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Generation and Characterization of a Monoclonal Antibody with Specificity for *Mycoplasma arginini*

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Previously, we generated monoclonal antibodies (MAbs) that bound to the surface of human embryonic stem cells (hESCs) in an attempt to discover new hESC-specific surface markers. In this study, MAb 47-235 (IgG1, κ) was selected for further characterization. The MAb bound to the surface of undifferentiated hESCs but did not bind to mouse ESCs or mouse embryonic fibroblast cells in flow cytometric analysis. The antibody immunoprecipitated a 47 kDa protein from the lysates of cell surface-biotinylated hESCs. Identification of the protein by quadrupole time of flight tandem mass spectrometry revealed that 47-235 binds to Ag 243-5 protein of *Mycoplasma arginini*. BM-Cyclin treatment of the hESCs that reacted with 47-235 resulted in loss of mycoplasma DNA and the reactivity to 47-235. Nevertheless, the hESCs that were reactive to 47-235 maintained self-renewal and pluripotency and thus could be differentiated into three embryonic germ layers.

Keywords: human embryonic stem cells, monoclonal antibody, *Mycoplasma arginini*, cell surface protein

Human embryonic stem cells (hESCs) derived from the inner cell mass of preimplantation embryos have been shown to proliferate infinitely under specific culture condition and to differentiate into a wide range of cell types *in vitro* (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Park *et al.*, 2003). hESCs share some features in common with mouse embryonic stem cells (mESCs), for example, high level expression of alkaline phosphatase and stem cell transcription factor, Oct-4. Nevertheless, the hESCs show marked differences from their mouse counterparts. In addition to morphological differences, hESCs and mESCs differ in growth conditions and cytokine requirements to maintain self renewal and pluripotency in culture. Actually, the number of human stemness genes shared by mESCs appeared to be quite low by the recent microarray analysis (Bhattacharya *et al.*, 2004; Ginis *et al.*, 2004).

Cell-surface markers are used routinely to define undifferentiated hESCs and mESCs (Solter *et al.*, 1978; Shevinsky *et al.*, 1982). Stage specific embryonic antigen-1 (SSEA1) is expressed on undifferentiated mESCs or differentiated hESCs, while SSEA3 and SSEA4 are expressed on undifferentiated hESCs but not on undifferentiated mESCs (Henderson *et al.*, 2002; Laslett *et al.*, 2003). Two human EC cell antigens TRA-1-60 and TRA-1-81 are also used to identify undifferentiated hESCs (Andrews *et al.*, 1984a; Badcock *et al.*, 1999). However, the epitopes of the surface antigens are carried by carbohydrates and their exact functions in ESCs are not known (Kannagi *et al.*, 1983). Therefore, it would be very valuable to identify new cell surface markers of hESCs that can be practically used in purification of the cells and also play important roles in regulating the development and differentiation of hESCs for basic stem cell researches.

Previously, in an attempt to identify new cell surface markers of hESCs, we generated monoclonal antibodies (MAbs) binding to the cell surface of hESCs by immunizing mice with hESC clumps (Son *et al.*, 2005). In this study, a MAb (47-235) was selected and its target antigen was identified. In addition, the hESCs reactive to 47-235 were characterized.

Materials and Methods

Cell culture

Human embryonic stem cell lines (Miz-hES1 and HSF6) were cultured as described previously (Park *et al.*, 2003; Abeyta *et al.*, 2004). Briefly, cells were cultured on a feeder cell layer of irradiated mouse embryonic fibroblast (MEF) in DMEM/F12 medium (Invitrogen, USA), supplemented with 20% serum replacement (Invitrogen), 0.1 mM 2-mercaptoethanol, 1% nonessential amino acids, 1 mM glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 4 ng/ml basic fibroblast growth factor (bFGF, PeproTech). The hESC colonies were subcultured every 5 days by detaching the colonies with 1 mg/ml collagenase IV (Sigma, USA). Characterization of the cells was also carried out by the methods described previously (Park *et al.*, 2003).

mESC lines J1 (UCHC, USA) was cultured on a feeder cell layer of irradiated MEF in DMEM medium (Invitrogen), supplemented with 15% fetal bovine serum (Invitrogen), 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1 mM glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 500 units/ml leukemia inhibitory factor (Li *et al.*, 1992). STO cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured as described previously (Andrews *et al.*, 1984b; Park *et al.*, 2003).

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Purification and biotinylation of MAb
MAbs were purified from the culture supernatants of hybridomas by Protein G-Sepharose column chromatography as described previously (Ryu *et al.*, 1997). Biotinylation of the purified antibody was carried out by ECL protein biotinylation module (Amershambiosciences, Korea) according to the supplier's protocol.

Flow cytometry
Single cell suspensions for flow cytometry were made from the undifferentiated Miz-hES1 as described previously (Chadwick *et al.*, 2003). Cell colonies were treated with collagenase IV for 1 h in normal growth medium and treated with cell dissociation buffer (Invitrogen) for 20 min in a 37°C incubator. Cells were dissociated by gentle pipetting and filtered through a 40 μm cell strainer. The dissociated cells were immediately resuspended at approximately 2×10^5 cells/ml in PBA (1% BSA, 0.02% NaN_3 in PBS) and incubated with each MAb or anti-SSEA1, anti-SSEA3, or anti-SSEA4 antibody (DSHB, USA) for 30 min at 4°C. After washing twice with PBA, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Ig) (BD Pharmingen) for 30 min at 4°C. Propidium iodide (PI)-negative cells were analyzed for the antibody binding using FACSCalibur (BD Immunocytometry System) and Cell Quest software (BD Immunocytometry System).

Cell surface biotinylation and immunoprecipitation
Cell surface biotinylation was performed according to the supplier's protocol with EZ-Link Sulfo-NHS-LC-Biotin (Pierce, USA). Biotin-labeled 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 phenylmethylsulfonyl fluoride, 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. The protein in the cell lysates was quantitated using a Bradford protein assay kit (BIO-RAD, USA). To remove the cellular proteins which non-specifically bind to Protein G plus-Sepharose (Santa Cruz Biotechnology, USA), the cell lysate from around 1×10^7 cells was incubated with 20 μl of 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 binding experiment. To immunoprecipitate the antigen recognized by a MAb, the precleared lysate was incubated with approximately 1 μg of monoclonal antibody 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 precleared 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 immersed 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 HRP-conjugated strepavidin (1:1,500; Amershambiosciences) at room temperature for 1 h. After extensive washing, the biotinylated proteins were visualized by ECL detection reagent (Amershambiosciences).

Target identification of MAb 47-235
To characterize the antigen recognized by MAb 47-235, the cell lysates were prepared from around 1×10^8 Miz-hES1 cells and subjected to immunoprecipitation as described above. The protein immunoprecipitated by 47-235 was resolved on SDS-PAGE gel and stained with Coomassie G250 (BIO-RAD) according to the supplier's protocol. The protein band corresponding approximately 47 kDa was excised, washed, and completely destained with 30% methanol. Then, the gel pieces were dehydrated in 100% acetonitrile for 10 min and dried for 30 min in a vacuum centrifuge. The protein was digested with modified porcine trypsin (Promega, USA) in 50 mM ammonium bicarbonate for 16 h at 37°C. The peptides extracted from the gel were concentrated using C_{18}ZipTips (Milliphore), and eluted with 50% (v/v) acetonitrile water. Mass spectrometric analyses were performed using a Q-TOF MS (Micromass) equipped with a nano-ESI source. The peptide solution was sprayed at a potential of about 2 kV, leading to the production of molecular ions. To obtain fragment ions, the collision energy was increased to 30 eV from 10 eV for collision-induced dissociation experiments. Argon was introduced as a collision gas at a pressure of 10 psi. Masslynx (Micromass) program was used for data processing, and the MS-Tag search progra(web-based: prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm) was employed to identify proteins based on the sequence of peptide fragments.

Elimination of mycoplasmas
Mycoplasmas were eliminated using BM-Cyclin (Roche, 799050) according to the supplier's instruction.

Detection of mycoplasmas by Western blot analysis and PCR
To detect mycoplasma protein, cell lysates were prepared as described above without biotinylation and subjected to Western blot analysis with 47-235. The cell lysates 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 phenylmethylsulfonyl fluoride, 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. The protein in the cell lysates was quantitated using a Bradford protein assay kit (BIO-RAD). The quantitated cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel under denaturing conditions and transferred to Protran nitrocellulose membrane (Schleicher & Schuell, USA). The membranes were then incubated with 47-235 followed by HRP-conjugated anti-mouse IgG antibody (Santa Cruz). Finally, the immunoblots were visualized using ECL detection reagent (Amershambiosciences).

To detect the DNA of mycoplasma, PCR was performed with e-MycoTM Mycoplasma PCR detection Kit (iNtRON BIOTECHNOLOGY, Korea) according to the supplier's protocol.

Preparation of embryoid bodies from hESCs
Human embryoid bodies (EBs) were prepared from Miz-hES1 and HSF6 cells by a previously reported method

with minor modifications (Li *et al.*, 1992; Zhang *et al.*, 2001). Briefly, confluent ES cells in a 100 mm dish were treated with 1 mg/ml collagenase IV (Invitrogen); then, small clumps of cells were cultured in suspension in EB differentiation medium (knockout-Dulbecco's modified Eagle's medium, Invitrogen), 20% fetal bovine serum, 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 1% nonessential amino acids and 2 mM L-glutamine (Invitrogen). Cultures were maintained in suspension for six and 12 days.

RT-PCR analysis

Total RNA was isolated from ESCs and EBs using High Pure RNA Isolation kit (Roche Co., USA). Total RNAs were treated with DNase 1, followed by PCR amplification with Access RT-PCR System Kit (Promega). First strand cDNAs were reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) at 48°C for 45 min. PCR reactions were performed in a thermocycler (MJ research) with cycling parameters as follows; denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, and elongation at 68°C for 2 min. Final extension at 68°C for 7 min was terminated by rapid cooling at 4°C. Cycling times were determined for each primer set to be within the exponential phase of amplification. PCR products were resolved on 1.2% agarose gels containing 400 ng/ml ethidium bromide and visualized on a UV transilluminator. The sense and antisense primers used for amplification of the cDNAs encoding human Oct4, Sox2, Pax6, CD34, AFP, or β-actin are shown in Table 1.

Table 1. Primer sequences for RT-PCR analysis

Gene	Direction	Sequence
Oct4	Forward	5'-CGACCATCTGCCGCTTTGAG-3'
	Reverse	5'-CCCCCTGTCCCCCATTCCTA-3'
SOX2	Forward	5'-TACCTCTTCCTCCCACTCCA-3'
	Reverse	5'-ACTCTCCTCTTTTGCACCCC-3'
Pax6	Forward	5'-AACAGACACAGCCCTCACAAACA-3'
	Reverse	5'-CGGGAACTTGAACTGGAACTGAC-3'
CD34	Forward	5'-TGAAGCCTAGCCTGTCACCT-3'
	Reverse	5'-CGCACAGCTGGAGGTCTTAT-3'
AFP	Forward	5'-CCATGTACATGAGCACTGTTG-3'
	Reverse	5'-CTCCAATAACTCCTGGTATCC-3'
β-Actin	Forward	5'-CCACTGGCATCGTGATGGAC-3'
	Reverse	5'-GCGGATGTCCACGTCACACT-3'

Results and Discussion

Binding of MAb 47-235 to the surface of hESCs

Previously, we generated MAbs that bind to the surface of hESCs, but not to MEFs or mESCs by immunizing mice with Miz-hESC1 cells (Son *et al.*, 2005). In this study, we selected MAb 47-235 (IgG1, κ) and further characterized. The MAb bound to the surface of hESC lines Miz-hES1 and HSF6 (Fig. 1A) but did not bind to mESC line J1 and feeder cells (MEF and STO) in flow cytometric analysis (Fig. 1B). The hESCs expressed SSEA3 and SSEA4, but not SSEA1 (Fig. 1A), while the mESCs expressed SSEA1 (Fig. 1B), indicating that the hESCs and mESCs were in undifferentiated state. The result suggested that 47-235 binds to the surface of undifferentiated hESCs specifically.

Identification of 47-235 antigen

To identify the cell surface antigen recognized by 47-235, the surface proteins of Miz-hES1 cells were biotinylated and the biotinylated cell lysates were immunoprecipitated with 47-235, followed by Western blot analysis with Streptavidin-HRP (Fig. 2A). An approximately 47 kDa protein was detected (Fig. 2A). To identify the 47 kDa protein, Miz-hES1 cells were cultured on a large scale and the cell lysates were subjected to immunoprecipitation with 47-235 followed by SDS-PAGE. After Coomassie G250 staining of the gel (Fig. 2B), the 47 kDa protein band was cut out and subjected to Q-TOF tandem MS after in-gel digestion with trypsin, as described in the Materials and Methods. The sequence analysis of 29 peptides revealed that the 47 kDa protein was Ag 243-5 protein of *Mycoplasma arginini* (Fig. 2C). This result suggests that the Miz-hES1 cells were contaminated with *Mycoplasma arginini* during culture and the

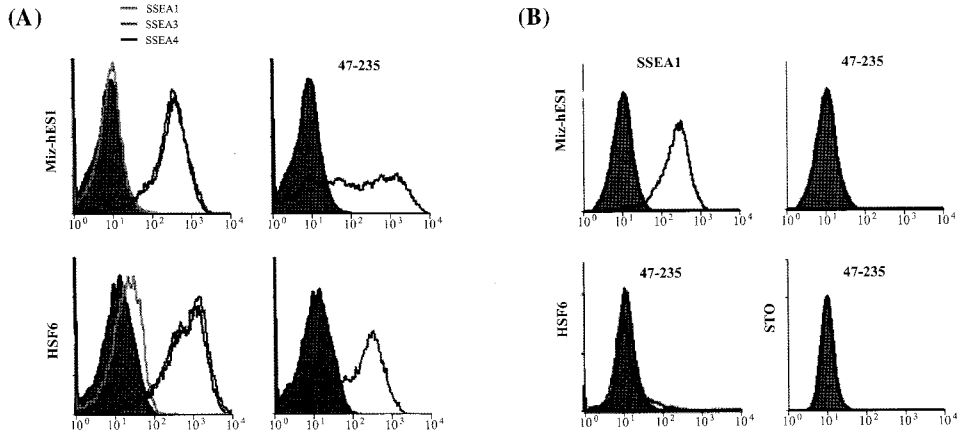


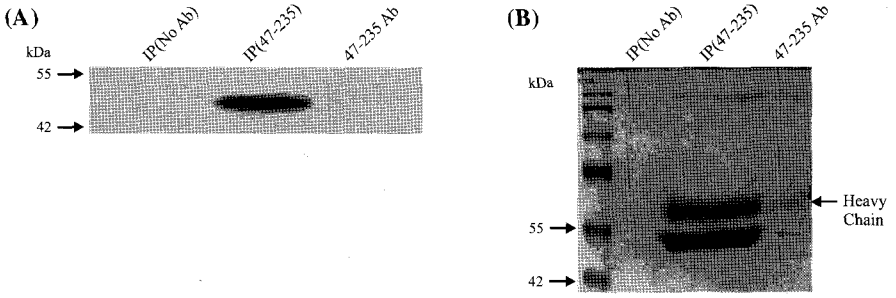
Fig. 1. Flow cytometric analysis of hESCs (A) and mESCs and feeder cells (B). Miz-hES1, HSF6, mES J1, MEF, and STO cells were stained with anti-SSEA1, anti-SSEA3, anti-SSEA4, and 47-235.

mycoplasma protein on the surface of the contaminated cells were immunogenic in mice to generate antibodies.

To confirm the antigen specificity of 47-235, the Miz-hES1 and HSF6 cells were treated with BM-Cyclin, which is antibiotic combination for the elimination of mycoplasmas, and together with untreated cells subjected to flow cytometric analysis using 47-235 and the ESC-specific surface markers. The result showed that 47-235 did not bind to the treated cells, while SSEA-3 and SSEA-4 markers were expressed on the cells (Fig. 3A), suggesting that 47-235 really bound to the mycoplasma protein. Contamination of the hESCs with mycoplasmas was also confirmed by Western blot analysis of the cell lysates using 47-235 (Fig. 3B) and PCR analysis for detection of the DNA of *Mycoplasma arginini* (Fig. 3C). The hESCs showed reactivity to 47-235 but the mESCs did not (Fig. 3B), indicating that the mESCs were not contaminated with mycoplasmas. This was consistent with the result of flow cytometry (Fig. 1). Also, the DNA of *Mycoplasma arginini* was detected from the contaminated cells (Fig. 3C). However, after treatment with BM-Cyclin, the DNA was not detected from the treated cells (Fig. 3C), as was seen in flow cytometric analysis (Fig. 3A).

Self- renewal and pluripotency of hESCs contaminated with mycoplasmas

The fact that the hESCs were contaminated with mycoplasmas provoked a question if the hESCs could maintain self-renewal and pluripotency during culture. To address this question, EBs were derived from the contaminated hESCs and cultured in bacterial petri dishes for six days. Then the hESCs and EBs were subjected to RT-PCR analysis for the expression of transcription factors (Oct4 and Sox2) essential for the maintenance of self-renewal and pluripotency in hESCs and markers indicative of differentiation (ectoderm,



(C)

Accession #	Name	Species	Found
gi|223095	Ag 243-5 protein precursor [Mycoplasma arginini]	Mycoplasma arginini	29

Matched Sequence	Delta Mass	Mod Subseq	# Mods	Score	Dist Score	Confidence	Cycle	Exp	Time
AGHFISLLYK	0.021		0	33	7	92	1111	3	22.996
AQEFLNEATADFK	0.040		0	34	12	98	1128	2	23.601
AQEFLNEATADFK	1.040	LN	0	18	5	85	1143	3	24.444
GISDDPVGVSNSTV	0.025		0	38	15	98	1111	2	22.961
IFSPATVFFTSIEK	0.021		0	36	13	98	1393	2	31.281
KVIIVAVDWDLSK	0.081		0	27	12	98	1170	2	25.464
LTNPANATVYK	0.010		0	24	8	94	1033	2	19.307
NFDFNTVLLTAGG	0.030		0	31	14	98	1279	2	28.569
NFDFNTVLLTAGG	0.090		0	22	8	94	1279	3	28.603
NVWILTGFQQGQE	0.035		0	24	6	86	1283	2	28.844
NVWILTGFQQGQE	0.115		0	20	7	92	1284	2	28.896
QIQANPTNYK	-16.995	IQ	0	25	4	76	1043	3	20.097
SFNQSIWEAVLEH	0.127		0	19	4	76	1334	2	29.976
SSLVLPVAGSLTSS	0.082		0	18	4	76	1382	2	30.952
SVLGIPTMLINDND	0.097		0	24	8	94	1266	2	28.136
SVLGIPTQLINDND	0.042		1	26	6	86	1201	2	26.281
TATFINGIVNK	0.049		0	31	7	92	1095	2	22.374
TATFINGIVNK	1.029	FI	0	22	3	71	1129	3	23.721
TEEAGFIAGYASSK	0.046		0	20	4	76	1079	3	21.786
TEEAGFIAGYASSK	0.046		0	31	9	95	1069	2	21.167
TEEAGFIAGYASSK	0.106		0	16	3	71	1071	2	21.236
TIAPFGGGHGAGV	0.112		0	19	8	94	1615	2	35.680
VIIVAVDWDLSK	0.056		0	16	4	76	1271	2	28.326
VIIVAVDWDLSK	0.056		0	32	10	97	1254	2	27.790
VSQETNNQSELIGI	0.014		0	22	7	92	1029	2	18.964
VTISDNNINIDTGFI	0.067		0	23	6	86	1152	2	24.978
YLIGVDTDQSK	0.020		0	22	5	85	1060	2	20.805
YLIGVDTDQSK	0.500		0	18	6	86	1061	3	20.925
YNNDNPTAK	0.057		0	22	4	76	676	2	12.001

Fig. 2. Target identification of MAb 47-235. (A) Cell lysates of biotinylated Miz-hES1 were immunoprecipitated with 47-235 [IP(47-235)] or no antibody [IP(No Ab)], and together with 47-235 antibody were subjected to Western blot analysis using Streptavidin-HRP. (B) Cell lysates of unbiotinylated Miz-hES1 were immunoprecipitated with 47-235 and the immunoprecipitated protein was subjected to 10% SDS-PAGE, which was then stained with Coomassie G250. The arrow head in the gel indicates a protein band immunoprecipitated with MAb 47-235. (C) Identification of 47-235 antigen as Ag 243-5 protein of *Mycoplasma arginini* by Q-TOF mass spectrometry. All 26 peptide sequences specify the protein of *Mycoplasma arginini*.

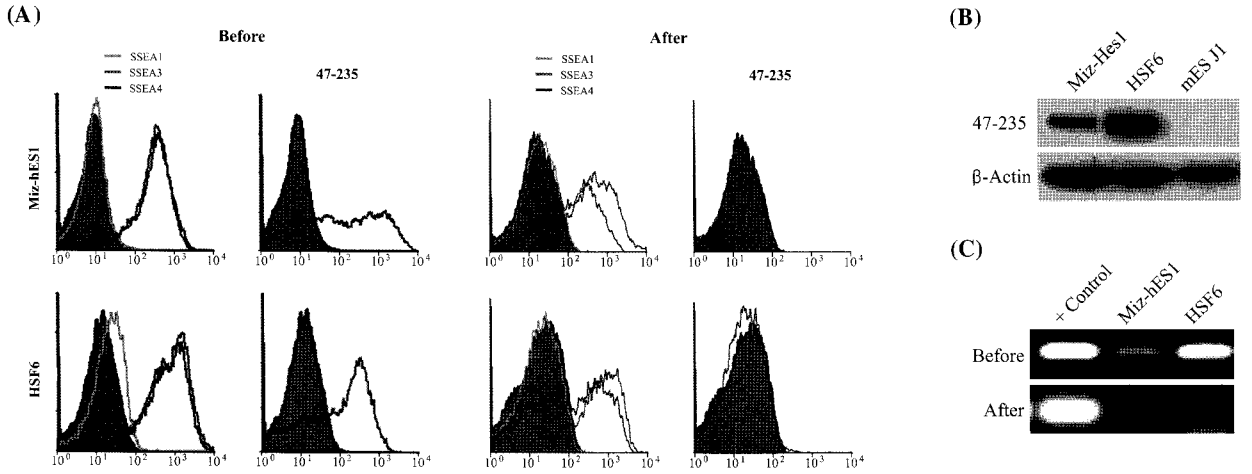


Fig. 3. Flow cytomtry (A), Western blot analysis (B), and PCR (C) of hESCs and mESCs before and after BM-Cyclin treatment.

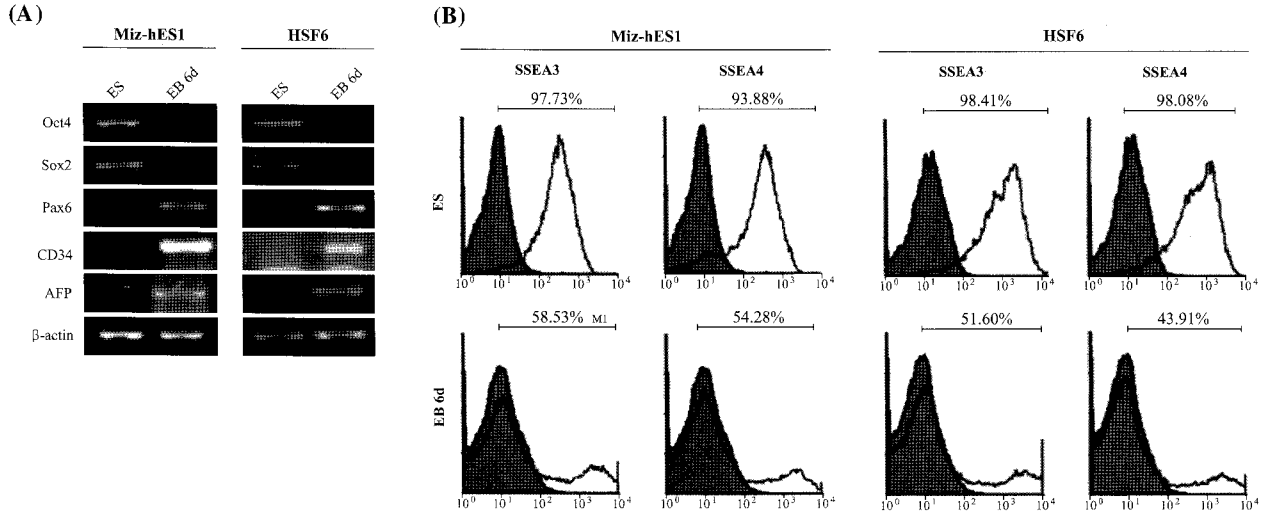


Fig. 4. RT-PCR analysis of expression of transcription factors and markers indicative of three embryonic germ layers (A) and flow cytometric analysis of hESC-specific cell-surface markers (B) in the contaminated hESCs and the EBs derived from the hESCs.

Pax6; mesoderm, CD34; and endoderm, AFP) using the PCR primers (Table 1). As shown in Fig. 4A, the hESC markers were detected in the undifferentiated hESCs, but hardly in the EB cells, while the three embryonic germ layer markers (Pax6, CD34, and AFP) were detected in the EB cells, but hardly in the hESCs. Likewise, expression of SSEA3 and SSEA4 was almost completely down-regulated upon differentiation (Fig. 4B). These results indicate that the contaminated hESCs were capable of differentiating into three embryonic germ layers, suggesting that self-renewal and pluripotency of the hESCs were not significantly impaired by the mycoplasma contamination.

Acknowledgments

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