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SIRPα1 and *SIRPα2*: Their role as tumor suppressors in breast carcinoma cells

Yukiko Yamasaki [a,b], Satoko Ito [b], Nobuyuki Tsunoda [a,b], Toshio Kokuryo [a,b], Kazuo Hara [b], Takeshi Senga [b], Reiji Kannagi [c], Tatsuyoshi Yamamoto [a], Koji Oda [a], Masato Nagino [a], Yuji Nimura [a], Michinari Hamaguchi [b,*]

[a] *Division of Surgical Oncology, Department of Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[b] *Division of Cancer Biology, Department of Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan*
[c] *The Program of Molecular Pathology, Aichi Cancer Center Research Institute, Nagoya 464-8681, Japan*

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Abstract

We have previously reported that expression of SIRPα1/SHPS-1 was strongly suppressed in v-Src-transformed cells and its forced expression resulted in the suppression of anchorage-independent growth of the cells [K. Machida, S. Matsuda, K. Yamaki, T. Senga, A.A. Thant, H. Kurata, K. Miyazaki, K. Hayashi, T. Okuda, T. Kitamura, T. Hayakawa, M. Hamaguchi, v-Src suppresses SHPS-1 expression via the Ras-MAP kinase pathway to promote the oncogenic growth of cells, Oncogene 19 (2000) 1710–1718]. We examined the effect of human SIRPα1 expression in breast cancer cell lines, Hs578T and MCF7, and compared with the effect of SIRPα2 expression in Hs578T. Forced expression of either SIRPα1 or SIRPα2 did not perturb the growth of Hs578T in a conventional attached condition. Their expression, however, enforced the actin stress fiber formation and induced activation of Rho, but not Rac, in Hs578T cells. Moreover, forced expression of either SIRPα1 or SIRPα2 displayed distinct suppressive effect on the anchorage-independent growth of Hs578T cells. Similarly, forced expression of SIRPα1 in MCF7 specifically suppressed the anchorage-independent growth of the cells. Taken together, our results strongly suggest the function of *SIRPα1* and *2* as type II tumor suppressors for human breast carcinoma.

Keywords: Breast carcinoma; SIRPα1/SHPS-1; SIRPα2/BIT; Anchorage-independent growth; Stress fiber

Signal regulatory protein (SIRP) α1 [1], also known as SHPS-1 [2], is a member of SIRP family of transmembrane glycoproteins [3,4]. In the extracellular domain, SIRPα1 has three immunoglobulin-like domains that mediate association with CD47, a ligand for SIRP [5,6]. In the cytoplasmic domain, SIRPα1 has two ITIM motifs with four tyrosine residues which are phosphorylated in response to various growth factor stimulation to mediate its association with the protein tyrosine phosphatases, SHP-1 and SHP-2 [7]. Kharitonenkow et al. [1] reported that overexpression of SIRPα1 inhibited the activation of MAP kinase in cells stimulated with insulin or EGF as well as in cells transformed by v-*fms*. In contrast, Takada et al. [7] reported that overexpression of wild-type SHPS-1, a rat homolog of SIRPα1, significantly increased mitogen-activated protein kinase (MAP kinase) activity in response to insulin. In addition, they reported that overexpression of mutant SIRPα1 /SHPS-1 lacking SHP-2-binding sites markedly inhibited the MAP kinase activation. Similarly, we observed critical role of SIRPα1 in the activation of MAP kinase by IL-1β or TNFα [8]. Although all these studies strongly suggest the importance of SIRPα1 in cell growth, whether SIRPα1 plays positive role or negative

* Corresponding author. Fax: +81 52 7442464.
E-mail address: mhamagu@med.nagoya-u.ac.jp (M. Hamaguchi).

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one remains unclear so that further study is required to clarify the overall picture of SIRPα1 in signal transduction.

Since the discovery of SIRPα1/SHPS-1, a growing number of SIRP family proteins have been identified [4]. Among them, SIRPα2, also known as BIT [9], is a member of SIRP family that shares close structural similarity with SIRPα1. SIRPα1 and SIRPα2 are structurally identical except for their N-terminal Ig-like domain (IgV) which is critical for the binding of CD47. Compared to SIRPα1, however, biological function of SIRPα2 and its difference with those of SIRPα1 remains largely unclear.

We have previously shown that forced expression of SIRPα1 /SHPS-1 in v-*src*-transformed 3Y1 suppressed the anchorage-independent growth of the cells [10]. Our preliminary study suggested that SIRPα1/SHPS-1 expression was decreased in human breast cancer tissues in a tumor-specific manner [11]. These results strongly suggest the important role of SIRPα1 in tumorigenesis of breast carcinoma. In spite of its importance, the effect of SIRPα1 in human cancer cell lines remains to be explored. To obtain more clues, we examined the effect of human SIRPα1 expression in human breast cancer cell lines, Hs578T and MCF7, and compared with the effect of SIRPα2 on Hs578T. We show here that both SIRPα1 and SIRPα2 have distinct effects on anchorage-independent growth of human breast carcinoma cell lines, whereas cell growth at attached condition remains unchanged.

Materials and methods

Antibodies and chemicals. Anti-SIRPα for immunoblotting, anti-SIRPα for immunohistochemical staining, and anti-Erk2 were purchased from ProSci (Poway, CA), LAB Vision (Fremont, CA), and Santa Cruz biotechnologies (Santa Cruz, CA), respectively. Anti-phospho Erk, anti-phospho Akt, and Anti-Akt antibodies were from Cell Signaling (Beverly, MA). Rhodamine-labeled Phalloidin was purchased from Molecular Probes (Eugene,OR).

Cell, plasmid construction, and transfection. Cell lines derived from human breast carcinoma, Hs578T, MCF-7, MDA-MB231, and human foreskin fibroblast (HFF) cells were maintained as previously described [12–14]. The complete human SIRPα cDNAs were generated from HFF total cDNA by RT-PCR. The amplified cDNA was cloned into pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen) and the BamHI/XhoI excised SIRPα fragments were subsequently subcloned into the pBabe-puro expression vector [15,16]. Orientation and sequence of the SIRPα1, α2 constructs were verified by DNA sequencing. Stable transfection of Hs578T with plasmid was carried out using GenePorter reagent (GTS, San Diego) as described by supplier and drug-resistant clones were selected [10,15].

Electrophoresis and immunoblotting. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were performed as described elsewhere [12]. Briefly, proteins were subjected to 10% SDS–PAGE and transferred to PVDF membrane. The membrane was blocked with 5% nonfat skimmed milk and incubated with the respective antibody followed by the secondary antibody. Proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence. Cells were fixed with 4% paraformaldehyde, treated with 0.5% Triton X-100, and incubated with indicated antibody followed by FITC-conjugated secondary antibody [13].

Rho and Rac activity assay. Rho and Rac activities were assayed as previously reported [17–19]. Briefly, cells were lysed in pull-down lysis buffer (5% glycerol, 1% NP-40, 150 mM NaCl, 25 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, protease inhibitor cocktail (Amersham Pharmacia Biotech), and 1 mM PMSF). Cell lysates were incubated with GST-Rhotekin RBD (residues 7–89) or GST-PAK PBD (residues 67–150) fusion protein bound to glutathion-agarose (Sigma–Aldrich) for 1 h at 4 °C to precipitate active Rho or Rac, respectively. Pull-down samples and total lysates were analyzed by immunoblotting with anti-Rho (Upstate Biotechnology) or anti-Rac (BD Biosciences) antibody.

MTT assay. Cell proliferation was measured by MTT assay as described previously [20]. Briefly, cells cultured in 96-well plates were treated with 0.4% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h. The medium was removed, and 100 ml of 0.04 N HCl in isopropanol was added. The color reaction was assayed by an automatic plate reader at 590 nm with a reference filter of 620 nm.

Soft agar colony assay. Cells (5×10^4) were mixed with 0.36% agar in DMEM supplemented with 5% FBS and overlaid onto a 0.72% agarose layer in 6-well plates. After 3 weeks of incubation, colonies in randomly selected fields (×40 magnification) were enumerated.

Suspension culture. Cells were mixed with 1.68% methyl cellulose (Sigma–Aldrich) in DMEM with 10% FBS. After indicated days of incubation, cells were collected by centrifugation and subjected to flow cytometric analysis.

FACS analysis. Cells were cultured in liquid medium or in methyl cellulose for several days and collected. Cells were fixed with 70% ethanol and stained in PBS containing propidium iodide and RNase. FACS analysis was performed with an EPIC/XL cell analyzer (Coulter).

Results and discussion

To confirm and extend our previous observations, we first examined the expression of SIRPα in human breast carcinoma tissues and cell lines with anti-SIRPα antibody that recognizes the cytoplasmic domains of SIRPα1 and SIRPα2. Of 48 cases we examined, 18 cases of breast carcinoma showed suppression of SIRPα1 and α2 expressions in a tumor-specific manner. Fig. 1A shows the representative five cases that show clear suppression of SIRPα1. Fig. 1B shows the expression of SIRPα1 and α2 in cell lines derived from human breast carcinoma. Comparing with human foreskin fibroblast cells (HFF), three breast carcinoma cell lines, Hs578T, MCF-7, and MDA-MB231, showed substantial suppression of SIRPα expression. With Hs578T, we next examined the effect of SIRPα1 and SIRPα2 expression. As shown in Fig. 1B, human SIRPα1 and SIRPα2 have close structural similarity but differ in their Ig V domains. Both *SIRPα1* and *SIRPα2* were ligated into pBabe vector and transfected into Hs578T cells. Empty vector was also transfected as a control (Control cells). As shown in Fig. 1D and E, both SIRPα1 and SIRPα2 were expressed well in Hs578T and associated with the plasma membrane. Interestingly, both Hs578T cells expressing SIRPα1 (SIRPα1 cells) and those expressing SIRPα2 (SIRPα2 cells) showed flattened morphology when compared with that of control Hs578T cells.

Since both of SIRPα-transfected cells showed change in their morphology, we next examined the actin stress fiber formation in these cells (Fig. 2A). Both SIRPα1 cells and SIRPα2 cells showed fine stress fiber formation, whereas control cells showed limited formation of the stress fiber. To confirm these observations, we examined relative activities of Rho and Rac by pull-down assay as described in

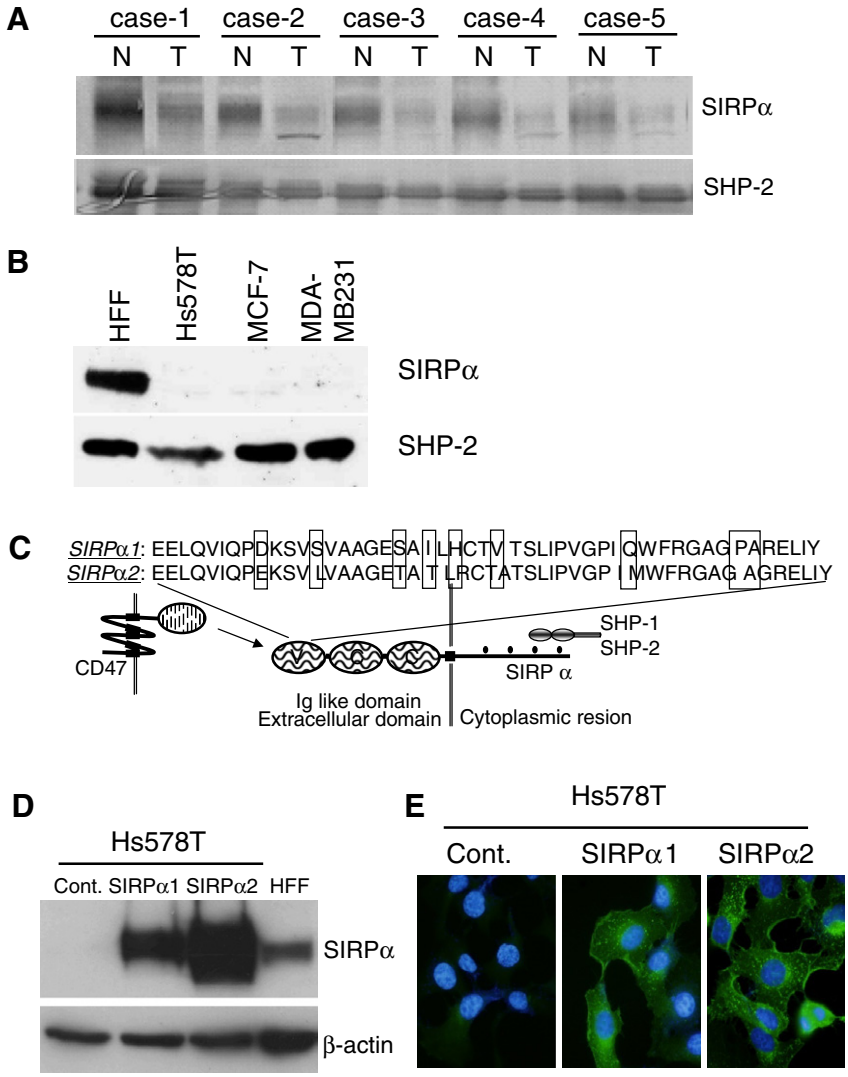

Fig. 1. Expression of SIRPα in human breast cancer tissues and cell lines. (A) Expression of SIRPα in paired normal (N) and tumor (T) tissues was examined by immunoblotting with anti-SIRPα. (B) Expression of SIRPα in cell lines derived from human breast carcinoma, Hs578T, MCF-7, and MDA-MB231. Human foreskin fibroblast (HFF) was used as a control. (C) Domain structure of SIRPα and amino-acid sequences in the first immunoglobulin-like domains of SIRPα1 and SIRPα2. Differences in the amino-acid sequence between SIRPα1 and SIRPα2 are indicated by boxes. (D) Lysates from control, SIRPα1, and SIRPα2 cells were probed by immunoblotting with anti- human SIRPα antibody. (E) Exogenous expression of SIRPα1 and SIRPα2 in Hs578T cells was confirmed by immunofluorescent staining with anti-SIRPα (green). Nuclei were costained with DAPI (blue). (For interpretation of color in this figure legand, the reader is referred to the web version of this figure.)

Materials and methods. As shown in Fig. 2B and C, relative ratio of active Rho, but not Rac, was substantially increased in SIRPα-transfected Hs578T cells as compared with those in control Hs578T cells.

We next examined the growth of the cells. In a conventional adherent culture condition, both SIRPα1 cells and SIRPα2 cells did not show any growth retardation or activation as compared with those of control cells (Fig. 2D). We found, however, clear effect of exogenous SIRPα expression on the anchorage-independent growth of Hs578T cells. Cells were suspended in soft agar to allow anchorage-independent growth as described in Materials and methods. As shown in Fig. 3A, control cells showed clear soft agar colony formation. In contrast, colony formation in SIRPα1 cells and SIRPα2 cells was substantially suppressed. Colonies greater than 50 μm in diameter in the fields were counted to confirm the effect of SIRPα expression. We found that both SIRPα1 cells and SIRPα2 cells showed similar levels of suppression in soft agar colony formation (Fig. 3B). To confirm these observations, we next examined the effect of SIRPα1 expression in MCF-7 cells. *SIRPα1* gene ligated with pBabe-puro was transfected into MCF-7 and drug-resistant colonies were isolated. Fig. 3D shows expression of SIRPα1 in two representative clones of transfected MCF-7 (CL-2 and CL-11). In these *SIRPα1*-transfected MCF-7 cell lines, anchorage-independent cell growth was substantially suppressed (Fig. 3 C and D).

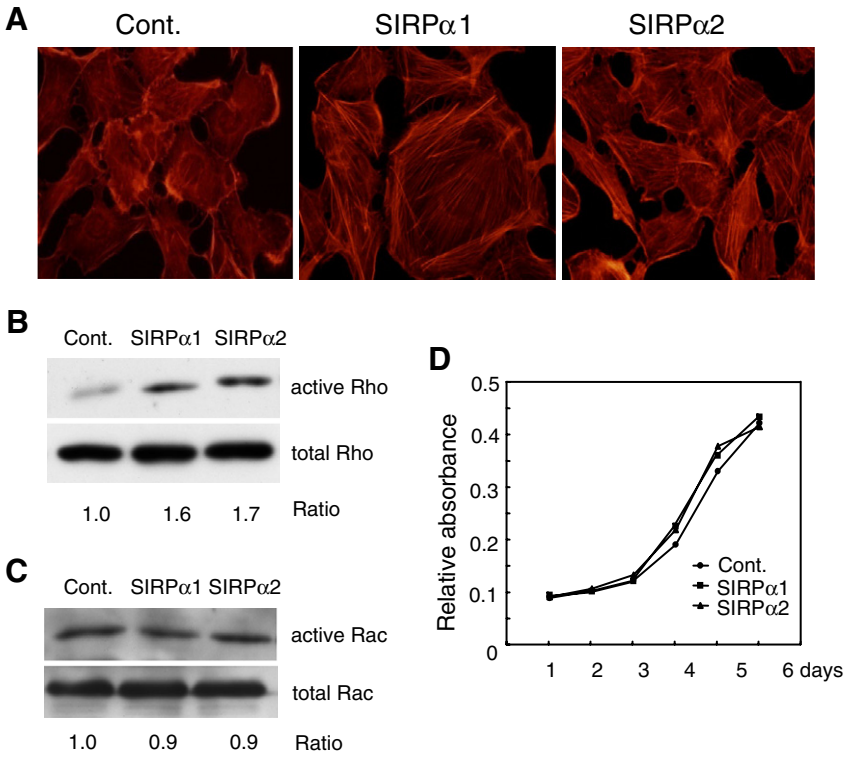

Fig. 2. Effect of SIRPα expression on the stress fiber formation and cell growth. (A) Actin stress fiber formation in control, SIRPα1, and SIRPα2 cells was examined by staining with rhodamine-conjugated phalloidin. (B) Relative activities of Rho in control, SIRPα1, and SIRPα2 cells were assessed with pull-down assay. GTP-bound active Rho and total Rho in the lysates were examined by immunoblotting with anti- Rho antibody. Relative amounts of active Rho were measured and are indicated. (C) Relative activities of Rac in control, SIRPα1, and SIRPα2 cells were assessed with pull-down assay. GTP-bound active Rac and total Rac in the lysates were examined by immunoblotting with anti-Rac antibody. Relative amounts of active Rac were measured and are indicated. (D) Cell growth in adherent condition was assessed by MTT assay as described in Materials and methods. The results represent mean values of three independent experiments.

We next examined the effect of SIRPα1 expression on the cell cycle of Hs578T cultured in suspended condition by flow cytometric analysis. As shown in Fig. 4A, control cells and SIRPα1 cells showed similar pattern of cell cycle when they were cultured in a conventional condition. SIRPα1cells, however, showed drastic change in cell cycle pattern when they were cultured in a suspended condition. After 10 days in suspended culture, SIRPα1 cells in G1, S, and G2/M phases almost completely disappeared and, in turn, large amount of subG1 peak appeared. In contrast to SIRPα1 cells, substantial number of cells remained in G1, S, and G2/M phases in control cells cultured in suspension, although subG1 peak was observed to some extent in the suspended condition. SIRPα1 cells in suspended condition were confirmed to have SIRPα1 expression to the levels similar to those of attached condition (Fig. 4B).

We examined the two major signaling pathways, Erk and Akt, which play important roles in cell growth and death, in suspended culture condition (Fig. 4C). In a conventional culture condition, both SIRPα1 cells and SIRPα2 cells had phosphorylation of Erk and Akt to the levels similar to those of control cells. After incubation in suspended condition for 2 days, relative phosphorylation levels of Erk and Akt simultaneously decreased in all cells regardless of SIRPα expression. Although decrease in Akt phosphorylation was slightly larger in SIRPα1 cells and SIRPα2 cells as compared to that in control cells, both SIRPα1 cells and SIRPα2 cells cultured in suspended condition had phosphorylated Akt to some extent, suggesting that SIRPα does not have direct suppressive effect on Erk and Akt signaling.

Hs578T, established by Hackett et al. [21], is a cell line derived from human breast carcinosarcoma that lacks estrogen receptor but expresses vimentin and has invasive capability [22]. On the other hand, MCF-7 is a breast carcinoma cell line obtained originally by pleural effusion of patient. In contrast to Hs578T, MCF-7 shows epithelioid morphology and has estrogen receptor [23], although both of them have malignant phenotype. With these cell lines that have distinct biological character, we showed that both SIRPα1 and SIRPα2 had strong suppressive effect on the anchorage-independent growth of the cells, whereas cell growth in a conventional condition remained unchanged under the expression of SIRPα1 or SIRPα2. These results strongly suggest that both types of SIRPα, SIRPα1 and SIRPα2, are candidates of tumor suppressor genes for human breast carcinoma.

By their function, tumor suppressors are classified into two types, type I and II. In contrast to type I tumor sup-

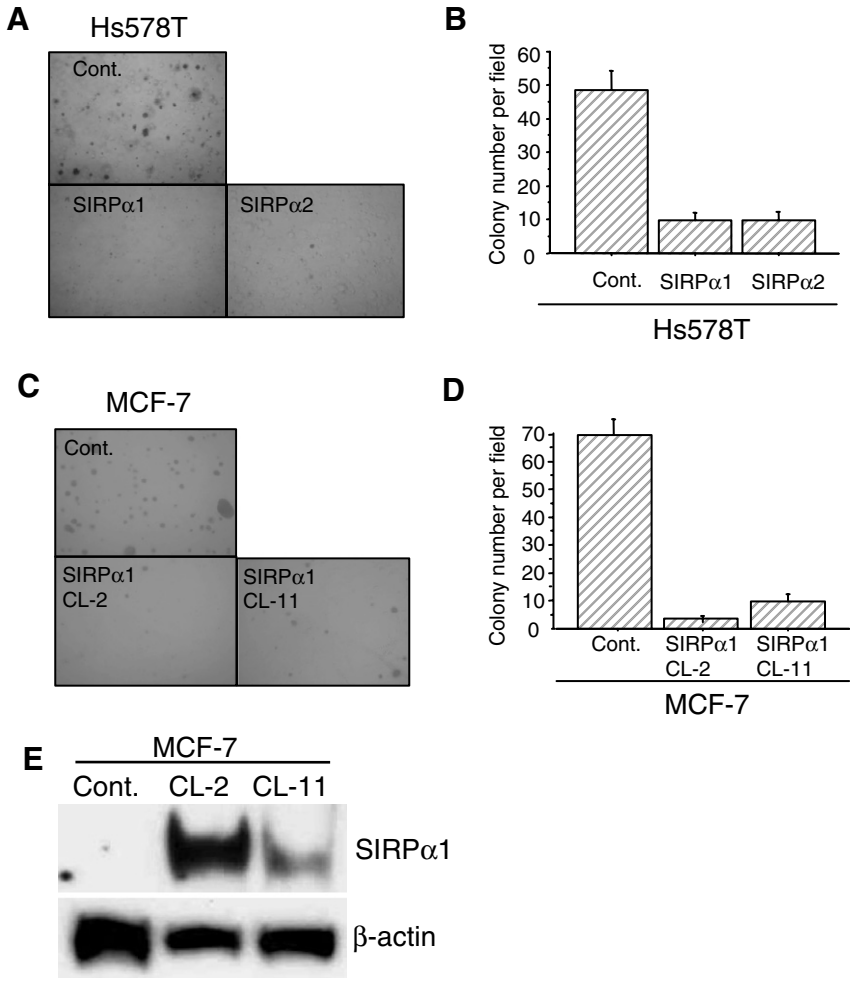

Fig. 3. Effect of SIRPα expression on soft agar colony formation. (A,B) Soft agar colony assay of Hs578T transfected with control vector (Cont), *SIRPα1* (SIRPα1) or *SIRPα2* (SIRPα2) was performed as described in Materials and methods. (C,D) Soft agar colony assay of MCF7 transfected with control vector (Cont) or SIRPα1 (CL-2 and CL-11) was performed. (B,D) The numbers of colonies were counted and mean averages ± SD from three independent experiments are indicated. (E) Expression of SIRPα1 in SIRPα1-transfected MCF-7 (CL-2 and CL-11) was confirmed by immunoblotting.

pressor genes such as p53 and Rb which are mutated or deleted in tumor cells, type II tumor suppressors are proposed to be transcriptionally down-regulated in specific tumor cell lines [24]. Candidate type II tumor suppressor genes such as *maspin* [25], *elafin* [26], *tropomyosin* 1 and 2 [27,28], and *SSeCKS* [29] have been isolated. Although none of their anti-tumor functions has been completely clarified yet, their definition as tumor suppressors is strengthened by at least two criteria [29]. First, demonstration of down-regulated expression in tumor cells is required. Second, tumor-suppressive activity by the ectopic expression in tumor cell lines should be demonstrated. As we showed in this report, expression of SIRPα1 in breast carcinoma tissues as well as breast carcinoma cell lines was substantially suppressed, whereas exogenous expression of SIRPα1 or SIRPα2 in Hs578T led to the suppression of the anchorage-independent growth of the cells. These results suggest that both *SIRPα1* and *SIRPα2* may be a candidate of type II tumor suppressor for human breast carcinoma. Our results also demonstrated that difference in the primary structure of SIRPα1 and SIRPα2 in their IgV domain is negligible for their tumor suppressive effect.

It should be noted that, both in SIRPα1 cells and SIRPα2 cells, suppression of anchorage-independent growth was associated with the recovery of fine stress fiber formation. Simultaneously, Rho signaling was activated in SIRPα1 cells and SIRPα2 cells as compared with those of control cells. While the mechanism that exhibits these biological effects remains unclear, our results indicate that morphological change and uncontrolled growth acceleration are closely associated phenotypes in breast carcinoma cells. Although the mechanism how SIRPα1 and SIRPα2 suppress the anchorage-independent growth of breast carcinoma cells remains to be clarified, our results suggest that both SIRPα1 and SIRPα2 can be important therapeutic tools for the suppression of uncontrolled growth of breast carcinoma.

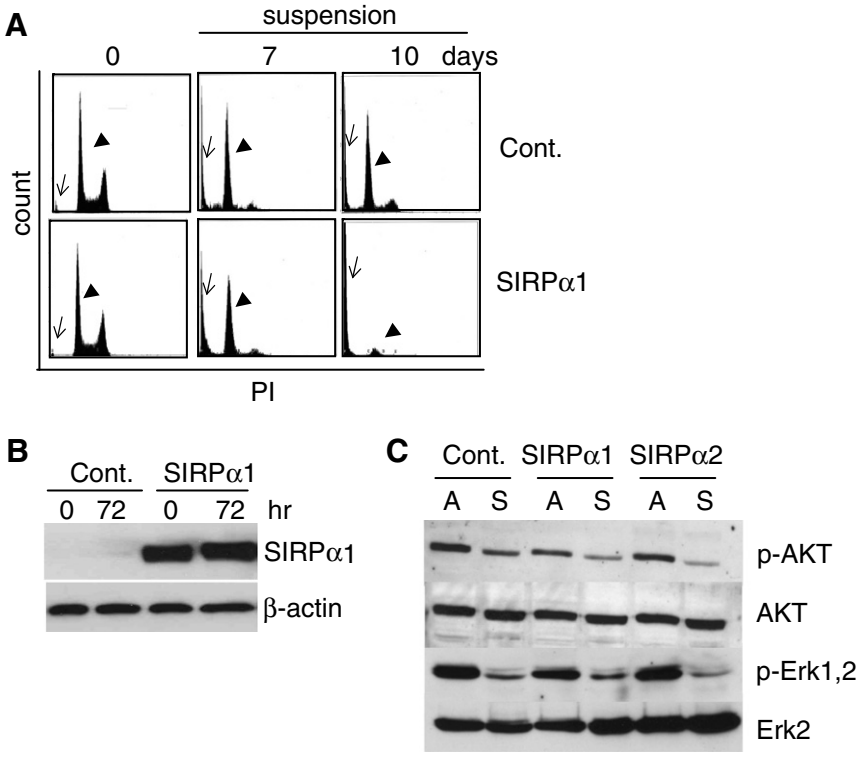

Fig. 4. Effect of SIRPα expression in suspended culture condition. (A) FACS analysis of SIRPα1 cells in attached condition (day 0) or in suspended condition (suspension) incubated for 7 or 10 days. Cells in G0/G1 fraction and sub-G0/G1 fraction are indicated by arrowheads and arrows, respectively. (B) Relative levels of SIRPα1 in SIRPα1 cells suspended for 0 h or 72 h were assessed by immunoblotting. (C) Control, SIRPα1, and SIRPα2 cells were cultured in either attached (A) or suspended (S) conditions. After incubation for 48 h, lysates from these cells were probed by immunoblotting with indicated antibodies.

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