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Spa-1 regulates the maintenance and differentiation of human embryonic stem cells

YOUNG-JIN LEE[1,2], HEE-YOUNG NAH[1], SEOK-HO HONG[1], JI-WON LEE[1], ILKYUNG JEON[1], JHANG HO PAK[3], JOO-RYUNG HUH[4], SUNG-HOON KIM[1], HEE-DONG CHAE[1], BYUNG-MOON KANG[1], CHUL GEUN KIM[2] and CHUNG-HOON KIM[1,*]

[1]Dept. of Obstetrics & Gynecology, College of Medicine, University of Ulsan, Asan Medical Center, [2]Dept. of Life Science, College of Natural Sciences and Stem Cell Research Center, Hanyang University, [3]Dept. of Obstetrics & Gynecology, College of Medicine, Asan Institute for Life Science, Asan Medical Center and [4]Dept. of Pathology, College of Medicine, University of Ulsan, Asan Medical Center, Seoul, Republic of Korea

ABSTRACT Human embryonic stem cells (hESCs) are pluripotent, whereby they can proliferate endlessly and differentiate into many different cell types. At the molecular level, little is known of the mechanisms underlying their capability for self-renewal and differentiation. In the present study, we established two new hESC lines (AMC-hES1 and AMC-hES2) and demonstrated the existence of a regulator that may be a key molecule in hESC dynamics. Spa-1 is a principal Ras-proximate 1 (Rap1) GTPase-activating protein in hematopoietic progenitor cells that regulates Rap1-related signal transduction and is expressed restrictively in human adult tissues (bone marrow, thymus, and spleen). To investigate its functions in hESCs, we examined *spa-1* expression profiles during hESC differentiation and used RNA interference (RNAi) to downregulate *spa-1* in these cells. Our results show that Spa-1 is expressed in undifferentiated hESCs and is downregulated during hESC differentiation. In addition, the process of passing from the mode of self-renewal to that of differentiation in hESCs was regulated by spa-1 via Rap1/Raf/mitogen-activated protein kinase kinase/extracellular signal-related kinase signaling. An RNAi expression vector against spa-1 (pSUPER.retro.puro) was transfected into hESCs, which were seen to differentiate into three germ layers in spite of being in the undifferentiated condition. Based on our findings, therefore, it appears that spa-1 may be involved in hESC dynamics, and our results provide fundamental information regarding the self-renewal and differentiation of hESCs.

KEY WORDS: *signal transduction, fate decision, gene regulation, differentiation*

Introduction

Human embryonic stem cells (hESCs), which are being considered as a new therapeutic material for the treatment of degenerative disorders, have specific properties that allow them to differentiate into several cell lineages, and can divide indefinitely when in the undifferentiated condition. hESC lines were first established in 1998 (Thomson *et al.*, 1998), and these pluripotent cells have been isolated from germ cells and blastocysts (Papaioannou *et al.*, 1984; Rossant and Papaioannou, 1984; Shamblott *et al.*, 1998; Thomson and Odorico, 2000).

It was thought that the self-renewal and differentiation activities of mouse embryonic stem cells (mESCs) and hESCs are regulated by the coordinated interaction between several intrinsic and extrinsic factors. Janus kinase-signal transducer and activator of transduction (Jak-STAT) signaling, which is activated by leukemia inhibitory factor (LIF), is crucial for the maintenance of the self-renewal activity of mESCs (Yoshida *et al.*, 1994; Niwa *et al.*, 1998; Burdon *et al.*, 1999; Matsuda *et al.*, 1999; Niwa, 2001).

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; CM, conditioned medium; EB, embroyid body; hESC, human embryonic stem cell; ICM, inner cell mass; Jak, janus kinase; LIF, leukemia inhibitory factor; Rap, ras-proximate; siRNA, small interference RNA; spa, signal-induced proliferation-associated gene; STAT, signal transducer and activator of transduction.

***Address correspondence to:** Chung-Hoon Kim. Dept. of Obstetrics and Gynecology, College of Medicine, University of Ulsan, Asan Medical Center, 388-1 Pungnap-2dong, Songpa-gu, Seoul 138-736, Korea. Fax: +82-2-3010-5059. e-mail: yjleelee@amc.seoul.kr

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In addition, a combination of the LIF and bone morphogenic protein signaling pathways (Ying *et al.*, 2003), as well as transcription factor Nanog (Mitsui *et al.*, 2003) are required for the self-renewal of mESCs, but it is unclear that whether LIF acts alone or with other (unknown) factors. By comparison, the self-renewal activity of hESCs is independent of LIF signaling (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Schuringa *et al.*, 2002), and they can be cultured on feeder cells, which are mitotically inactivated, with hESC culture medium and basic fibroblast growth factor (bFGF and FGF2), which is essential for the maintenance of hESCs (Amit *et al.*, 2004). It has been reported that hESCs can be cultured and maintained with LIF and/or transforming growth factor β-1 (TGFβ-1), but these results were not seen as a significant advancement in the culturing of hESCs (Amit *et al.*, 2004). Recently, it has been demonstrated that wnt pathway genes may also be regulators involved in hESC dynamics (Walsh *et al.,* 2003; Sato *et al.,* 2004; James *et. al.,* 2005; Prowse *et. al.,* 2005). Boyer *et. al.* (2005) identified OCT4, SOX2, and NANOG target genes using genome-scale location analysis, and oct4-dependent transcriptional networks (Babaie *et al.,* 2007) as well as fibroblast growth factor 2 (FGF2) signaling mechanism involved in transforming growth factor β (Greber *et al.,* 2007) were reported. Despite these reports, there are much uncovered knowledge about the molecular mechanisms that regulate the maintenance and differentiation of hESCs. Thus, we scanned genes that are expressed uniquely in undifferentiated hESCs by oligo DNA chip, which resulted in the identification of signal-induced proliferation-associated gene-1 (spa-1) as an overexpressed gene in undifferentiated hESCs compared to differentiated hESCs (day-20 embryoid bodies; Supplementary Table 1).

Spa-1 is a principal Rap1 (Ras-proximate 1) GTPase-activating protein in hematopoietic progenitor cells, and regulates Rap1-related signal transduction (Kurachi *et al.*, 1997; Gao *et al.*, 1999; Pak *et al.*, 2001; Roy *et al.*, 2002). The Spa-1 family consists of several distinct proteins, including spa-1, E6-targeted protein 1 (E6TP1), spine-associated RapGAP (SPAR), and spa-1-like proteins (SPA-Ls), all of which show a specific cellular distribution in tissues (Kurachi *et al.*, 1997; Gao *et al.*, 1999; Pak *et al.*, 2001). In Spa-1-deficient mice, there appears to be an increase in the expression of Rap1GTP in progenitor cells in the bone marrow, and this is associated with an increase in the number of blood leukocytes following constitutive activation of extracellular signal-related kinase (ERK). The increased population of leukocytes was comprised predominantly of mature granulocytes, closely resembling the condition of human chronic myelogenous leukemia (CML) in the chronic phase (Ishida *et al.*, 2003). It has been suggested that this phenomenon is mediated, at least in part, by Rap1-B-raf-mediated activation of ERK (Kometani *et al.,* 2004). In humans, the spa-1 gene is located on chromosome 11q13.3, which is one of the hot spots in human hematologic malignancies (Wada *et al.*, 1997; Wong, 1999).

We have investigated the expression of spa-1 and spa-1 effectors to define the possible influence of spa-1-involved signaling on the maintenance and differentiation of hESCs. In addition, we examined the spa-1 signaling pathway to determine the molecular mechanisms that control the self-renewal activity of hESCs. The results of this study suggest that spa-1 can influence the maintenance and differentiation of hESCs via the Rap1/Raf/

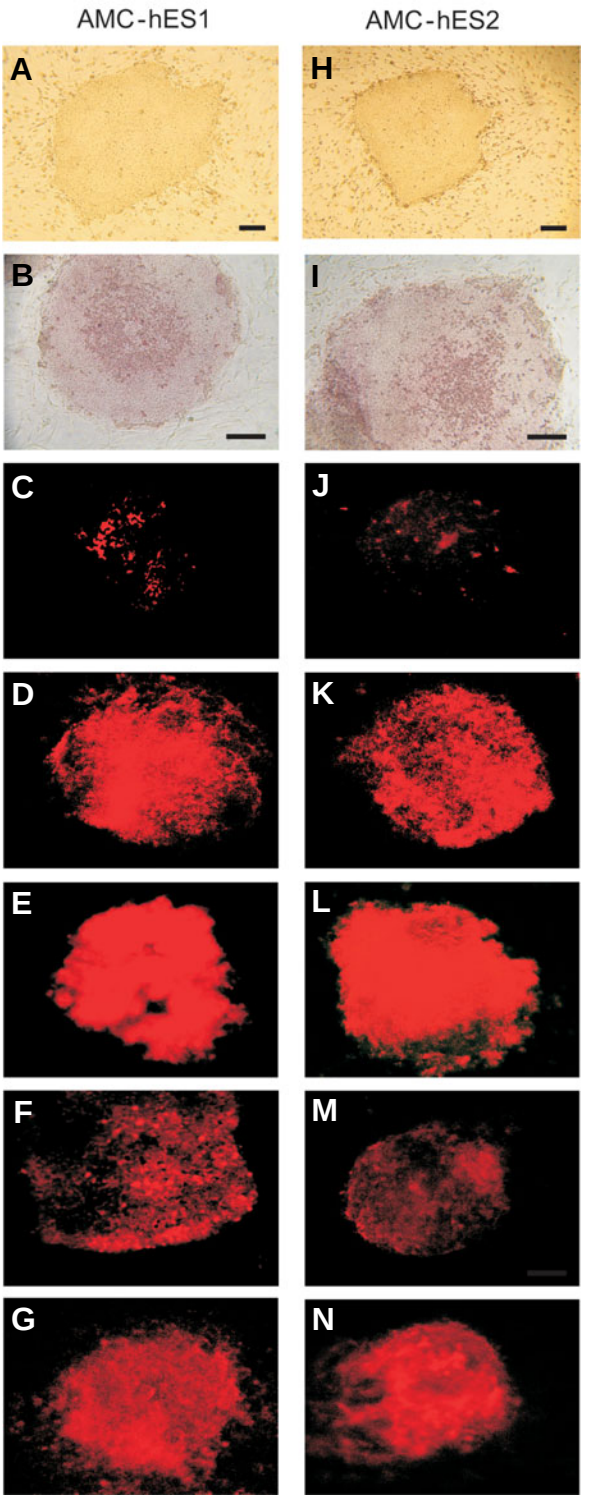

Fig. 1. **Derivation and characterization of human embryonic stem cell (hESC) lines. (A,H)** *Colonies of established and undifferentiated hESCs. Expression analyses of hESC marker proteins for the two novel hESC lines established (AMC-hES1 and AMC-hES2): alkaline phosphatase* **(B,I)**, *SSEA-1* **(C,J)**, *SSEA-3* **(D,K)**, *SSEA-4* **(E,L)**, *TRA-1-60* **(F,M)**, *and TRA-1-81* **(G,N)**. *Scale bars, 100 μm.*

mitogen-activated protein kinase kinase (MEK)/ERK pathway.

Results

Derivation and cultivation of two new human embryonic stem cell (hESC) lines (AMC-hES1 and AMC-hES2)

Two novel hESC lines (AMC-hES1 and AMC-hES2) were derived from *in-vitro*-cultured human blastocysts by isolation of the ICM. ICMs were immunosurgically isolated from five blastocysts and cultured onto MEF feeder layers (Solter and Knowles, 1975). After 5–7 days of cultivation, clumps of tightly associated cells were observed, and these clumps were mechanically dissociated and replated onto MEF feeder layers. Two cell lines, AMC-hES1 and AMC-hES2, were grown for 40 passages *in vitro*, at which time they still consisted mainly of cells with the morphology of undifferentiated hESCs (Fig. 1A and 1H). Established hESCs exhibited a large, compact, multicellular morphology with a distinguishable border toward the MEF feeder layers, and had a high nucleus:cytoplasm ratio and prominent nucleoli at high magnification. During the maintenance of hESCs, regions where cells exhibited differentiated morphology were mechanically removed before passaging. The growth rate of each of these two cell lines (24–48 h) was comparable to that of other hESC lines (Miz-hES4 and Miz-hES5) and these lines were successfully frozen and thawed.

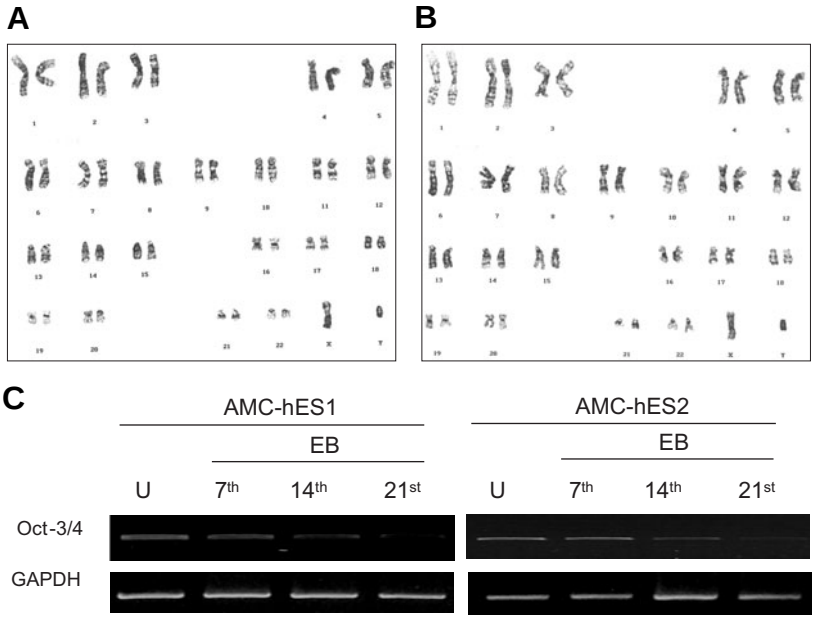

Fig. 2. Characterization of hESC lines. *Karyotypes of AMC-hES1 and AMC-hES2 after 32 passages;* **(A)** *AMC-hES1, 46 XY and* **(B)** *AMC-hES2, 46 XY.* **(C)** *Expression patterns of* octamer-binding transcription factor (oct-3/4) *mRNAs (self-renewal marker) during hESC differentiation (U, undifferentiated; EB, embryoid bodies at day-7, -14, and -21).*

Characterization of hESC lines

The undifferentiated state of the embryonic stem cell is characterized by a high level of expression of AP and the stem cell transcription factor octamer-binding transcription factor 3/4 (oct-3/4). hESCs typically express SSEA-3 and SSEA-4 but not SSEA-1, and express the keratin-sulfate-associated antigens TRA-1-60 and TRA-1-81 (Andrews, 1984; Andrews *et al.,* 1984; Fenderson *et al*., 1987; Pease *et al*., 1990; Thomson *et al*., 1998; Reubinoff *et al*., 2000). An evaluation of hESC lines for surface markers that characterize undifferentiated hESCs showed that two hESC lines were positive for AP (Fig. 1B and 1I), SSEA-3 (Fig. 1D and 1K), and SSEA-4 (Fig. 1E and 1L), but negative for SSEA-1 (Fig. 1C and 1J). The two cell lines were also found to be positive for TRA-1-60 (Fig. 1F and 1M) and TRA-1-81 (Fig. 1G and 1N).

Karyotypic analysis was performed at 32 passages, and both hESC lines were found to have an apparently normal XY human karyotype (Fig. 2A and 2B).

The POU transcription factor oct-3/4 is a stem-cell marker that is expressed in undifferentiated hESCs and downregulated upon differentiation (Rao *et al*., 2004). Using RT-PCR we showed that both AMC-hES1 and AMC-hES 2 expressed oct-3/4, and that its expression decreased upon differentiation (Fig. 2C).

To test for pluripotency *in vivo*, AMC-hES1 and AMC-hES2 were injected into the testicular capsule of SCID mice. Twelve weeks later, on autopsy, the resulting lesions were multicystic tumors with solid areas (Fig. 3A) that had replaced the testicular tissue, although a rim of residual seminiferous tubules was observed in smaller tumors (Fig. 3B). No metastatic spread was identified outside the testicles. Histologically, the tumors were composed of a mixture of tissues derived from all three germ layers, including cartilage, muscle, bone, glandular epithelium, squamous epithelium, and primitive neuroectoderm (Fig. 3C–H). The characteristics of the two newly established hESC lines (AMC-hES1 and AMC-hES2) are summarized in Table 1.

TABLE 1

OVERVIEW OF CHARACTERIZATION FOR THE TWO NEW HUMAN EMBRYONIC STEM CELL (hESC) LINES (AMC-HES1 AND AMC-HES2)

Marker	AMC-hES1*	AMC-hES2*
SSEA-1	«	«
SSEA-3	+	+
SSEA-4	+	+
TRA-1-60	+	+
TRA-1-81	+	+
Karyotyping	46, XY	46, XY
Oct-3/4	+	+
Teratomas	+	+
Freeze/thaw	+	+

*Characterization of hESC lines on a mouse embryonic fibroblast in main text feeder layer.

Large-scale analysis of gene expression in undifferentiated and differentiated hESCs

The mean values of the intensities of each spot in the three experiments were calculated, and shown in Fig. 4A. Of 19104 known genes examined (Supplementary table 1), changes in mRNA expression were detected in 1731 genes: 564 (Supplementary table 2) or 1173 (Supplementary table 3) were activated in undifferentiated hESCs or differentiated hESCs, respectively, and

Only 9% of all examined genes were activated or repressed more than two folds (Fig. 4B). These genes were classified into ten functional categories based on biological functions: apoptosis-, cell cycle-, cell death- cellular-physiological process-, immune response-, response to stress-, signal transduction-, transcription-, chromatin structure-related and not determined (ND) genes.

Expression profiles of Spa-1 during hESC differentiation and in adult tissues

Expression analyses of human spa-1 were performed in undifferentiated or differentiated hESCs and in human adult tissues using RT-PCR. First of all, we examined the differentiation potential of the AMC-hES1 line by examining the expressions patterns of germ-layer marker genes such as alpha-1 antitrypsin (*α1AT*), albumin, carnitine-acylcarnitine translocase (*cACT*), brachyury (*brachyury*), and the 68-kD neurofilament subunit (*NF-68kD*). Spa-1 transcripts were decreased following AMC-hES1 differentiation, as were the pluripotency marker genes, oct-3/4, nanog and sox2 (Fig. 5A). The expression profile of spa-1 was confirmed in three other hESC lines (AMC-hES2, Miz-hES4, and Miz-hES5; Fig. 5B). In human adult tissues, spa-1 was expressed more strongly in the cervix, heart, kidney, lung, ovary, placenta, spleen, testis, and thymus compared to adipose tissue, bladder, colon, esophagus, prostate tissue, skeletal muscle, and small intestine (Fig. 5C).

Spa-1-related Rap1 Signaling could be involved in hESC Maintenance and Differentiation

Spa-1 is a Rap1 GTPase-activating protein that is present in several cells and tissues, and is involved in the regulation of Rap1-regulated signal transduction (Harritori *et al.*, 1995; Kurachi *et al.*,

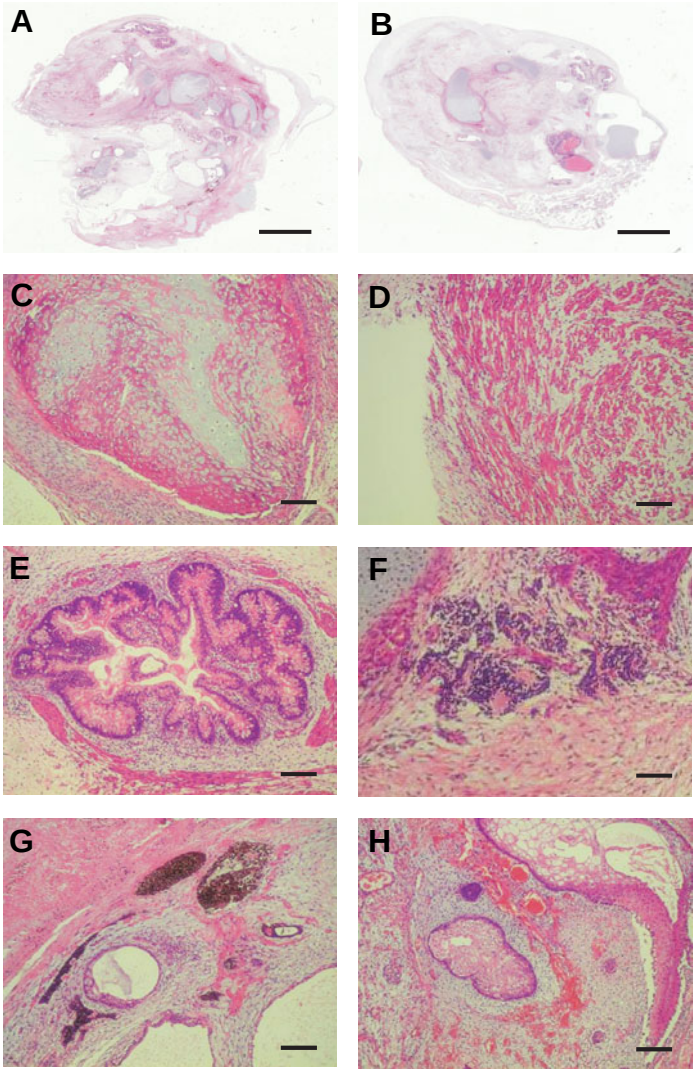

Fig. 3 (Left). *In vivo* differentiation of AMC-hES1 and AMC-hES2. *Whole-mount section of a testicular lesion from an SCID mouse inoculated with AMC-hES1* **(A)** *or AMC-hES2* **(B)**. *The tumors are solid and cystic, and have largely replaced the testicular tissue; scale bars, 2 mm for (A,B). Histologic appearance of the elements in the teratomas formed by AMC-hES1 and AMC-hES2:* **(C)** *cartilage and bone,* **(D)** *smooth muscle bundles,* **(E)** *gut-like tubular structures with goblet cells, containing glandular epithelium and smooth muscle layers,* **(F)** *primitive neural epithelium,* **(G)** *retina-like structure with melanin-pigmented choroid epithelium, and* **(H)** *squamous epithelium; scale bars, 100 μm.*

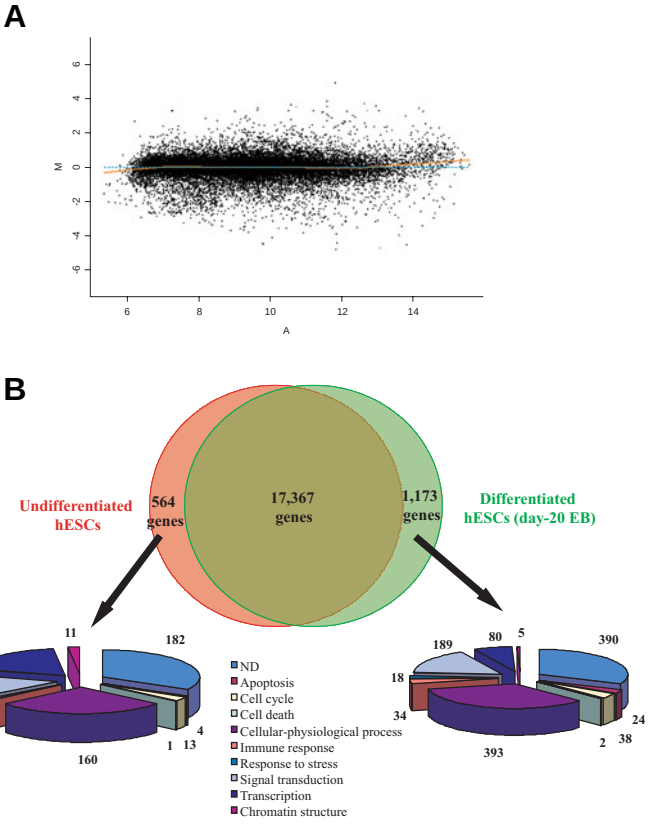

Fig. 4 (Right). Expression profiles of genes in hESCs and differentiated hESCs *(20-day EB).* **(A)** *MA plot (M, exression ratio; A, signal intensity) represents genes activated and repressed in undifferentiated hESCs and differentiated hESCs (20-day EB). The MA plot is used to represent the (R,G) data (R, red for Cy5; G, green for Cy3) where* $M=\log_2 R/G$ *and* $A=\log_2(RxG)$. **(B)** *Venn diagram of genes preferentially expressed in undifferentiated hESCs or differentiated hESCs. ND, not determined.*

1997). To evaluate whether spa-1 is capable of regulation in hESCs, we examined spa-1-associated signal regulation in undifferentiated and differentiated AMC-hES1 cells. Rap1 (Rousseau-Merck *et al.*, 1990), Raf-1 (Cook *et al.*, 1993; Schmitt and Stork, 2001) and B-raf (Vossler *et al.*, 1997) were expressed in both undifferentiated and differentiated hESCs, and exhibited no statistically certified change in their expressions (Fig. 6A). To quantify Rap1 activity, we used RalGDS-RBD protein, which binds preferentially to GTP-Rap1 (Mochizuki *et al.*, 1999). Rap1 was significantly activated by differentiation in the AMC-hES1 line compared to undifferentiated cells (Fig. 6B). Since Raf-1 and B-raf were activated when these proteins were phosphorylated at Ser^{338} (Roy *et al.*, 1998; Chiloeches *et al.*, 2001) and Thr^{598}/Ser^{601} (Zhang and Guan, 2000), respectively, we examined whether Rap1 stimulates the phosphorylation of endogenous Raf-1 or B-raf at the corresponding residues in undifferentiated and differentiated AMC-hES1 cells. As shown in Fig. 6C, the phosphorylation of B-Raf (Thr^{598}/Ser^{601}) was stimulated by differentiation of AMC-hES1, but that of Raf-1 (Ser^{338}) was decreased. Also, activation level of ERK1/2 was slightly decreased in differentiated AMC-hES1 (Fig. 6D). These data indicate that spa-1 is expressed in hESC lines and that phosphorylation-induced activation of Raf-1 or B-raf is dependent on Rap1 status, which is regulated by spa-1.

To study the role of spa-1 in the regulation of hESCs *in vitvo,* we used the pSUPER system to stably suppress the expression of the *spa-1* gene. The pSUPER construct consists of an H1-RNA promoter clone next to the 19-nucleotide *spa-1* sequence, separated by a short, 9-nucleotide spacer that forms the hairpin, followed by the reverse complement of the same nucleotide sequence (Fig. 7A). AMC-hES1 cells were transfected with pSUPER-spa-1 (used S1 target sequence) with or pSUPER-con (used C1 target sequence), and were selected with puromycin for 7 days. As shown in Fig. 7B, we observed that the cellular levels of spa-1 were diminished but not totally eliminated. In the pSUPER-spa-1 hESCs, Rap1 was significantly activated in the pSUPER-spa-1 cells compared to the wild-type cells and the pSUPER-Con, and the activity of Raf-1 or B-raf was decreased or increased in the pSUPER-spa-1 cells, respectively. In addition, self-renewal activity was decreased in pSUPER-spa-1-transfected hESCs compared to that of wild type and pSUPER-con-transfected cells (Fig. 7C). Endoderm and mesoderm marker genes were highly expressed but expression of ectoderm marker gene was decreased in spa-1-diminished hESCs compared to that of wild type and control hESCs (Fig. 7C). The similar results were observed in the other experiment used S2 and C2 target sequence for silencing of spa-1 expression (data not shown).

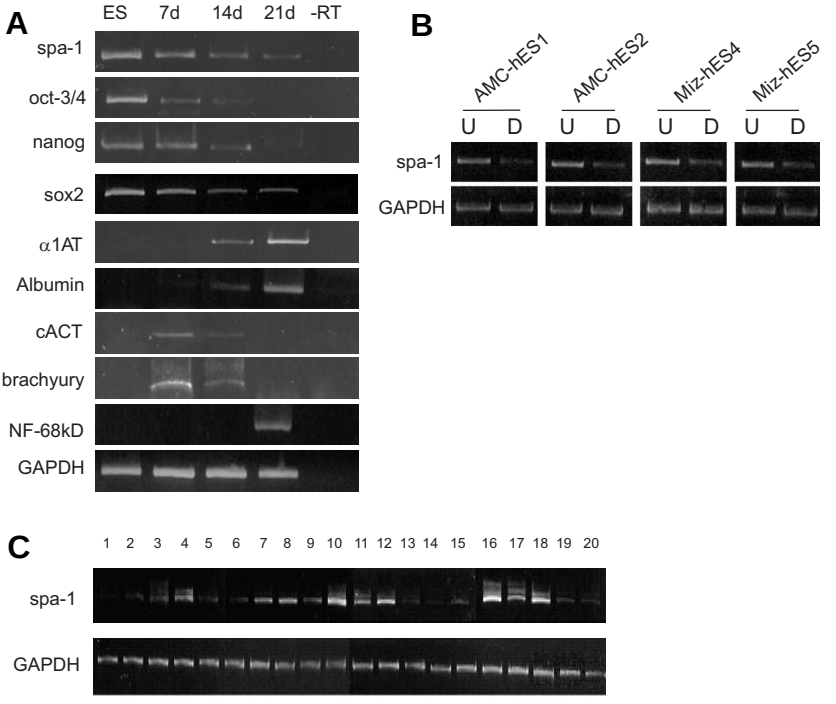

Fig. 5. Expression profile of *spa-1* in AMC-hES lines and human adult tissues. (A) *Expression profiles of* spa-1 *and germ-layer marker genes* [oct-3/4, nanog *and* sox-2 *for pluripotency;* alpha-1 antitrypsin (α1AT) *and* albumin *for endoderm;* carnitine-acylcarnitine translocase (cACT) *and* brachyury *for mesoderm;* 68-kD neurofilament subunit (NF-68kD) *for ectoderm] were analyzed in differentiated hESCs (day-7, -14, and -21 EB; AMC-hES1 line) using semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). During hESC differentiation,* spa-1 *transcripts decreased and, interestingly, the* spa-1 *expression pattern was similar to that of the pluripotent marker genes* (oct-3/4, nanog *and* sox-2). GAPDH *was used as a loading control. Semiquantitative RT-PCR analysis of* spa-1 *expression in* **(B)** *four hESC lines (U, undifferentiated; D, differentiated state, day-14 EB) and* **(C)** *human adult tissues (lane 1, adipose; lane 2, bladder; lane 3, brain; lane 4, cervix; lane 5, colon; lane 6, esophagus; lane 7, heart; lane 8, kidney; lane 9, liver; lane 10, lung; lane 11, ovary; lane 12, placenta; lane 13, prostate; lane 14, skeletal muscle; lane 15, small intestine; lane 16, spleen; lane 17, testis; lane 18, thymus; lane 19, thyroid; lane 20, trachea).*

Discussion

Human embryonic and adult stem cells have been considered as candidate materials of cell therapy for degenerative diseases. To be applied to cell therapy, the mechanisms underlying the self-renewal and differentiation of these stem cells, as well as their involvement in other cellular events, must be clearly defined. In the study described here, we established two novel hESC lines (AMC-hES1 and AMC-hES2) and analyzed their capability for self-renewal and multilineage differentiation. Examination of the two newly established hESC lines revealed that both exhibited common features of hESCs, such as expression of certain cell surface epitopes (SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), high AP activity, cell morphology (Fig. 1), a normal karyotype (AMC-hES1, 46XY; AMC-hES2, 46XY; Fig 2A and 2B), and the ability to differentiate into three germ layers (Fig. 3). In addition, these two new cell lines expressed oct-3/4, a transcription factor that is expressed only in pluripotent cells and, as also seen in these cell lines, is downregulated during differentiation (Fig. 2C). Our established hESC lines have properties that have also been described by other research groups (Thomson *et al.*, 1998; Park *et al.*, 2003), and could provide us with fundamental information for hESC research.

If hESCs are to be useful in further applications, it is necessary

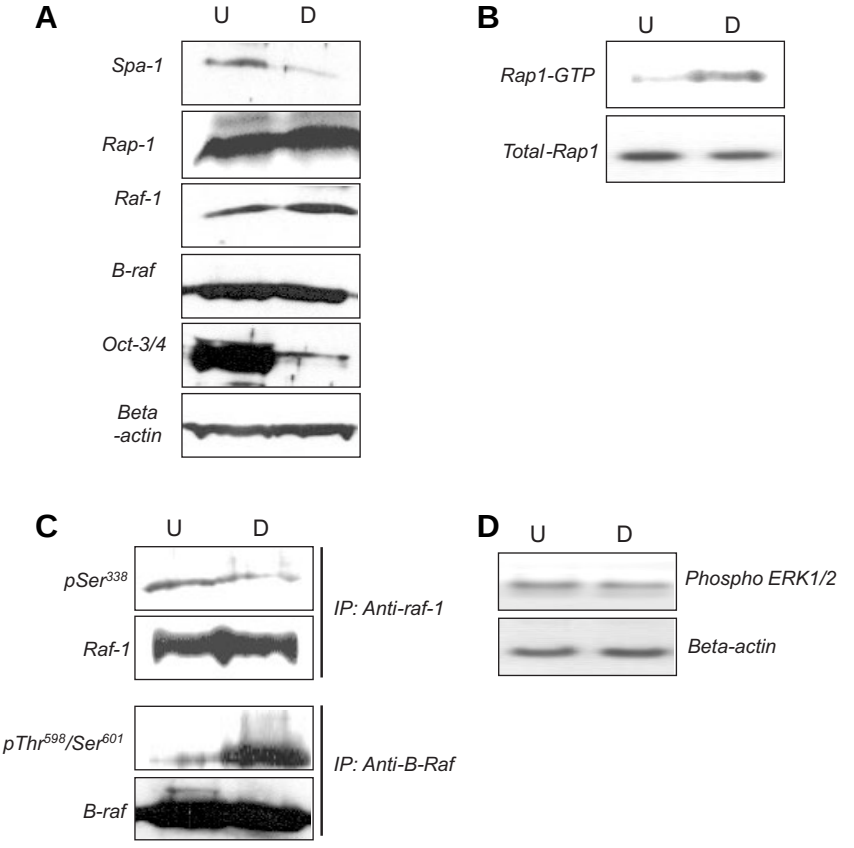

Fig. 6. Switching of Raf-1 and B-raf activation between undifferentiated and differentiated hESCs. (A) *Expression profiles of spa-1 downstream signaling molecules by Western blotting. Oct-3/4 was used as a control for differentiation of the AMC-hES1 line and beta-actin was used as a loading control.* **(B)** *Rap-1 activation was analyzed in undifferentiated (U) and differentiated (D) AMC-hES1 cells using RalGDS-RBD.* **(C)** *Undifferentiated and differentiated AMC-hES1 lysates were immunoprecipitated (IP) with anti-Raf-1 or anti-B-raf antibodies and immunoblotted with antiphosphoserine^{338} antibody or antiphospho-Thr^{598}/Ser^{601} antibody, respectively.* **(D)** *Levels of phosphorylated ERK1/2 between U and D cells. U, undifferentiated AMC-hES1 line; D, day-21 EB derived from the AMC-hES1 line.*

to understand the molecular nature of their capability for self-renewal and differentiation. In the present study, we identified genes that were preferentially expressed in undifferentiated or differentiated hESCs using cDNA microarray. Microarray results showed that only 9% (1737 of 19104) of all examined genes spotted on the microarray slide were activated or repressed more than twofold in undifferentiated or differentiated hESCs, respectively (Fig. 4). As known, self-renewal marker genes (oct-4, nanog and sox2) were activated in undifferentiated hESC and repressed in differentiated hESCs in our data. Also, 3 germ layer marker genes were activated in differentiated hESCs (Supplementary Table 1), so, our microarray experiment was confirmed based on published data. In our microarray results, spa-1 gene was activated in undifferentiated hESCs compared to differentiated hESCs, and have demonstrated in the present study that spa-1 signaling is needed for the maintenance of hESCs.

Spa-1 is a mitogen-induced GTPase-activating protein and was isolated from a fetal liver-derived immature cell line; its expression is induced when cells are in a proliferating state (Hattori *et al.*, 1995). Spa-1 encodes a principal Rap1-specific GAP in lymphohematopoietic tissues (Kurachi *et al.*, 1997), and its family regulates Rap1-associated signal transduction (Gao *et al.*, 1999; Pak *et al.*, 2001; Roy *et al.*, 2002). As seen in Fig. 5A and 5B, spa-1 was expressed in four hESC lines (AMC-hES1, AMC-hES2, Miz-hES4, and Miz-hES5) and was downregulated during hESC differentiation (day-14 EB). These results suggest that spa-1 plays an important role in hESC self-renewal via the Rap1/Raf/ERK signal pathway, and that it is one of the candidate factors in the regulation of hESC dynamics.

Based on the expression of spa-1, we focused on Rap1/Raf/ERK signal pathways in undifferentiated and differentiated hESCs. Expression of Rap1, Raf-1 and B-raf was not changed. In addition, Rap1 and B-raf were activated, whereas Raf-1 was inactivated during differentiation (Fig. 6). Several studies have documented that Rap1 attenuates Ras-mediated ERK activation in certain cell models and inactivates Raf-1 by sequestering it, while Rap1 activates B-raf (Bos *et al.*, 2001; Caron, 2003; Carey *et al.*, 2003; Stork, 2003). Importantly, cell fate is determined by the duration of ERK signaling by Raf-1 and B-raf as a result of differential activation kinetics on their substrate MEK in the PC12 cell model (O'Neill and Kolch, 2004). From these results we suggest that for hESCs, passage from the self-renewal mode to the differentiation mode is dependent on the activity of Raf-1 or B-raf on Rap1 regulation.

As seen in Fig. 7, we isolated spa-1-suppressed hESCs using the siRNA technique; these cells were seen to differentiate into endoderm and mesoderm cell types and rarely expressed the pluripotent marker genes (oct-3/4, nanog and sox-2). In addition, in spa-1 suppressed hESCs, Rap1 was activated and the activity of Raf-1 or B-raf was decreased or increased, respectively, in spite of the prevailing undifferentiated culture conditions. Also, the phosphorylated form of ERK1/2 was slightly decreased in spa-1 suppressd hESCs (Fig. 7B). Interestingly, spa-1 suppressed hESCs were differentiated into endoderm and mesoderm cell types not ectoderm (Fig. 7C). This result suggested that at least spa-1 involved signaling pathways maintain self-renewal activity and can be a factor of determination for hESCs differentiation, in addition, these pathways (Fig. 8) are needed to differentiate into ectoderm cell types. It was reported that spa-1-deficient mice developed myeloid disorders that resemble chronic-phase human CML and myeloproliferative stem-cell disorders (Ishida *et al.*, 2003). Several reports have also suggested that deregulation of Rap1 signaling is£involved in malignancy in epithelial cell transformation (Gao *et al.*, 2001), leukemia in mice (Dupuy *et al.*, 2001), and in human cancer cell lines (Yajnik *et al.*, 2003). These findings suggest that regulation of Rap1 activation by spa-1 or Rap1-activating proteins is crucial for cell fate decisions and tumorigenesis and, as such, spa-1 could be one of the molecules that regulate hESCs via Rap1/Raf/MEK/ERK signaling.

It is generally accepted that hESCs are independent of LIF signaling (Humphrey *et al.*, 2004) and that wnt and FGF2/PI3K/Akt/PKB positively regulate the expression of key molecules involved in hESC self-renewal, although the signaling pathways remain unclear (Mitalipova *et al.*, 2003; Amit *et al.*, 2004; Sato *et al.*, 2004; Dvorak *et al.*, 2005). It was reported that bFGF (FGF2) prevents the apoptosis induced by death-receptor stimulation, via Raf-1 activation in a MEK-independent manner (Alavi *et al.*, 2003), and that B-raf is a crucial effecter for Ras-mediated tumorigenesis in several tumor types (Mercer and Pritchard, 2003). Also, Amstrong *et al.* (2006) reported that PI3K/AKT, MARK/ERK and NFkappaβ signallings are necessary to maintain the pluripotency of hESCs.

These findings and ours suggest that spa-1 is expressed in hESC lines and regulates Rap1 activity by modulating the activities of Raf-1 or B-raf, thus determining whether cells enter the self-

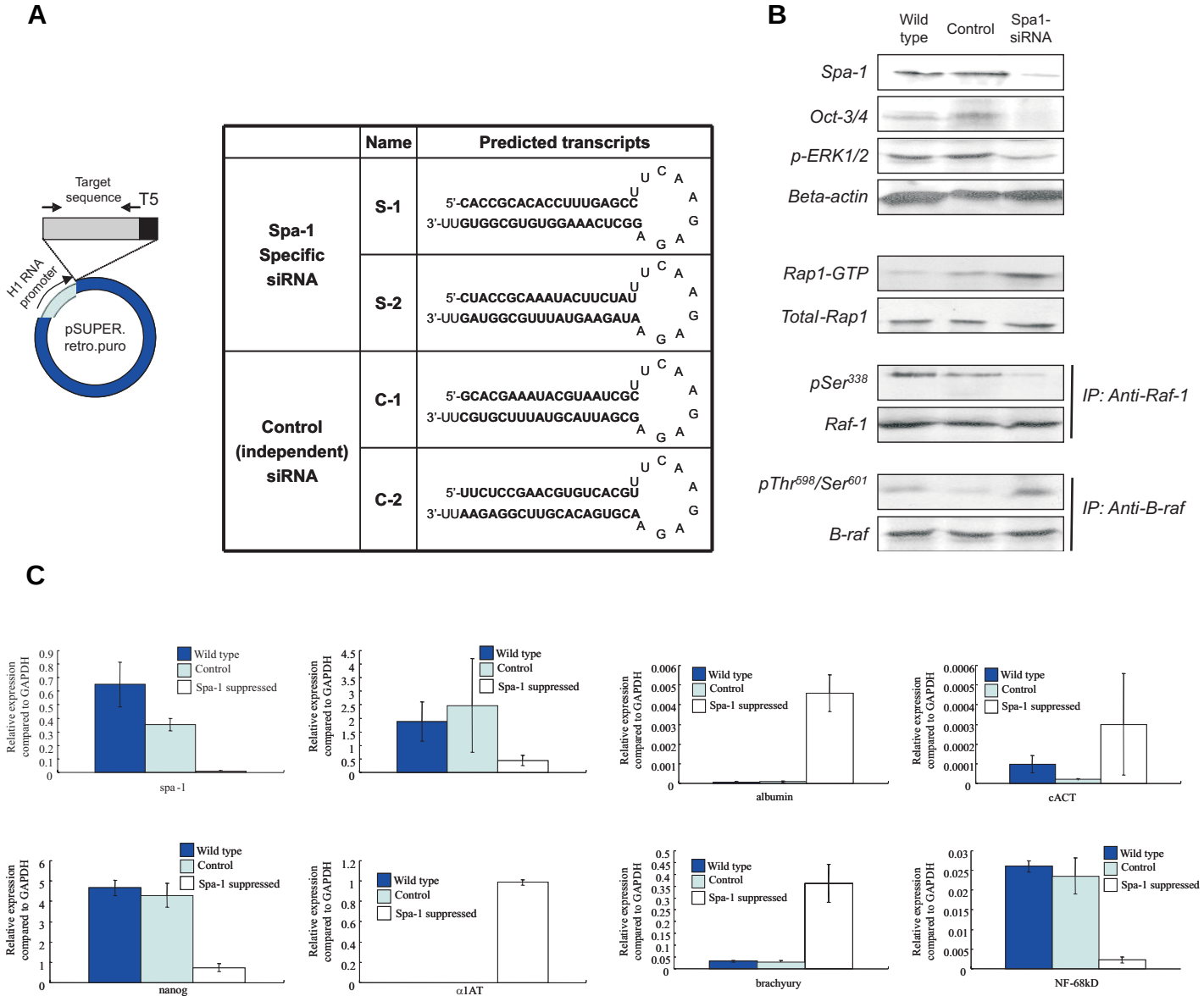

Fig. 7. **Vector-based suppression of gene expression in hESCs. (A)** *Schematic of the pSUPER.retro.puro vector. The H1-RNA promoter cloned in front of the gene-specific targeting sequence (19-nucleotide, 19 nt, sequences from the target transcripts separated by a short spacer from the reverse complement of the same sequence) and 5 thymidines (T5) as a termination signal. The predicted secondary structures of pSUPER.retro.puro-spa-1 or pSUPER.retro.puro-control transcript and the synthetic siRNA used to target spa-1 are depicted.* **(B)** *Western blot analysis for activation level for ERK1/2, Rap1, Raf-1 and B-raf in wild or control or spa-1 suppressed AMC-hES1 cells. Stable suppression of gene expression. hESCs were cultured in Matrigel-coated plates and transfected with pSUPER.retro.puro-spa-1 (pSUPER-spa-1; used S1 target sequence) or pSUPER.retro.puro-control (used C1 oligo target sequence) vector. U, undifferentiated AMC-hES1 cells; Control, pSUPER.retro.puro-control transfected AMC-hES1 line; spa-1 –/–, stably small interference (si)RNA expression vectors against the spa-1-transfected AMC-hES1 line.* **(C)** *After transfection, cells were harvested and spa-1 expression analyzed using quantitative real-time PCR to analyze their differentiation profiles. Values on the ordinate represent relative expression compared to GAPDH.*

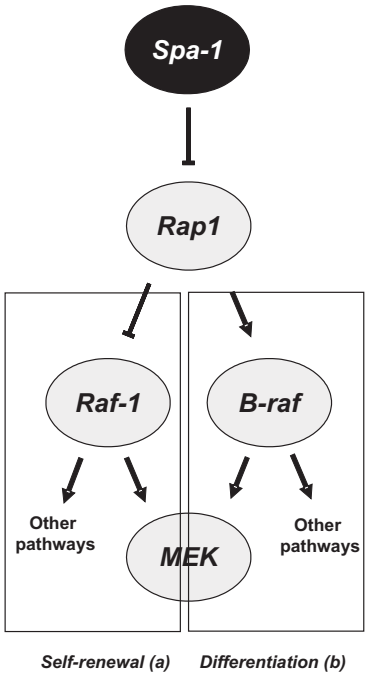

Fig. 8. A model of signaling among Spa-1, Rap1 and Rafs (Raf-1 and B-raf) for self-renewal and differentiation of hESCs. *Depending upon the relative expression of spa-1 and rap1, raf-1 and B-raf was either phosphorylated (activation) or dephosphorylated (inactivation); these modulations could be one of the determinants of hESC self-renewal and differentiation.*

renewal or differentiating mode. In the undifferentiated state, Raf-1 is activated by inhibition of Rap1, which is regulated by spa-1, whereas B-raf-1 is activated by the activation of Rap1.

The cellular mechanisms involved in the control of hESCs seem more complicated than those of mESCs, and the molecular mechanisms underlying the activity of key molecules (e.g., FGF2, LIF, Wnt, Oct-3/4, and Nanog) in the maintenance and differentiation of hESCs have yet to be clearly established. The results presented here, however, provide us with fundamental information regarding the cellular dynamics involved in the maintenance and differentiation of hESCs.

Materials and Methods

Human embryo culture and isolation of the inner cell mass (ICM)

hESC lines were established from six cryopreserved embryos that were donated for study with the approval of the institutional review board and after obtaining the informed consent of patients undergoing *in vitro* fertilization treatment. Embryos were cultured to blastocyst stage in G1.3/G2.3 medium (Vitrolife, Sweden) in accordance with the manufacturer's instructions.

In vitro cultured blastocysts were removed from the zona pellucida with 0.5% pronase E (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% knockout serum replacement (Invitrogen, Carlsbad, CA). ICMs were isolated from the blastocysts by immunosurgical removal of the trophectoderm using rabbit antihuman whole serum (Sigma-Aldrich) and guinea-pig complement (Sigma-Aldrich), and transferred on to a mitomycin C (Sigma-Aldrich)-treated mouse embryonic fibroblast (MEF) feeder layer (Solter *et al.*, 1975). ICM outgrowths were passaged to plates with fresh medium and new primary MEF by mechanical dissection.

Culture of hESCs and embryoid body formation

hESCs were maintained on MEF feeder layers in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% knockout serum replacement (Invitrogen, Carlsbad, CA), 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA), 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA), 100 U/ml penicillin G (Sigma Aldrich), 100 μg/ml streptomycin (Sigma Aldrich), and 4 ng/ml human recombinant bFGF (Invitrogen, Carlsbad, CA) at 37ºC and in an atmosphere of 5% CO_2. MEFs were cultured primarily from 13.5-day postcoitum fetuses of CF-1 mice, as reported previously (Thomson *et al.,* 1998), and hESC colonies were subcultured on new feeders every 5–7 days. Feeder-free culture of hESCs was performed as follows. Plates were incubated with growth-factor-reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ) diluted to 1:30 in cold DMEM/F12 at 4ºC for at least overnight, and conditioned medium (CM) prepared from MEF was used for maintenance of the hESCs under feeder-free conditions.

Mechanically dissociating hESCs induced embryoid body (EB) formation, and harvested hESCs were grown in suspension culture with the same hESC culture medium except that it lacked bFGF. At days 7, 14, and 21 after the beginning of culture, EB was prepared for RNA and protein isolation.

Characterization of established hESCs

Immunocytochemistry: Alkaline phosphatase (AP) activity and the expression of hESC-surface, stage-specific antigens were measured using an embryonic stem cell characterization kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Briefly, cultured hESCs were washed with PBS and fixed in a mixture of 90% methanol and 10% formalin. AP staining solutions (Chemicon, Temecula, CA) were added to fixed hESCs at room temperature for 15 min. For analyses of hESC-surface, stage-specific antigen expressions, hESC colonies were washed in PBS, fixed with 4% formaldehyde for 15 min at room temperature, and then washed again three times in PBS. The following primary antibodies were used: SSEA-1, SSEA-3, SSEA-4, Tra-1-60, and Tra-1-81 (Chemicon, Temecula, CA). The primary antibodies were detected using CY™5-conjugated goat anti-mouse or anti-rat IgG (Zymed, San Francisco, CA) as the secondary antibody.

Karyotyping: The hESCs were incubated in hESC culture medium with 0.1 μg/ml colcemid (KaryoMax colcemid solution; Invitrogen, Carlsbad, CA) for 3 h, dissociated (trypsinized), fixed in methanol:acetic acid (3:1, v/v), and mounted on glass slides. The chromosomes were visualized by Giemsa staining and the karyotype of hESCs determined by cytogeneticists

In Vivo Differentiation (Teratoma Assay) Analyses: For the teratoma assay, immunodeficient SCID mice (NOD.CB17-SCID/J, Charles River Laboratories, Wilmington, MA) were used. hESC colonies were mechanically detached from the surface and dissociated into small cell aggregates (clumps of 400–500 undifferentiated hESCs). Clumps were injected with a sterile 26-G needle into the testicular capsule of 5-week-old SCID mice. The resulting teratomas were analyzed histologically 12 weeks later.

Oligo microarray

Total RNA was isolated from undifferentiated hESCs and hESC-derived differentiated cells (20-day EB) by using TriZol-reagent (GIBCO-Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and purified by RNeasy mini kit (Qiagen, Valencia, CA). Fluorescently-labeled probes for oligo microarray analysis were prepared by Amino allyl MessageAmp™ aRNA kit (Ambion Inc., Texas). Labeled probes (Cy5, hESCs; Cy3, 20-day EB) were hybridized to a Human 1A(V2) Oligo Microarray kit(Agilent Technologies, Palo Alto, CA) at 60°C for 16h. Slides were washed twice in 6x SSC / 0.005% Triton X-102 at 60°C for 20 min and once in 0.1x SSC / 0.005% Triton X-102 at RT for 10 min and four times in D.W for 1 min and spin dried. DNA chips were scanned using

ScanArray Lite (PerkinElmer Life Sciences, Billerica, MA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA) to obtain gene expression ratios. Logged gene expression ratios were normalized by LOWESS regression (Yang *et al.,* 2002). The statistical significance of the differential expression was assessed by computing a q-value for each gene. To determine the q-value we used a permutation procedure, and for each permutation two-sample t-statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1 or less than -1, i.e. a twofold difference in expression level, and when the q-value was < 0.1.

Semiquantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from undifferentiated or differentiated hESCs using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with RNase-Free DNase set (Qiagen, Valencia, CA). Briefly, lysis and homogenization of the hESCs and binding of RNA to the silica-gel membrane were performed according to the provided standard protocols, After washing with a reduced volume of Buffer RW1, the RNA was treated with DNase I while bound to the silica-gel membrane. The DNase was removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution were then performed according to the provided standard protocol. RNA quality was ensured by gel visualization and spectrophotometric analysis ($OD_{260/280}$). First-strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan) and random hexamers from 1 μg of each total RNA. cDNA samples were subjected to PCR amplification with specific primers (Table 2) under appropriate conditions. The human glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control. All PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide (Sigma Aldrich). FirstChoice Human Total RNA Survey Panel (Ambion, Austin, TX) was used for total RNA of human adult tissues.

TABLE 2

PRIMER SEQUENCES FOR REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Gene	Forward (F) and reverse (R) primer sequences	Product size (bp)
spa-1	F: 5'-caccgcacacctttgagcc-3' R: 5' -gtggctgcactctcagactc-3'	461
Undifferentiated cell markers		
Oct-3/4	F: 5'-cttgctgcagaagtgggtggaggaa-3' R: 5'-ctgcagtgtgggtttcgggca-3'	169
Nanog	F: 5'-tgcctcacacggagactgtc-3' R: 5'-tgctattcttcggccagttg-3'	354
Sox2	F: 5'-acaccaatcccatccacact-3' R: 5'-gcaaacttcctgcaaagctc-3'	224
Endoderm		
α1AT	F: 5'-actgtcaacttcggggacac-3' R: 5'-ccccattgctgaagaccttа-3'	517
Albumin	F: 5'-cttcctgggcatgtttttgt-3' R: 5'-ggttcaggaccacggataga-3'	401
Mesoderm		
cACT	F: 5'-tatttgctcccttgcttgga-3' R: 5'-cctaccccaaaaacaaacga-3'	415
Brachyury	F: 5'-acgccatgtactccttcctg-3' R: 5'-tgagcttgttggtgagcttg-3'	204
Ectoderm		
NF-68kD	F: 5'-acgctgaggaatggttcaag-3' R: 5'-tagacgcctcaatggtttcc-3'	561
Housekeeping gene		
GAPDH	F: 5'-gctgtgggcaaggtcatcc-3' R: 5'-cttgctggggctggtggtc-3'	391

**oct-3/4*, octamer-binding transcription factor 3/4; sox2, SRY (sex determining region Y)-box 2; *α1AT*, alpha-1 antitrypsin; *cACT*, carnitine-acylcarnitine translocase; *NF-68kD*, 68-kD neurofilament subunit;Ä*GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *bp*, base pairs.

Western blotting

Undifferentiated, differentiated, and transient spa-1 –/– hESCs were lysed in Triton X-100 lysis buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 50 mM NaF, and a protease inhibitor mixture; Calbiochem, Darmstadt, Germany), and insoluble materials were precipitated by centrifugation at 16,000 X g for 10 min at 4ºC. The supernatant was transferred to a new tube and protein concentrations were determined by using a protein assay dye reagent (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. Twelve micrograms of each hESC lysate was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and then immunoblotted with anti-Spa-1 (BD Transduction Laboratories, San Jose, CA), anti-Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-B-raf (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Oct-3/4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-p44/42 MAP kinase (ERK1/2; Cell Signaling Technologies, MA) or anti-Beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. Binding of primary antibodies was detected by incubating blots with horseradish-peroxidase-conjugated goat antirabbit or antimouse antibody, and blots were developed using enhanced chemiluminescence (Western blotting detection reagents; Amersham Biosciences, Piscataway, NJ).

Rap-1 activation assay using RalGDS-RBD

Lysates derived from undifferentiated or differentiated (day-21 EBs) hESCs were clarified by centrifugation at 16,000 X g for 10 min at 4ºC. Thirteen micrograms of RalGDS-RBD, coupled to agarose beads (Upstate Biotechnology, Lake Placid, NY), were added to lysates and incubated at 4ºC for 45 min with slight agitation. After brief centrifugation, the resulting pellet was washed three times with 1 X Tris lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1% NP-40, 2.5 mM $MgCl_2$, and 5% glycerol). After the final wash, protein sample buffer was added to the samples, and proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. The blot was probed with rabbit anti-Rap1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to determine the degree of Rap1 activation.

Immunoprecipitation

Total cell lysates were obtained with Triton X-100 lysis buffer from undifferentiated and differentiated (day-21 EBs) hESCs. Raf-1 and B-raf protein was immunoprecipitated with rabbit-anti-raf-1 or rabbit anti-B-raf antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively, and protein A-Sepharose beads. Immunoprecipitates were washed three times with 1 ml of high-salt buffer and once with 1 ml of 20 mM Tris-Cl (pH 8.0), resuspended in 5 X protein sample buffer, resolved by 12% SDS-PAGE, and then transferred to nitrocellulose membranes. Phosphorylation of Ser^{338} (for Raf-1) or Thr^{598}/Ser^{601} (for B-raf) was detected by immunoblotting using mouse anti-phospho-Ser^{338} Raf-1 antibody (Upstate Biotechnology, Lake Placid, NY) or goat anti-phospho-Thr^{598}/Ser^{601} B-raf antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Blots were reprobed with rabbit anti-Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-B-raf (Santa Cruz Biotechnology, Santa Cruz, CA) antibody to determine the amount of immunoprecipitated Raf-1 or B-raf protein, respectively.

Small interference RNA (siRNA)

pSUPER.retro.puro vector (mammalian expression vector; OligoEngine, Seattle, WA) was used for the expression of siRNA in hESCs. The spa-1-specific insert specified a 19-nucleotide sequence corresponding to nucleotides 107–125 (5'-caccgcacacctttgagcc-3') or 651–669 (5'-ctaccgcaaatacttctat-3') downstream of the translation start codon of spa-1 (NCBI GenBank, AF029789), which are separated by a 9-

nucleotide noncomplementary spacer (5'-ttcaagaga-3') from the reverse complement of the same 19-nucleotide sequence (pSUPER-spa-1). Two control vectors were constructed using a 19-nucleotide sequence (5'-gcacgaaatacgtaatcgc-3' and 5'-ttctccgaacgtgtcacgt-3') with no homology to any human mRNA sequence in GenBank. siRNA expression vectors were constructed as follows: 19 nucleotide sequences were inserted into the pSUPER.retro.puro backbone after digestion with BglII and Hind III (New England Biolabs, Beverly, MA) and transformed into competent JM109 cells (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The confirmation of constructs was performed by digestion with several restriction enzymes, electrophoresis, and nucleotide sequencing.

Transfection into hESCs

Transfection of hESCs was carried out in 12-well plates (Nunc, Rochester, NY) under feeder-free culture conditions, as per the manufacturer's protocol. Briefly, 2 μg of vector constructs was diluted in 50 μl of 0.15 M NaCl, and 6.6 μl Exgen 500 solution (Fermentas, Ontario, Canada) was added to 43.4 μl of 0.15 M NaCl. Diluted DNA and Exgen 500 solution were mixed well and incubated at room temperature for 10 min. Each feeder-free cultured hESC was transfected with DNA/Exgen 500 solution and incubated at 37ºC in an atmosphere of 5% CO_2. For stable expression, the cells were passed 3 days after transfection onto Matrigel-coated, feeder-free culture plates with CM, and after 3 days, puromycin (1 μg/ml final concentration) was added for 7 days. Puromycin-resistant hESCs were selected, dissociated, and plated onto MEF-coated plates for further studies.

Quantitative real-time analysis

To analyze differentiation profile of spa-1-suppressed hESCs, the quantitative real-time PCR was performed in an ABI Prism 7000 Sequence Detector (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems) as the detector according to the manufacturer's instruction. A PCR reaction mixture of 20 μl containing 10 μl of SYBR Green PCR Master Mix, 3 μl of each cDNA and a pair of primers (Table 2). Specific PCR products were detected with the fluorescent double stranded DNA-binding dye, SYBR Green (Morrison *et al.,* 1998). PCR amplification was performed in triplicate and replicated in three independent experiments. Gel electrophoresis and melting curve analyses were performed to confirm correct PCR product sizes and absence of nonspecific bands. The expression levels of each gene were normalized against gapdh using the comparative C_T method according to the manufacturer's protocols (Applied Biosystems, Sequence Detection system User Bulletin #2; Livak *et al.,* 2001).

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