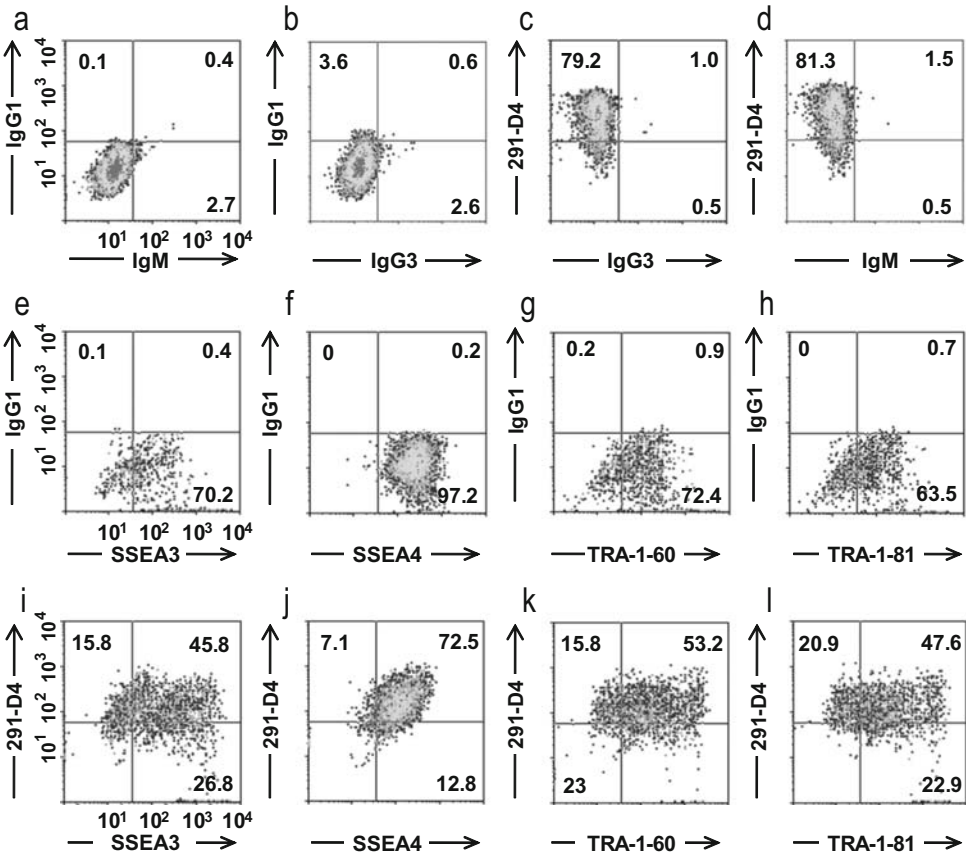


Fig. 4 Multi-color flow cytometric analysis of H9 cells with MAb 291-D4 and hESC-specific antibodies. H9 cells were incubated with one of hESC-specific antibodies, viz., SSEA3, SSEA4, TRA-1–60, or TRA-1–81, and further incubated with appropriate secondary antibodies, viz., FITC-conjugated anti-mouse IgG, anti-mouse IgM, or anti-rat IgM. Then, the cells were incubated with biotin-conjugated 291-D4 followed by with streptavidin-PE (**i–l**). Appropriate isotype-matched control staining was also performed to establish no cross-reactivity between the antibodies used (**a–h**). Values in each quadrant indicate the percentage of positive cells

for the investigation of surface molecules on hESCs in the pluripotent and undifferentiated state, for the identification of hESC characteristics, and for the assessment of differentiation.

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