

RESEARCH ARTICLE

Downregulation of Signal Regulatory Protein Alfa 1 in K562 Cells Results in the Aberrant Cell Growth in Low Serum Culture

Shinichiro Takahashi

Signal regulatory protein (SIRP)- α 1 is a myeloid inhibitory immunoreceptor. SIRP α 1 expression is significantly reduced in the majority of myeloid malignancies. SIRP α 1 is a negative regulator of signaling and the reduced expression is considered to play a role in the pathogenesis of these diseases through aberrant signaling, but the biological roles of SIRP α 1 remain largely unknown. In this study, by using SIRP- α 1-knockdown chronic myeloid leukemia K562 (K562SIRP- α 1KD) cells, I found that in low (0.1, 0.3%) serum media, the cell growth was increased until day 5, whereas control cells did not proliferate beyond day 2 under the same serum conditions. Consistently, in K562SIRP- α 1KD cells, 2 days of culture in 0.3% serum increased the proportion of S-phase cells, as well as bromodeoxyuridine (BrdU) incorporation compared to their controls. Moreover, serum stimulation experiment revealed that Brd-U positive cells were significantly increased in K562SIRP α 1 KD cells. Furthermore, in K562SIRP- α 1KD cells, phosphorylation of extracellular regulated kinase (ERK) and Akt was potently increased under low serum conditions. Collectively, these data suggested that down-regulation of SIRP α 1 led to the constitutive activation of these aberrant signaling, as well as abnormal growth, in K562 cells.

Keywords: signal regulatory protein- α 1; serum starvation; cell survival; extracellular regulated kinase; leukemia

Introduction

The signal regulatory protein (SIRP) α 1, also termed CD172a or Src homology 2 domain-containing phosphatase substrate-1 (SHPS-1), is a cell surface receptor expressed predominantly in monocytes, granulocytes, dendritic cells, as well as hematopoietic stem cells [1, 11, 25]. SIRP α 1 is the most characterized member of the human SIRP family. Its overexpression leads to a reduced responsiveness to tyrosine kinase ligands, such as epidermal growth factor, platelet-derived growth factor and insulin [11]. These suggest that, SIRP α 1 is considered as a negative regulator of signaling. In addition to this, SIRP α 1 expression is significantly reduced in the majority of acute and chronic myeloid leukemias [23]. Therefore, the reduced expression is considered to play a role in the pathogenesis of these diseases through aberrant signaling, such as SHP-1 and its downstream signaling KIT pathway [26].

It was reported that, CD47, a ligand for SIRP α 1, was identified as an important “don’t eat me” signal expressed on tumor cells [9, 16]. CD47 is constitutively upregulated

on human myeloid leukemias, and over-expression of CD47 on a myeloid leukemia line increases its pathogenicity by allowing it to evade phagocytosis [9]. Therefore, the blockade of the CD47: SIRP α 1 axis between tumor cells and immune cells has been attracted attention [12, 3]. However, the precise biological mechanisms of the downstream effects of SIRP α 1 suppression in leukemia cells have not been clarified.

My group previously established SIRP α 1-knockdown chronic myeloid leukemia K562 (K562SIRP α 1KD) cells expressing reduced levels of SIRP α 1 by stably transfecting SIRP α 1 siRNAs, and found that expression of β -catenin is increased in these cells [15]. Phosphorylation of Ser 9 of glycogen synthase kinase (GSK)-3 β , results in the activation of this protein, leading to the up-regulation of β -catenin [5]. We revealed that downregulation of SIRP α 1 results in the aberrant phosphorylation of several signaling pathway components, such as extracellular regulated kinase (ERK), Akt and GSK-3 β , which may lead to the induction of β -catenin.

In this study, I examined whether the serum deprivation enhances the activity of downstream signaling pathways, affect to the cell survival. As a result, I revealed that in serum starvation conditions, K562SIRP α 1KD cells showed aberrant cell proliferation.

Materials and Methods

Cell growth assay

K562 SIRP α 1KD cells and their control cells were previously established in our laboratory [15] and employed in this study. Exponential phase cells were collected and counted, washed twice with phosphate buffered saline (PBS) and seeded 5000 cells/well at 96 well plate in indicated serum concentrations. At indicated times, cell growth was determined by means of dye reduction assay involving a tetrazolium salt WST-8 (Dojindo, Tokyo, Japan).

Cell cycle analysis

Cell cycle profiles were determined by analyzing the DNA contents using propidium iodide (Dojindo) staining and flow cytometry as described [7]. Briefly, 48 hours after the indicated proportions of the serum in the culture, the cells were washed, fixed, and stored at -20°C until analysis. These cells were collected, suspended in 300 μl of RNase/PBS solution and incubated at 37°C for 30 min. Then, the cells were resuspended in 300 μl of propidium iodide/PBS solution, and incubated in the dark at room temperature for 15 min. After filtration, all samples were applied to an Epics XL (Beckman Coulter, Nyon, Switzerland). To analyze the data, ModFitLT (Verity Software, Topsham, ME, USA) were employed.

Measurement of proliferation by BrdU incorporation

BrdU incorporation assay was carried out as per the BrdU Staining Kit for Flow Cytometry FITC (eBioscience, San Diego, CA, USA). To examine cell proliferation in low serum condition, exponentially grown cells are incubated for 48 hours in 0.3% serum in culture. For serum stimulation assay, exponentially grown cells were cultured and collected and counted, washed twice with PBS, cultured 24 hours in serum free RPMI. Then FBS was added to a final 10% volume into the medium and incubated for 16–20 hours. These cells were washed twice with PBS and 10 μM of BrdU was added to the cell medium for 45 minutes and then the cells were washed with flow cytometry staining buffer. Cells were fixed and stained for BrdU using a FITC conjugate anti-BrdU FITC antibody (clone BU20A). Samples were analyzed using MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). Negative control samples without BrdU were used to establish levels of background autofluorescence and to define the BrdU positive gate.

Western Blot

Western blot was performed as described previously [15, 8]. Briefly, total cellular extracts were prepared from the cells cultured with 0.3% FBS for indicated period. To examine the signaling, anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), anti-phospho-p38 (T180/Y182), anti-p-Akt (S473), anti-Akt1 rabbit polyclonal antibodies and p44/42 MAPK mouse monoclonal antibody were purchased from Cell Signaling Technology (Beverly, USA). Anti-38 α /stress-activated protein kinase (SAPK) 2 α mouse monoclonal antibody was obtained from

BD Biosciences (San Jose, CA, USA) and employed for this study.

Results

Cell proliferation of K562SIRP α 1KD cells is significantly increased in low serum conditions

I first examined the cell proliferation characteristics of K562SIRP α 1KD cells in various concentrations of serum. As my group reported previously, there are sufficient expression of SIRP α 1 mRNA and protein in K562 cells and these were obviously suppressed by the stable transfection of SIRP α 1 siRNA into K562 cells [15]. By employing these cells, I observed that in low serum culture from 0.1% to 0.3%, there is a marked increment of the cell proliferation in both (1–2, 2–6) K562SIRP α 1KD cells, compared to their controls (vec 5, vec 6) (**Figure 1**). The cell growth was increased until day 5 in K562SIRP α 1KD cells, whereas control cells did not proliferate beyond day 2 under the same serum conditions (**Figure 1**). This phenomenon was not observed in more than 1% serum in the medium. Consistently, cell cycle analyses after low serum (0.1% to 0.3%) culture for 48 hours revealed that the proportions of S-phase cells are significantly increased in K562SIRP α 1KD cells, but not in their controls (**Figure 2A, 2B**). This was also not observed in more than 1% serum. I next examined the percentages of BrdU incorporated cells to examine the cell proliferation. After 48h incubation with low serum culture (0.3% serum), the percentages of BrdU incorporated cells were slightly increased in K562SIRP α 1KD cells (**Figure 3A**). Moreover, serum stimulation experiment was also performed, and found that Brd-U positive cells were significantly increased in K562SIRP α 1 KD cells (**Figure 3B, 3C**). Collectively, these suggest that in the low serum condition, downregulation of SIRP α 1 result in the increase of the viable cells.

Activation of ERK and Akt in serum starved condition

I next examined the signaling status in these conditions, because there are difference of viability and cell growth between K562SIRP α 1 KD cells and their controls. Leukemia cell growth and survival responses to environmental triggers are controlled by the activation of multiple intracellular signaling pathways, in which ERK, Akt and p38 mitogen-activated protein kinases (p38MAPK). The function of the p38 kinases is required for the generation of various activities, including regulation of apoptosis, cell-cycle arrest and differentiation [20]. Among these kinase pathways examined, as shown in **Figure 4**, I found that in this low serum culture (0.3% serum), ERK is potently phosphorylated in K562SIRP α 1KD cells. I also observed obvious phosphorylation of Akt in K562SIRP α 1KD cells. In addition, there are some increase of p38 phosphorylation in 1–2 at 2 and 5 days, and slight increase in 2–6 at 5 days. These suggest that suppression of SIRP α 1 may have marginal effect on p38 phosphorylation. This issue will be discussed in the next section.

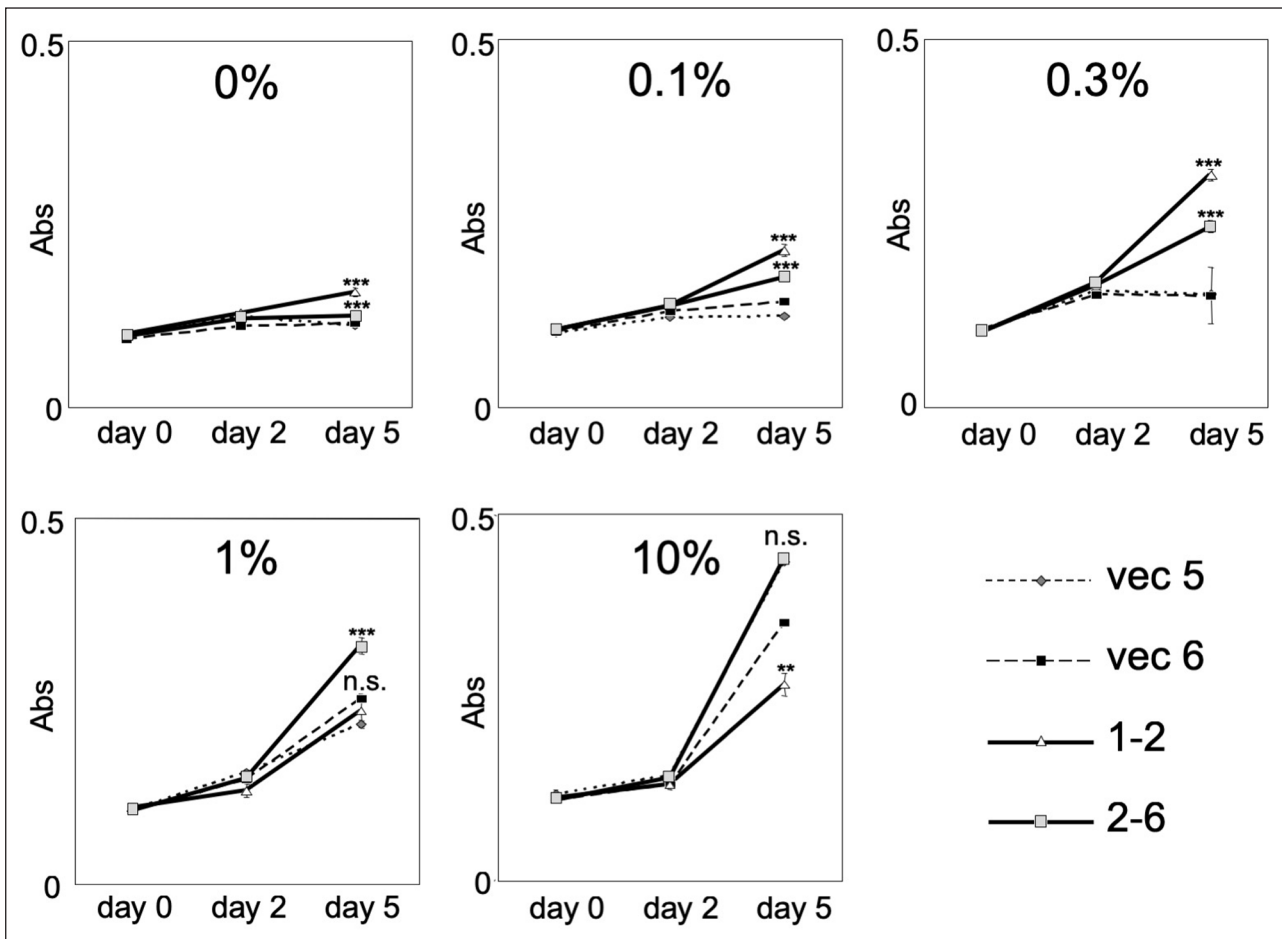


Figure 1: Growth of K562 SIRP α 1KD cells and their controls on the various concentrations of serum in the medium. Cell proliferation was quantified by the WST-8 assay. Indicated amounts of serum were added to the medium and the WST-8 assay was performed at the times indicated. 1-2 and 2-6 are K562 SIRP α 1KD cells. vec5 and vec 6 are control cells. Data were calculated from the average $\pm$ standard deviations from at least three samples and shown are representative of three independent experiments. P-value was calculated from student's t-test, by comparing controls (vec 5, vec 6) and each (1-2, 2-6) K562 SIRP α 1KD cells (** $p < 0.01$, *** $p < 0.001$, n.s: not significant).

Discussion

Mitui *et al.* [18], previously reported that MEK-ERK signaling pathway is required for serum-dependent survival of liver cancer HepG2 cells. They also revealed that transient expression of active MEK1 prevented apoptosis in serum-deprived condition [18]. I speculate that activation of ERK (Figure 4), observed in low serum culture, may leads to the escape from the cell death by serum withdrawal. Although there were no growth differences between K562SIRP α 1KD cells and their controls in the full culture condition (Figure 1, 10%), I observed obvious difference for the growth in low serum culture (Figure 1, 0.1–0.3%). Serum is a complex mix of albumins, growth factors and inhibitors [27]. I speculate that in the full culture condition, redundant signals mask the important SIRP α 1 related signal, which is necessary for cell growth and survival, such as ERK signaling. This may be explained by the fact that there was very modest difference for ERK phosphorylation between K562 SIRP α 1KD cells and their controls in normal culture conditions [15], whereas very strong difference for phosphorylated ERK in low serum culture (Figure 4) in the present study. In addition, I

observed marginal phosphorylation of p38 MAPK in K562 SIRP α 1KD cells in low serum culture (Figure 4). In general, the activation of the p38 MAPK pathway tends to promote apoptosis whereas the activation of the ERK pathway tends to be anti-apoptotic [6]. Birkenkamp *et al.* [2], have previously demonstrated by using TF-1 human erythroleukemia cells, that the effects of the ERK signaling pathway can overcome the pro-apoptotic effects of the p38 signaling pathway. They revealed that although the activation of the p38 pathway may be required for growth factor withdrawal-induced apoptosis, in the presence of high enough levels of ERK activation, p38 activation may not be sufficient in itself for apoptosis to occur [2]. Based on my findings (Figures 1 and 4) and a literature [2], these suggest that survival effect of SIRP α 1KD (Figure 1) is mainly regulated by ERK activation.

The Ras-ERK-MAPK pathways are frequently activated in hematological malignancies [17, 20]. Lunghi *et al.*, [14] previously reported that among 20 AML patients, 14 cases of AML patients had high ERK1/2 activity whereas only four cases of AML had low ERK1/2 activity. It is possible to

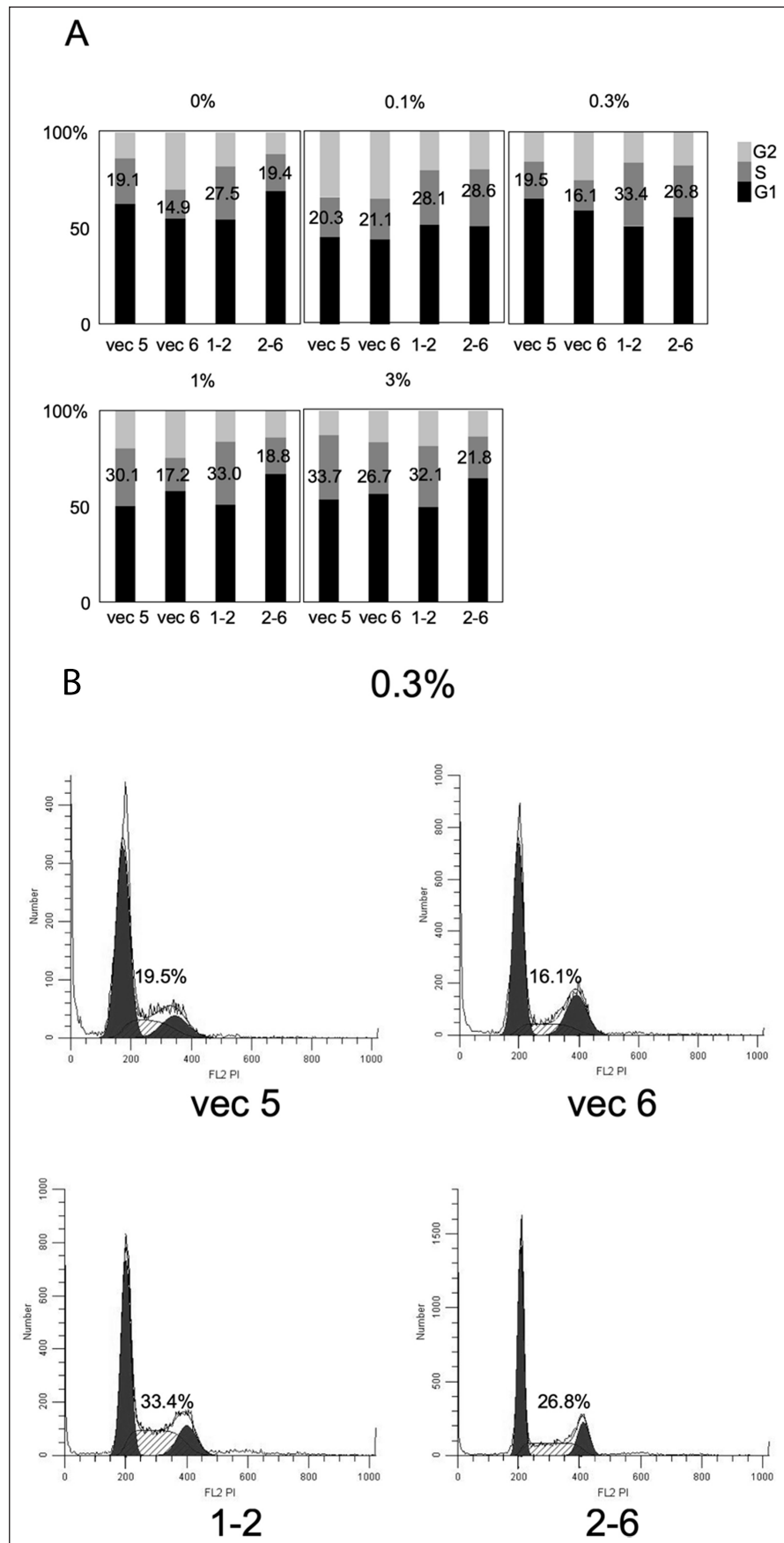


Figure 2: Effects of the change of serum concentration in the culture for 48 hours on the cell cycle for K562 SIRP α 1KD cells and their controls. The data shown are representative of three independent experiments, and reproducibility was confirmed. **(A)** Summary of the percentages of cells in each phase of the cell cycle (G1, S and G2/M) of the data are presented. **(B)** Flow cytometry images of cell cycle analysis using propidium iodide staining. Depicted are cells incubated with 0.3% serum in culture.

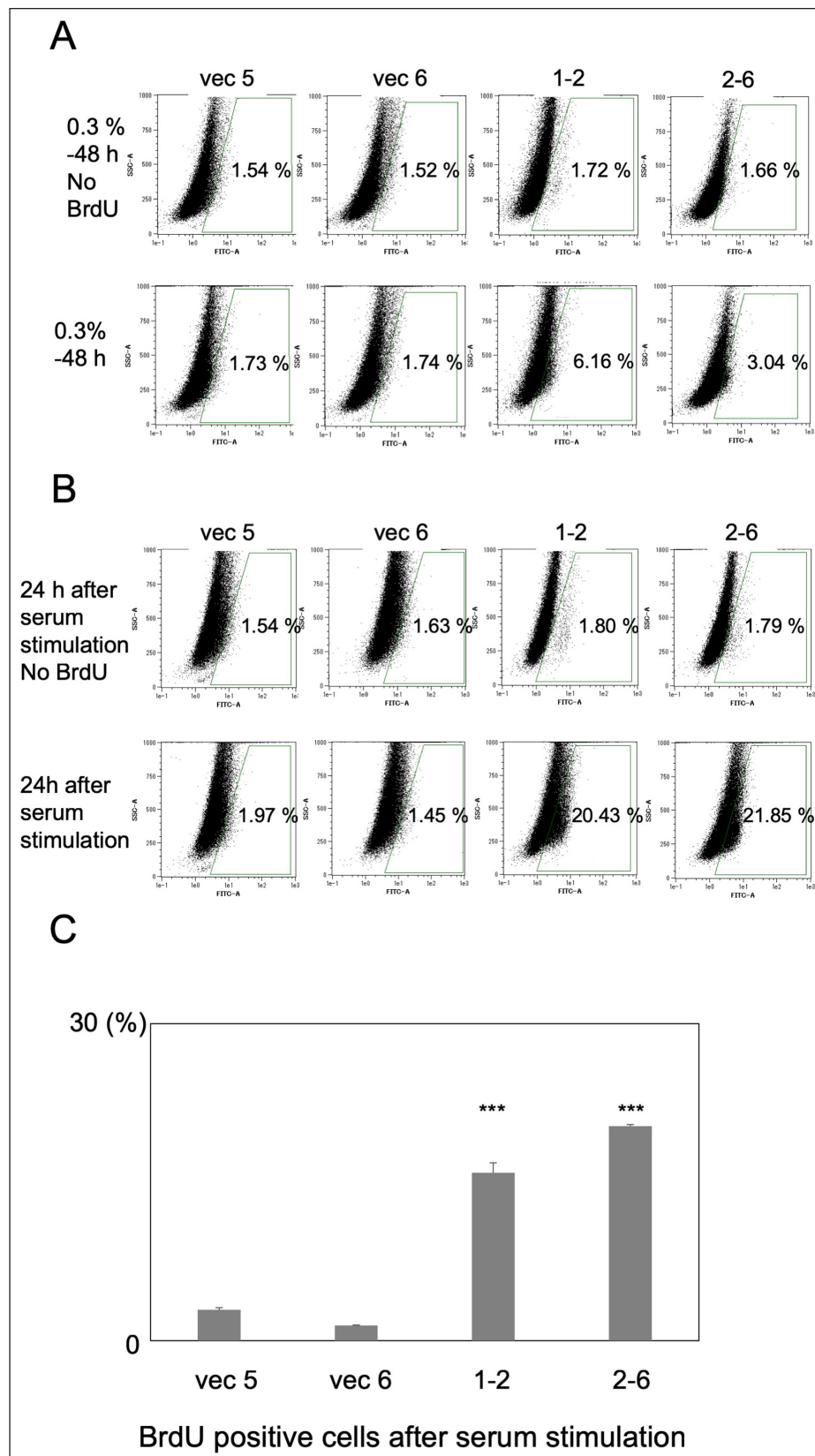


Figure 3: BrdU incorporation measured using flow cytometry. **(A)** Flow cytometry plots of side scatter (SSC) vs. BrdU fluorescence. Result of BrdU positive cells incubated for 48 hours in 0.3% serum in culture. Upper panel indicates negative control samples without BrdU. They were used to establish levels of background autofluorescence and to define the BrdU positive gate. Numbers denote percent of cells in the indicated area. **(B)** Flow cytometry plots, similar to Figure 3A. Result of BrdU positive cells after serum stimulation. Shown are representative of three independent experiments and reproducibility was confirmed. **(C)** Summary of BrdU positive cells after serum stimulation, obtained from three independent experiments. Depicted are average $\pm$ standard deviations and P-value was calculated from student's t-test, by comparing controls and each (1–2, 2–6) K562 SIRP α 1KD cells (** $p < 0.001$).

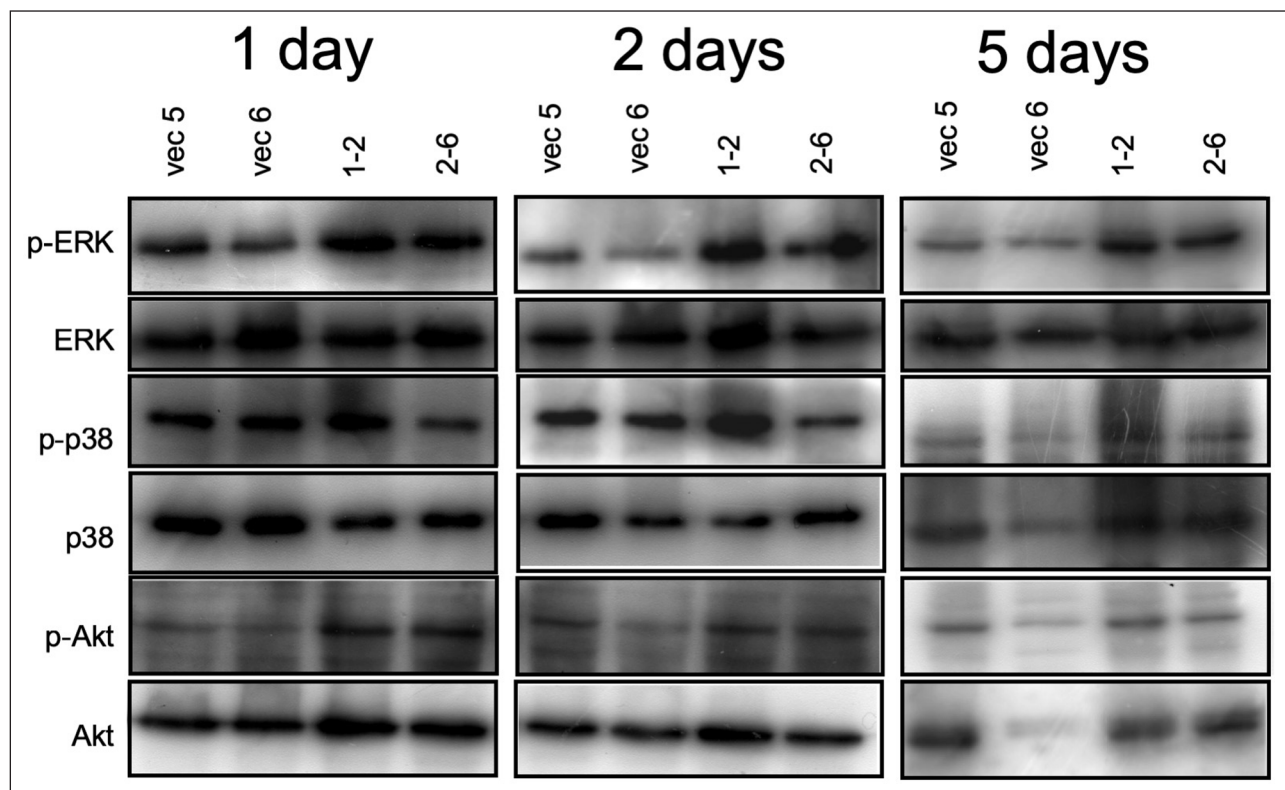


Figure 4: Down-regulation of SIRP α 1 results in the constitutive phosphorylation of ERK and Akt in low serum culture. The indicated K562 SIRP α 1KD cells were cultured with 0.3% FBS for indicated period and total cell lysates were harvested. After blotting, the blots were stained with the indicated activation-specific antibodies. These are, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38 (T180/Y182), anti-p-Akt (S473) rabbit polyclonal antibodies. Subsequently, the blots were stripped and stained with the indicated antibodies, (i.e., p44/42 MAPK mouse monoclonal antibody, anti-Akt1 rabbit polyclonal antibodies, anti-38 α /SAPK2 α mouse monoclonal antibody) recognizing above proteins in their nonactivated state demonstrate equal loading.

speculate that the down-regulation of SIRP α 1 is contributing to the activation of ERK1/2 in primary AML cells. Cortez *et al.*, [4] demonstrated that BCR-ABL expression activates Ras, ERK and Jnk pathways as a primary consequence of expression, induces G1-to-S phase transition, DNA synthesis, and activation of cyclin dependent kinases in cells that were arrested in G0 by growth factor deprivation [4]. The baseline activation of ERK by BCR-ABL in K562 cells may not enough to confer serum starvation, but additional aberration, such as down-regulation of SIRP α 1, result in further activation of ERK and several other pathways, may be required to obtain resistance to serum deprivation. Although alterations affecting the functions of transcription factors that regulate myeloid maturation play important roles in leukemogenesis [22, 29], inappropriate MAPK may also play a role in leukemic transformation [17, 20]. It has been shown that the interruption of this pathway profoundly affects the growth of AML cell lines and primary AML samples with constitutive MAPK activation [17, 24]. As down-regulation of SIRP α 1, as well as inappropriate MAPK activation, is frequently observed in hematological malignancies, our study may shed light on the importance of SIRP α 1 expression, which is relatively easy to examine by flow cytometry, in predicting the efficacy to employ molecularly targeted therapies including MAPK inhibitors.

Although SIRP α 1 expression is widely expressed, the down regulation of SIRP α 1 is also reported in various cancers. In prostate cancer, SIRP α 1 expression is downregulated in prostate cancer tissues and cell lines, and over-expression of SIRP α 1 resulted in the reduction of the number of live prostate cancer tissues, whereas SIRP α 1 silencing increased prostate cancer proliferation [30]. Consistently, it was demonstrated that SIRP α 1 receptors interact with SHP-2 to inhibit wild-type epidermal growth factor receptor (EGFR) mediated tumor migration, survival and cell transformation [28]. Same group also demonstrated that SIRP α 1 receptors interfere with EGFR to inhibit glioblastoma cell transformation and migration [10]. The expression of SIRP α 1 was down-regulated in hepatocellular carcinoma tissues and cells, and over-expression of SIRP α 1 in these cells results in a decrease in the growth of hepatocellular carcinoma cells [21]. Oshima *et al.* [19] demonstrated SHPS-1 (SIRP α) is down-regulated in breast cancer tissues compared with the matched normal tissues. In v-src-transformed BALB/c3T3 cells, over-expression of SHPS-1 resulted in the suppression of anchorage independent cell growth in soft agar, as well as peritoneal dissemination in nude mice [19]. This is quite consistent with the fact that the inhibition was observed for human neutrophil migration in the presence of soluble, recombinant CD47 consisting SIRP α binding loop [13]. As SIRP α signal is inhibition of cell growth, the addition of

CD47 ligand expected to inhibit the growth of serum starved cells. Seiffert *et al.* [23], reported that SIRP α 1 expression is reduced in myeloid malignancies. Concretely, no expression in 4 out of 4 chronic myeloid leukemia patients and 26 out of 59 acute myeloid leukemia patients analyzed, while there is sufficient expression in normal myelo-monocytic cells, as well as with bone marrow hematopoietic stem/progenitor cells [23]. However, the biological significance of this down-regulation of SIRP α 1 in myeloid leukemias has not been clarified until now.

My current study revealed the contribution of the down-regulation of SIRP α 1 to the constitutive activation of these aberrant signaling, as well as abnormal growth, in leukemia cells. To clarify the mechanisms the down-regulation of SIRP α 1 in myeloid malignancies, as well as solid cancer, and to develop the restoration of SIRP α 1 expression in these tumors, may open a door to new therapeutic strategies.

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Competing Interests

The author has no competing interests to declare.

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