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CD9 Is Associated with Leukemia Inhibitory Factor-mediated Maintenance of Embryonic Stem Cells

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Mouse embryonic stem (ES) cells can proliferate indefinitely in an undifferentiated state in the presence of leukemia inhibitory factor (LIF), or differentiate into all three germ layers upon removal of this factor. To determine cellular factors associated with self-renewal of undifferentiated ES cells, we used polymerase chain reaction-assisted cDNA subtraction to screen genes that are expressed in undifferentiated ES cells and down-regulated after incubating these cells in a differentiation medium without LIF for 48 h. The mRNA expression of a tetraspanin transmembrane protein, CD9, was high in undifferentiated ES cells and decreased shortly after cell differentiation. An immunohistochemical analysis confirmed that plasma membrane-associated CD9 was expressed in undifferentiated ES cells but low in the differentiated cells. Addition of LIF to differentiating ES cells reinduced mRNA expression of CD9, and CD9 expression was accompanied with a reappearance of undifferentiated ES cells. Furthermore, activation of STAT3 induced the expression of CD9, indicating the LIF/STAT3 pathway is critical for maintaining CD9 expression. Finally, addition of anti-CD9 antibody blocked ES cell colony formation and reduced cell viability. These results indicate that CD9 may play a role in LIF-mediated maintenance of undifferentiated ES cells.

INTRODUCTION

Mouse embryonic stem (ES) cells, which originally derived from inner cell mass of an early embryo named blastocyst, are able to sustain their pluripotency in in vitro cell culture (Evans and Kaufman, 1981; Martin, 1981). Undifferentiated mouse ES cells can be maintained for a long time in media containing the cytokine leukemia inhibitory factor (LIF) (Smith *et al.*, 1988; Williams *et al.*, 1988). Pluripotency of such cultured ES cells has been demonstrated in both in vivo and in vitro experiments. When injected into blastocysts, ES cells participate in embryonic development involving all three germ layers producing chimeric mice (Bradley *et al.*, 1984). ES cells also form teratomas containing various mature tissues when injected into immunocompromised mice (Evans and Kaufman, 1981; Martin, 1981). In vitro, mouse ES cells start to differentiate to every possible lineage upon removal of LIF from the culture medium. The mechanism by which LIF maintains ES cells in undifferentiated state is not completely understood. The transcription factor STAT3 is a downstream target of LIF and its receptor interaction (Niwa *et al.*, 1998; Matsuda *et al.*, 1999). It has been shown that the activity of STAT3 is necessary and sufficient for LIF-induced self-renewal of mouse ES cells (Matsuda *et al.*, 1999). It remains unclear, however, which genes are regulated by the STAT3 transcription factor in mouse ES cells and play actual roles in the maintenance of stem cells. Indeed, there is no generally accepted mechanism by which stem cells are maintained as undifferentiated cells. Human ES cell cultures have been recently established using mouse fibroblasts as feeder cells (Shamblott *et al.*, 1998; Thomson *et al.*, 1998). In contrast to mouse ES cells, LIF cannot replace such feeder cells to maintain self-renewal of human ES cells (Thomson *et al.*, 1998). In addition, some of the adult stem cells may be maintained in a multipotent status in vitro for extended intervals (Pittenger *et al.*, 1999), but the in vitro culture of others such as hematopoietic and neural stem cells have not yet been successfully established as homogeneous stem cell

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populations. Elucidating how different types of stem cells can be maintained in culture may provide important clues into the regulation of stem cell self-renewal, and may prove critical to facilitate in studies of adult stem cells. It is important to understand the molecular mechanisms of in vitro maintenance of stem cells, particularly when clinical application of such stem cells into various diseases now becomes promising (Petersen and Terada, 2001).

To identify candidate genes that play a role in stem cell maintenance, we attempted to isolate genes that were highly expressed in undifferentiated ES cells but rapidly down-regulated after cell differentiation. We used a polymerase chain reaction (PCR)-assisted cDNA subtraction method that has been successfully applied to enrich/identify genes that are selectively expressed in other cells (Seale *et al.*, 2000; Geschwind *et al.*, 2001; Terskikh *et al.*, 2001). Through this method, a plasma membrane-associated molecule, CD9, was determined to be a gene selectively expressed in undifferentiated ES cells. Additionally, the potential regulation and role of CD9 in mouse ES cells were explored.

MATERIALS AND METHODS

ES Cell Culture

Mouse ES cell lines R1 (a gift from A. Nagy, Toronto, Ontario, Canada) or EB3/5 (a gift from H. Niwa, Osaka, Japan) were maintained on gelatin-coated dish in a medium containing 1000 U/ml recombinant mouse LIF (ESGRO; Chemicon International, Temecula, CA) as described previously (Hamazaki *et al.*, 2001). In vitro ES cell differentiation was induced using a standard method as we described previously (Minamino *et al.*, 1999; Hamazaki *et al.*, 2001). Briefly, ES cells were washed twice with phosphate-buffered saline (PBS), and resuspended in a differentiation medium (Iscove's modified Dulbecco's medium [Invitrogen, Carlsbad, CA] containing 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 300 μM monothioglycerol). Cells were cultured on Petri dish in which ES cells form aggregated embryoid body. In experiments of readdition of LIF, ES cells were differentiated in the ES differentiation medium described above for initial 3 d, and LIF (or 4-hydroxy tamoxifen [4-HT]) was added back directly to the culture.

PCR-assisted cDNA Subtraction

PCR-assisted cDNA subtraction was performed based on the manufacturer's manual for the PCR-Select cDNA subtraction kit (CLONTECH, Palo Alto, CA). Total RNA was prepared from undifferentiated ES cells (tester) and 48-h differentiated ES cells (driver) by using an RNA aqueous kit (Ambion, Austin, TX). The mRNA was purified using Poly (A) Pure kit (Ambion).

Cloning and Sequencing of Subtracted cDNA

A subtracted cDNA library was cloned into TOPO TA-cloning vector (Invitrogen). After transformation, insert cDNAs were amplified by colony PCR with nested PCR primers 1 and 2R provided with the PCR-Select cDNA subtraction kit (CLONTECH). Amplified PCR fragments were purified with PCR purification kit (QIAGEN, Valenica, CA) and sequenced.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from ES cells by using an Aqueous kit (Ambion). Total RNA (2 μg) was used for cDNA synthesis by using SuperScript II first-strand synthesis system with oligo(dT) (Invitrogen). Final products of reverse transcriptase reaction were filled up to 200 μl with H_2O, and 5 μl was used for each PCR reaction. PCR amplification was performed using *Taq* DNA polymerase (Eppendorf, Westbury, NY). The PCR reaction consisted of 25–30 cycles (specified below) of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Sequence of upstream and downstream primers pair and cycle numbers used for each gene were as follows: CD9 (CAGTGCTTGCTATTGGACTATG, GCCACAGCAGTCCAACGCCATA, 30), osteopontin (GCAGACACTTTCACTCCAATCG, GCCCTTTCCGTTGTTGTCCTG, 30), CD81 (CCATCCAGGAGTCCCAGTGTCT, GAGCATGGTGCTGTGCTGTGGC, 30), platelet endothelial cell adhesion molecule-1 (PECAM-1) (AGGGGACCAGCTGCACATTAGG, AGGCCGCTTCTCTTGACCACTT, 30), E-cadherin (GTCAACACCTACAACGCTGCC, CTTGGCCTCAAAATCCAAGCC, 25), β1 integrin (AATGTTTCAGTGCAGAGCC, ATTGGGATGATGTCGGGAC, 30), α3 integrin (AACAGCGCTACCTCCTTCTG, GTCCTTCCGCTGAATCATGT, 30), α5 integrin (GCTGGACTGTGGTGAAGACA, CAGTCGCTGACTGGGAAAAT, 30), α6 integrin (AGGAGTCGCGGGATATCTTT, CAGGCCTTCTCCGTCAAATA, 30), heparin binding-epidermal growth factor (HB-EGF) (GTTGGTGACCGGTGAGAGTC, TGCAAGAGGGAGTACGGAAC, 30), brachyury (AAGGAACCACCGGTCATC, GTGTGCGTCAGTGGTGTGTAATG, 30), β-actin (TTCCTTCTTGGGTATGGAAT, GAGCAATGATCTTGATCTTC, 25), Oct-4 (TGGAGACTTTGCAGCCTGAG, TGAATGCATGGGAGAGCCCA, 30), UTF1 (GCCAACT-CATGGGGCTATTG, CGTGGAAGAACTGAATCTGAGC, 30), FGF4 (TACTGCAACGTGGGCATCGGA, GTGGGTTACCTTCATGGTAGG, 30), Rex-1 (CGTGTAACATACACCATCCG, GAAATCCTCTTCCAGAATGG, 30), and FGF5 (AAAGTCAATGGCTCCCACGAA, CTTCAGTCTGTACTTCACTGG, 30).

For each set of PCR primers, RT-PCR without reverse transcriptase was conducted to confirm that no genomic DNA was amplified.

Immunofluorescence Staining

ES cells were cultured on gelatin-coated plate, washed once with PBS, and fixed in 3.7% formamide/PBS for 15 min at room temperature. Cells were then treated with 0.5% Triton X/PBS for 5 min and with 5% bovine serum albumin/PBS for 1 h at room temperature. Cells were further incubated with either anti-SSEA1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-mouse osteopontin (R & D Systems, Minneapolis, MN), or anti-mouse CD9 (KMC8) (BD PharMingen, San Diego, CA) for 2 h at room temperature. After four times washing with PBS, cells were incubated with anti-mouse IgG, anti-goat IgG, or anti-rat IgG antibodies conjugated to fluorescein isothiocyanate (Jackson Immunoresearch Laboratories, West Grove, PA). After four times washing with PBS, cells were mounted by Vectashield containing 4,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

Propidium Iodide Staining

Propidium iodide was added (final 10 μg/ml) directly to the culture medium for staining cells with low viability. After a 30-min incubation at room temperature, staining was observed under a fluorescent microscope (IX70; Olympus, Tokyo, Japan).

RESULTS

To identify genes that may be involved in maintenance of undifferentiated ES cells, we attempted to isolate genes that are highly expressed in ES cells and rapidly down-regulated after cell differentiation. Herein, we used a PCR-assisted subtraction method. The cDNA prepared from undifferentiated ES cells was subtracted by the cDNA from differentiating ES cells in a differentiation medium without LIF for 48 h. Of the subtracted cDNA clones, 304 cDNAs have been sequenced. Of these, 98 genes were cloned more than two times, and the rest were unique. Osteopontin was the most

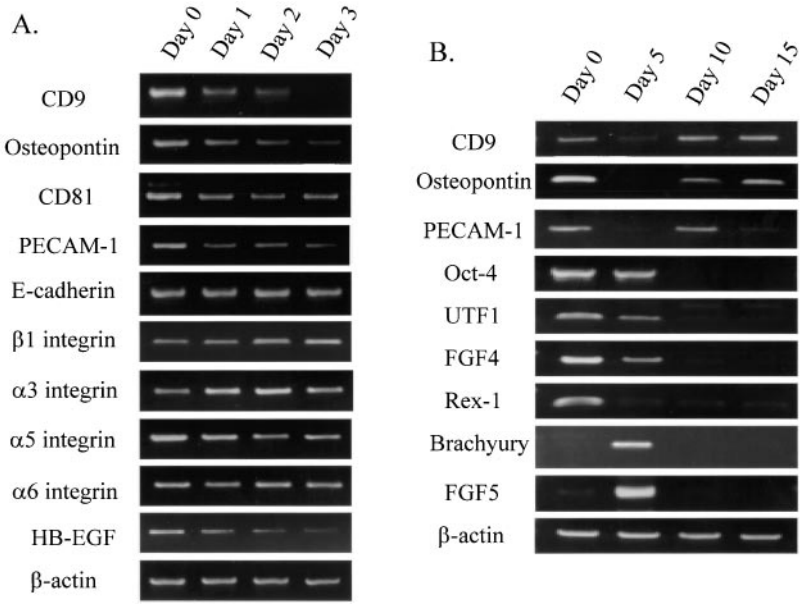

Figure 1. (A) Expression pattern of CD9 and other cell adhesion-related molecules during initial ES cell differentiation. Total RNA was isolated from undifferentiated ES cells (day 0) and differentiated ES cells (days 1–3 after cultured in the differentiation medium). (B) Expression pattern of CD9 and other molecules during long-term ES cell differentiation. Total RNA was isolated from undifferentiated ES cells (day 0) and differentiated ES cells (days 5, 10, and 15 after cell differentiation). RNA was subjected to RT-PCR analysis. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

frequently cloned gene, and a total of nine cDNA clones (corresponding to a total of three different cDNA fragments) was found among the 304 sequenced genes. Osteopontin, one of the extracellular matrix proteins, has been shown to be highly expressed in ES cells and decreased after ES cell differentiation (Botquin *et al.*, 1998). In the present screening, we also cloned other genes previously identified as ones highly expressed in ES cells and down-regulated after cell differentiation, such as Rex-1 (Hosler *et al.*, 1989), KLF4 (Kelly and Rizzino, 2000), and stathmin (Doye *et al.*, 1992) (one clone each). These results indicate that the method effectively identifies undifferentiated ES cell-associated genes.

CD9 was among the genes identified, and its expression in ES cells has not been reported previously. CD9 is a type III membrane protein with four transmembrane domains (tetraspanin) and proposed to be involved in cell adhesion, migration, proliferation, and fusion (Ikeyama *et al.*, 1993; Masellis-Smith and Shaw, 1994; Hadjiargyrou and Patterson, 1995; Maecker *et al.*, 1997; Tachibana and Hemler, 1999). Using RT-PCR, we confirmed that the mRNA expression of CD9 was down-regulated within 24 h of ES cell differentiation (Figure 1A). The CD9 expression was further decreased until day 3 in the differentiation medium. In the same system, we also examined other cell adhesion-related molecules such as osteopontin and PECAM-1, which have been reported to be expressed in undifferentiated ES cells (Botquin *et al.*, 1998; Robson *et al.*, 2001). The mRNAs of both osteopontin and PECAM-1 were expressed in undifferentiated ES cells and down-regulated after differentiation as well. In contrast, the expression of other cell adhesion-related molecules including E-cadherin, and β1, α3, α5, and α6 integrins was not significantly changed throughout the time course of differentiation (days 0–3). Expression of HB-EGF, which is known to associate with CD9 (Iwamoto *et al.*, 1994), also down-regulated during ES cell differentiation. The expression of CD81, another tetraspanin molecule closely related to CD9 (Maecker *et al.*, 1997), was also detected in undifferentiated ES cells and modestly down-regulated after differentiation.

We also examined the mRNA expression of CD9 in a longer time course of ES cell differentiation, up to day 15 (Figure 1B). CD9 mRNA was expressed in undifferentiated ES cells (day 0) and down-regulated at day 5 of differentiation. The expression was increased again at day 10 and 15 of differentiation. Osteopontin and PECAM-1 also highly expressed in ES cells, temporally decreased by day 5, and increased again at day 10. Accordingly, those cell adhesion-related molecules (CD9, osteopontin, and PECAM-1) were highly expressed in undifferentiated ES cells and rapidly decreased during initial cell differentiation. However, their expression was not ES cell specific in contrast to germ cell/early embryonic cell-specific genes such as Oct-4, UTF1, FGF4, and Rex-1 (Rogers *et al.*, 1991; Niswander and Martin, 1992; Okuda *et al.*, 1998; Pesce *et al.*, 1998). Oct-4, known to be required for inner cell mass formation in blastocysts (Nichols *et al.*, 1998), was expressed until day 5 of the ES cell differentiation and eliminated by day 10. Expression of UTF1, FGF4 and Rex-1 was also eliminated by days 5–10. Markers for early mesodermal differentiation, such as brachyury and FGF5 (Haub and Goldfarb, 1991) expression, were high at day 5 and decreased thereafter in the system. In addition, contractile cardiac myocytes were observed under microscope by day 8–10, and expression of albumin mRNA was detected by day 12 as we demonstrated previously (Hamazaki *et al.*, 2001).

The protein expression of CD9 and osteopontin was then examined using immunofluorescence staining. CD9 was localized at cell surface of ES cells, as expected, when they were maintained in the ES maintenance medium containing LIF. However, within 5 d of cell differentiation, CD9 protein expression became almost undetectable (Figure 2). Osteopontin, which is a secreting protein, was detected at peri-nucleus, presumably endoplasmic reticulum or Golgi, in undifferentiated ES cells. The protein expression of os-

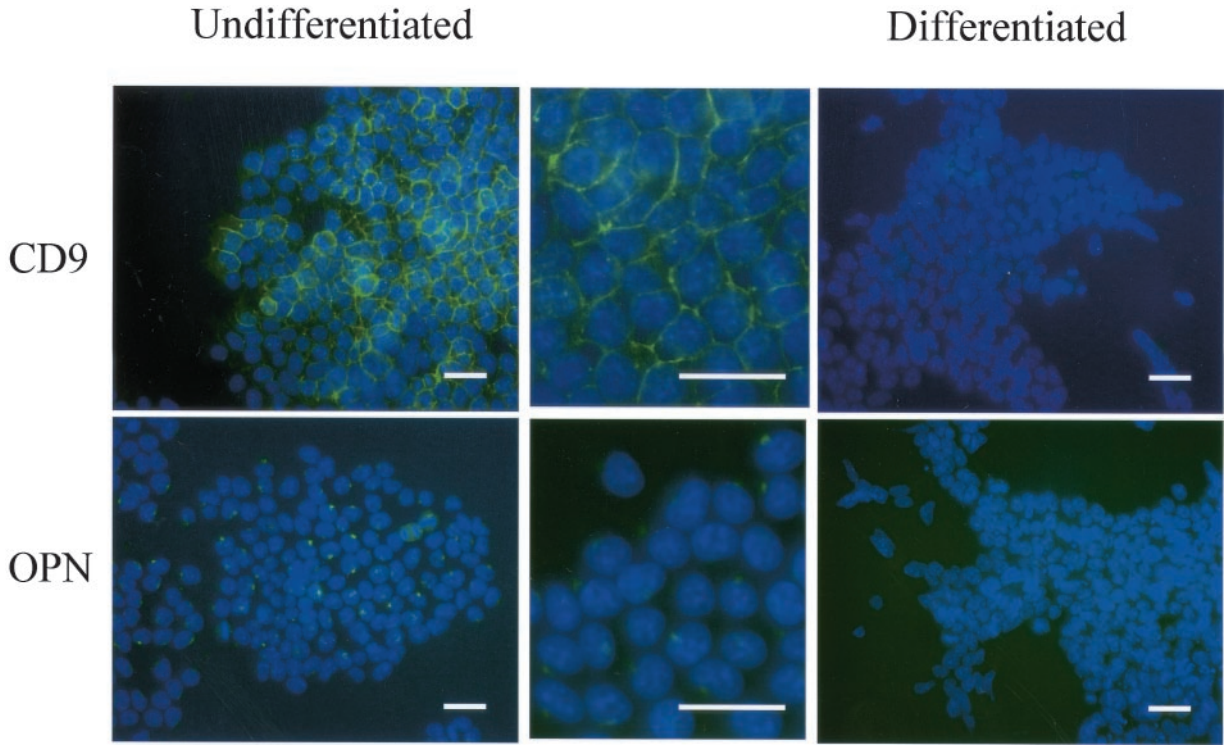

Figure 2. CD9 and osteopontin were selectively expressed in undifferentiated ES cells. The expression of CD9 and osteopontin in undifferentiated ES cells (day 0) or differentiated ES cells (day 5) was examined by indirect immunofluorescence analysis. Undifferentiated ES cells were maintained on gelatin-coated dish in the ES medium (left, low magnification; middle, high magnification). Differentiated ES cells were cultured for 5 d in the differentiation medium (right). Cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and incubated initially with anti-CD9 antibody (1:1000 dilution) or anti-osteopontin antibody (1:1000 dilution), and subsequently with fluorescein isothiocyanate-conjugated anti-rat IgG (1:100 dilution; for CD9) or anti-goat IgG (1:100 dilution; for osteopontin). Cells were costained with 4,6 diamidino-2-phenylindole to demonstrate nuclei. Bar, 50 μm.

teopontin was similarly decreased within 5 d of ES cell differentiation.

To further confirm the association of CD9 and osteopontin expression with undifferentiated status of ES cells, we examined their protein expression when LIF was added back to differentiating ES cells (Figure 3A). ES cells were incubated in the differentiation medium for 4 d, treated with trypsin, and then incubated in the ES maintenance medium containing LIF again. Most of the cells remained differentiated in this condition, but, of interest, several undifferentiated ES cell-like colonies (growing as a compact colony with tight cell-to-cell conjunctions) appeared in the culture within 4 d after switching to the LIF-containing medium (Figure 3B). These undifferentiated ES cell-like colonies were morphologically distinguishable from the other differentiated cells. Moreover, SSEA1, which is a surface marker for undifferentiated mouse ES cells (Solter and Knowles, 1978), was exclusively expressed in these compact colonies. Anti-CD9 staining revealed that these undifferentiated ES-like colonies also strongly expressed CD9. Importantly, the expression of CD9 was low or not observed in surrounding differentiated cells. Expression of osteopontin was also found exclusively in those ES-like colonies. These results indicate that CD9 expression as well as osteopontin expression is associated with the undifferentiated phenotype during early differentiation of ES cells.

To examine whether LIF is a factor important for CD9 expression in the ES maintenance medium, we incubated ES cells in LIF-free differentiation medium for 72 h. Then, LIF was added back to the culture for additional 24 h. CD9 mRNA was increased by this readdition of LIF (Figure 4). STAT3 is known as a downstream target transcription factor of the LIF receptor-mediated signaling. By using STAT3ER (STAT3 fused to estrogen-ligand binding domain), with which STAT3 activity could be modulated by concentration of 4-HT in the medium, we examined whether STAT3 activity was sufficient for the up-regulation of CD9 expression. ES cells constitutively expressing STAT3ER were maintained with the ES maintenance medium containing LIF. After LIF removal for 3 d, 4-HT was added. As shown in Figure 4, CD9 mRNA expression was induced by addition of 4-HT. These results indicate that the LIF/STAT3 pathway is important for expression of CD9 in ES cells.

Finally, we investigated whether CD9 expression is important in undifferentiated ES cells by using a neutralizing antibody against CD9, KMC8 (Oritani *et al.*, 1996). This antibody has been shown to block cell differentiation and cell fusion in other cell types, including myoblasts (Oritani *et al.*, 1996; Aoyama *et al.*, 1999; Tachibana and Hemler., 1999; Tanio *et al.*, 1999). ES cells were incubated in the medium containing LIF in the presence of the antibody for 24 h. The control cells were incubated with the identical concentration

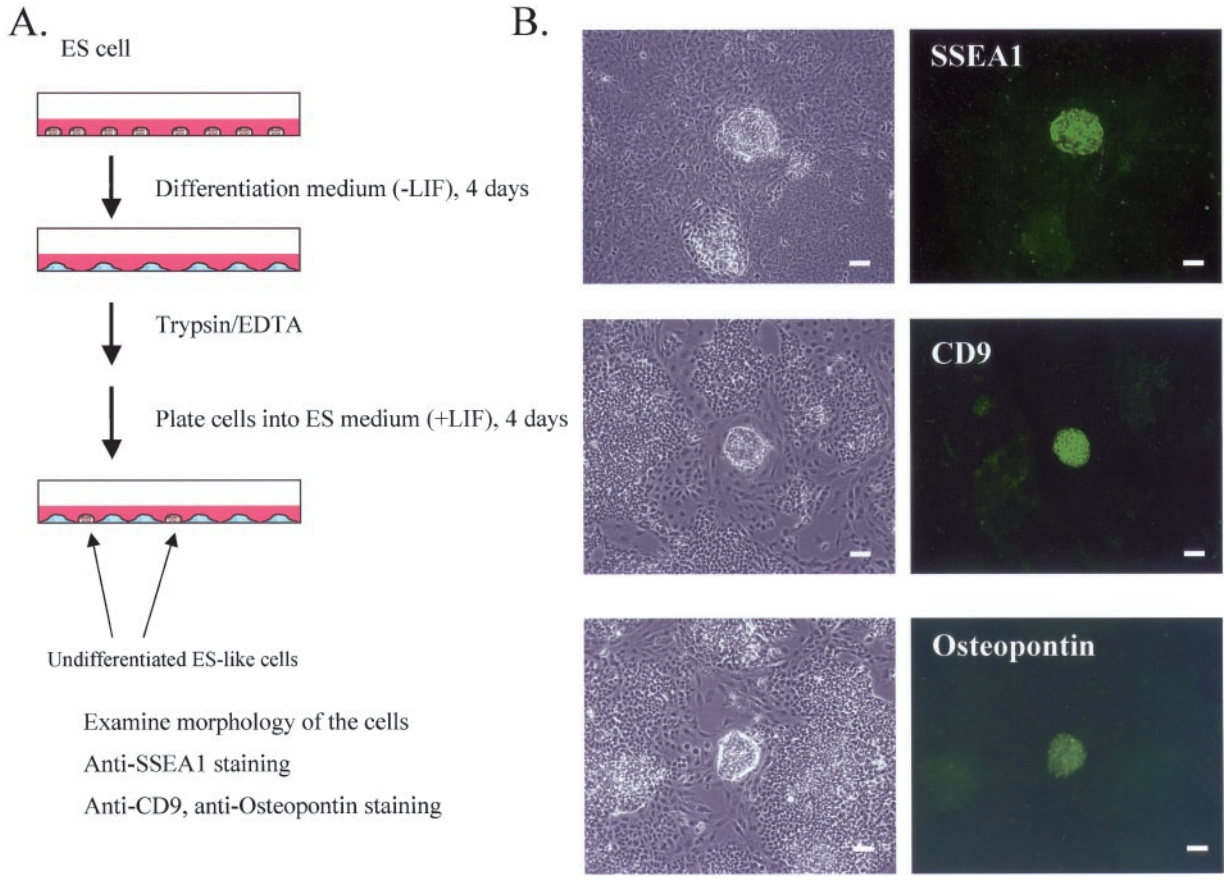

Figure 3. CD9 expression was detected exclusively in undifferentiated ES-like colonies. (A) ES cell differentiation was induced by removing LIF from the culture medium for 4 d. Cells were treated with trypsin, washed, and cultured again in the ES medium containing LIF. Within 4 d, undifferentiated ES-like colonies appeared in the culture. (B) Those mixed populations of the undifferentiated ES-like colonies and apparently differentiated cells were examined for the expression of SSEA1, CD9, and osteopontin by immunofluorescence staining as described in Figure 2 (right). Phase contrast images of the cells (left). Bar, 100 μm.

of an isotypic control antibody containing the identical concentration of azide in stock solution. Interestingly, the ES cells cultured in the presence of the anti-CD9 antibody did not form compact ES-like colonies (Figure 5). Moreover, cells appeared to be dead within 24 h of culture in the presence of the anti-CD9 antibody. The decrease in cell viability after treatment with the anti-CD9 antibody was confirmed by propidium staining of cells (Figure 5). Anti-CD9 antibody decreased the cell viability at the concentration as low as 1 μg/ml. In contrast, neither nonspecific rat IgG2a,κ nor anti-osteopontin antibody (up to 10 μg/ml) affected the colony formation or survival of ES cells. Specific interaction between SSEA1 molecules on cell surface (Lewis^X determinants) is critical for cell aggregation of embryonic carcinoma cells (Eggens *et al.*, 1989). An antibody against SSEA1, which blocks cell adhesion (Cao *et al.*, 2001), also caused decrease in cell viability of ES cells as seen with the anti-CD9 antibody.

DISCUSSION

A PCR-assisted subtraction method revealed that CD9 was among a group of genes highly expressed in ES cells but down-regulated shortly after cell differentiation. This group of genes includes several cell adhesion-related molecules such as osteopontin and PECAM-1, whereas others such as integrins β1, α3, α5, and α6 and E-cadherin were constitutively expressed during initial differentiation of ES cells. We also demonstrated that CD9 is likely under regulation of the LIF/STAT3 pathway in ES cells, which is critical for self-renewal of undifferentiated ES cells. Indeed, CD9 expression was exclusively associated with the undifferentiated phenotype of ES cells when such cells reappeared among differentiating ES cells by readdition of LIF to the culture. The expression of CD9 along with other cell adhesion-related molecules may be important for maintenance of undifferentiated ES cells. Furthermore, a blocking antibody against CD9 (KMC8) inhibited colony formation and survival of ES cells, suggesting that CD9 is playing a role in maintenance of ES cells in vitro.

A potential mechanism by which the anti-CD9 antibody reduced cell survival may be its interference with cell adhesion of ES cells as seen with anti-SSEA1 antibody (Cao *et al.*, 2001). Although CD9 itself is not considered as a cell adhesion molecule, CD9 has been proposed to play a role in

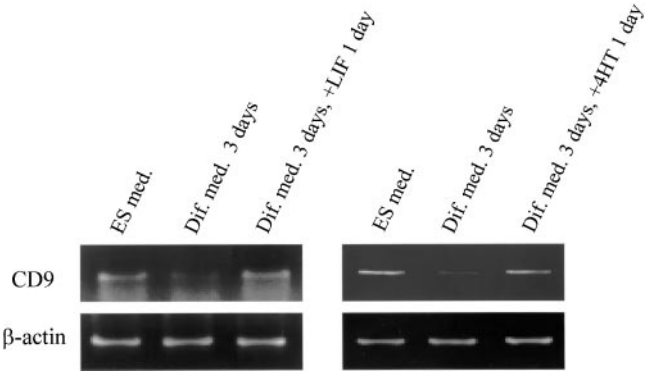

Figure 4. CD9 expression was dependent on the LIF/STAT3 pathway. ES cells constitutively expressing STAT3ER were maintained in the ES medium containing LIF (ES med.). The cells were then cultured in the differentiation medium for 3 d (Dif. med. 3 d), and 1000 U/ml LIF or 1 μM 4-HT was added directly to the medium and cultured for another day (Dif. med. 3 d + LIF/4HT 1 d). RNA prepared from the cells was subjected to RT-PCR. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

cell-extracellular matrix or cell–cell interactions as a cofactor of integrin (Rubinstein *et al.*, 1994; Nakamura *et al.*, 1995; Berditchevski *et al.*, 1996). It still remains unclear, however, how the association of CD9 with integrin regulates cell–cell/cell–extracellular matrix interactions or modifies signal transduction through the integrin/integrin–ligand interaction. Osteopontin, one of the components of the extracellular matrix, can serve as a ligand of integrin (Denhardt *et al.*, 2001), and PECAM-1 binds to integrins and enhances their function (Tanaka *et al.*, 1992; Leavesley *et al.*, 1994; Piali *et al.*, 1995; Buckley *et al.*, 1996; Newman, 1997). Collectively, integrin-associated signals may be important in self-renewal of ES cells.

Cell adhesion, especially interactions among cells and components of their environmental niche, is considered to be important for stem cell maintenance of hematopoietic stem cells (HSCs) (Chan and Watt, 2001). It has been demonstrated that CD9 may be involved in hematopoiesis. The KMC8 antibody against CD9 inhibited production of myeloid cells in long-term bone marrow cell cultures (Oritani *et al.*, 1996) and expansion of erythroid progenitor cells (Aoyama *et al.*, 1999) or osteoclasts (Tanio *et al.*, 1999) when cocultured with stromal cells. Accordingly, CD9 might be important for colony formation or maintenance of HSCs. In this context, it is tempting to examine the expression of CD9 in adult stem cells other than HSCs, such as neuronal stem cells or liver stem cells.

CD9 is also known to associate with HB-EGF (Iwamoto *et al.*, 1994), which is proposed to be important in cell survival under stressed conditions (Miyoshi *et al.*, 1997; Takemura *et al.*, 1997; Horikawa *et al.*, 1999). Because induced expression of CD9 can potentiate the function of HB-EGF (Iwamoto *et al.*, 1994; Higashiyama *et al.*, 1995), CD9 may support cell survival through HB-EGF. HB-EGF mRNA was also expressed in ES cells and down-regulated during initial differentiation. It should be noted that CD9 may be involved in cell-cell fusion as well. CD9−/− female is sterile because CD9 null oocyte cannot fuse to sperm (Miyado *et al.*, 2000; Le Naour *et al.*, 2000), and overexpression of CD9 in myoblast-derived RD sarcoma cell facilitates the formation of multinucleated cells (Tachibana and Hemler, 1999).

Despite the potential role of CD9 in ES cells we propose herein, CD9 null mice are viable without an apparent abnormality except female infertility. One hypothesis would be that closely related tetraspanins such as CD81, which was

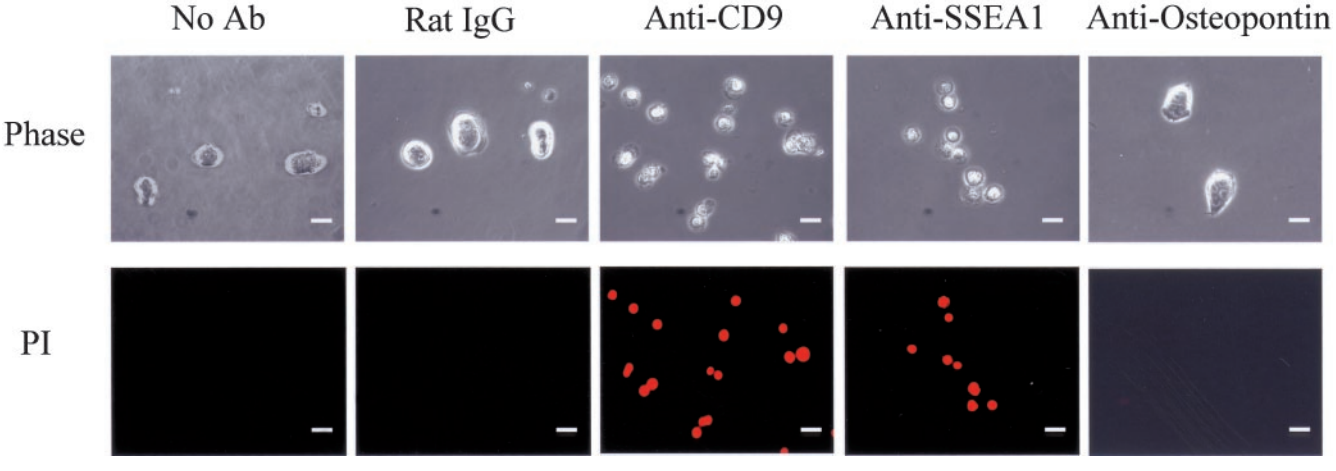

Figure 5. Effect of anti-CD9 antibody on colony formation and viability of ES cells. ES cells cultured in the ES medium were washed with PBS and treated with trypsin for 10 min at 37°C in CO_2 incubator. The cells were plated at the cell concentration of 2×10^3 cell/ml on gelatin-coated 24-well dish, and incubated for 24 h in ES medium without addition of any antibody, or in the presence (final concentration of 10 μg/ml) of control rat IgG2a,κ antibody (#555841; BD PharMingen), anti-CD9 antibody (KMC8, #558748; BD PharMingen), anti-SSEA1 antibody (MC-480; Developmental Studies Hybridoma Bank, University of Iowa), or anti-osteopontin antibody (#AF808; R & D Systems). Phase contrast images of cells are shown in top panels. Cell viability was monitored by staining cells with propidium iodide as described in MATERIALS AND METHODS (bottom). Bar, 20 μm.

also expressed in ES cells, may compensate function of CD9 during early embryonic development. Alternatively, CD9 may be required more stringently in in vitro maintenance of ES cells. Such a gap between in vitro ES cell maintenance and in vivo development has been observed previously with deficiencies of other molecules. The LIF/STAT3 signaling mediated by the LIF receptor/gp130 complex is critical for maintenance of ES cells in vitro; however, embryos lacking LIF, the LIF receptor, or gp130 develop normally, at least until midgestation (Stewart *et al*., 1992; Ware *et al*., 1995; Yoshida *et al*., 1996). Recently, Nichols *et al*. (2001) found that gp130−/− embryos were unable to resume embryogenesis after delayed implantation. Moreover, pluripotent cells were absent in delayed gp130−/− blastocysts, and they had reduced number of ICM cells due to apoptotic cell death. These results imply the importance of stem cell maintenance under suboptimal conditions even although it is not necessary for normal development. CD9 may be one of the factors downstream of the LIF/gp130/STAT3 pathway, critical for stem cell maintenance under such suboptimal conditions or stem cell maintenance in vitro. Maintenance of stem cells in vitro is important particularly when we consider clinical application of stem cells. Expansion of adult normal adult stem cells in vitro as a homogeneous population would facilitate application of such stem cells. The study of factors necessary for ES cell maintenance may contribute to a discovery of common mechanisms by which stem cells can be sustained as stem cells in vitro.

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