

# Correlation of PU.1 and signal regulatory protein $\alpha 1$ expression in PU.1 transgenic K562 cells

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**Abstract.** PU.1 is a key transcription factor for hematopoiesis and the reduction of this protein expression plays important roles in various hematological malignancies. To identify PU.1 downstream target genes, we recently reported dual microarray analyses, using PU.1 knockdown K562 (K562PU.1KD) cells stably expressing short inhibitory RNAs versus control cells and PU.1-overexpressing K562 (K562PU.1OE) cells versus control cells. Several PU.1 candidate target genes, including cell surface receptor, signal regulatory protein (SIRP)  $\alpha 1$ , were identified. In this study, we revealed that the expression of SIRP $\alpha 1$  is positively correlated with the expression of PU.1 in various K562PU.1KD and K562PU.1OE cells, shown by real-time PCR and flow cytometry analyses. SIRP $\alpha 1$  is a negative regulator of signaling and its reduced expression is considered to play a role in the pathogenesis of hematological malignancies through the activation of downstream signaling pathways. By comparing 3 different clones of K562PU.1KD cells to their controls, we found constitutive phosphorylation of the extracellular signal-regulated kinase (ERK), but not of Akt, in these cells. Taken together, the down-regulation of PU.1 expression suppresses the expression of SIRP $\alpha 1$ , which may play a role in the aberrant activation of ERK in these cells.

## Introduction

PU.1 is a member of the Ets transformation-specific sequence (Ets) family of transcription factors and is expressed in granulocytic, monocytic and B-lymphoid cells (1,2). PU.1 deficient

mice exhibit defects in the development of neutrophils, macrophages and B cells (3,4). It has been reported that mice carrying hypomorphic PU.1 alleles that reduce PU.1 expression, to 20% of normal levels, developed acute myeloid leukemia (AML) (5). Moreover, down-regulation of PU.1 has been reported to play a role in the pathogenesis of multiple myeloma (6) and is related to a poor prognosis in myelodysplastic syndrome (7). Therefore, characterizing the function and identifying the target genes of PU.1 are important for understanding the molecular biology of hematopoiesis and oncogenesis.

To identify target gene(s) regulated by PU.1, we previously employed dual microarray analyses to compare PU.1-knockdown K562 cells (K562PU.1KD) stably expressing PU.1 siRNAs versus control cells and PU.1 overexpressing K562 cells (K562PU.1OE) versus control cells. From these analyses, we identified metallothionein and vimentin as direct target genes negatively regulated by PU.1 (8). In addition to these, several genes, including signal regulatory protein (SIRP)  $\alpha 1$ , were identified as candidate target genes, suppressed by PU.1 down-regulation and induced by PU.1 up-regulation (8).

SIRP $\alpha 1$  is a cell surface receptor expressed predominantly in monocytes, granulocytes, dendritic cells, and their precursors, as well as in bone marrow hematopoietic stem cells (9,10). SIRP $\alpha 1$  is also termed Src homology 2 domain-containing phosphatase (SHP) substrate-1 (SHPS-1), brain immunoglobulin-like molecule with tyrosine-based activation motif (BIT), p84 and macrophage fusion receptor. In humans, there are at least 15 members designated as SIRPs (9). They consist of 2 subtypes distinguished by the presence or absence of a cytoplasmic domain containing immunoreceptor tyrosine-based inhibition motifs (9). SIRP $\alpha 1$  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). Therefore, SIRP $\alpha 1$  is considered as a negative regulator of signaling.

In this study, we examined whether the expression of PU.1 is indeed correlated with the expression of SIRP $\alpha 1$  in PU.1 transgenic K562 cells. As a result, we clearly revealed that the expression of SIRP $\alpha 1$  is tightly related to the level of PU.1 expression in these cells. Furthermore, we revealed aberrant signaling in K562PU.1KD cells.

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## Materials and methods

**Cell culture and generation of PU.1 knockdown cells.** K562PU.1KD cells (2-10 and 3-10) and their control cells (vec 5 and vec 6) were used as previously described (8,12). K562PU.1OE cells (A2 and H8) and their controls (V1 and V2) (8) were also employed for this study. To generate more K562PU.1KD cells (3-12), PU.1 siRNA expression vectors were transfected into K562 cells by electroporation as described previously (8,12). In addition, we employed pcPURU6 $\beta$ iSTOP vector (Takara) as a control to generate another control line, vec 4. K562 clones stably transfected with the PU.1 siRNA vectors and the PU.1 expression vector were maintained with 1  $\mu$ g/ml puromycin (for siRNA expression vectors) or 400  $\mu$ g/ml neomycin (for the PU.1 expression vector) containing RPMI with 10% fetal bovine serum.

**mRNA expression analysis.** cDNAs were prepared from cells using reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA). Quantitative PCR was performed using Quantitect SYBR-Green PCR reagent (Qiagen, Miami, FL) following the manufacturer's protocol employing an Opticon mini real-time PCR instrument (Bio-Rad) as previously described (13). Sequences of the primers were: SIRP $\alpha$ 1 forward, 5'-CAGTAACATCACCCAGCA-3' and reverse, 5'-GTCCACGTTGGTCTGGAAGT-3'; PU.1 forward, 5'-GTGCCCTATGACAACGGATCT-3' and reverse, 5'-GAAGCTCTCGAACTCGCTGT-3' (13); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GAAGGTGAAGGTCGGAGT-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3' (13). Thermal cycling conditions for PU.1 were: step 1, 95°C for 15 min; step 2, 95°C for 15 sec; step 3, 60°C for 1 min. Steps 2-3 were repeated for 35 cycles. The conditions for SIRP $\alpha$ 1 were, step 1, 95°C for 15 min; step 2, 95°C for 30 sec; step 3, 50°C for 30 sec; step 4, 72°C for 30 sec. Steps 2-4 were repeated for 35 cycles. The conditions for GAPDH were, step 1, 95°C for 15 min; step 2, 95°C for 30 sec; step 3, 55°C for 30 sec; step 4, 72°C for 30 sec. Steps 2-4 were repeated for 35 cycles. The copy number of each sample was calculated as previously described (14).

**Surface marker expression analysis by flow cytometry.** For flow cytometry analysis, the cells were incubated with PE-conjugated mouse anti-human CD172a (SIRP1 $\alpha$ ) (BioLegend, San Diego, CA) or PE mouse IgG (BioLegend), lysed with FACSlysis solution (Becton-Dickinson, Mountain View, CA) and applied to a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Switzerland), as described previously (15).

**Western blot analysis.** The number of the cells was adjusted in each sample at the day before experiment. Then, the day of the experiment, 1x10<sup>7</sup> cells were collected and lysed in buffer A [10 mM HEPES, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM DTT, 1X protease inhibitor cocktail (Roche, Indianapolis, IN)] for 10 min on ice. After 1,300 x g centrifugation for 10 min, the resulting supernatants were collected as cytosol extracts to examine the signaling molecules. The pellets were washed by buffer B (20 mM HEPES, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1X protease inhibitor cocktail), suspended and incubated on ice for 10 min, centrifuged at 8,000 x g

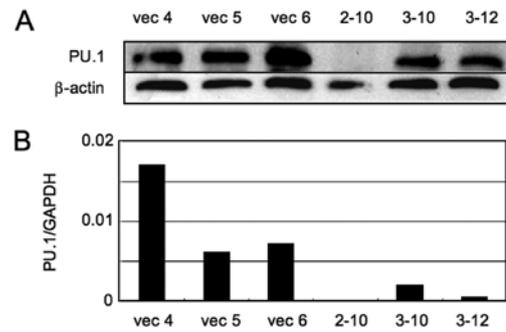


Figure 1. The expression of PU.1 in K562PU.1KD cells. (A) Western blot analysis. Equal amounts of soluble proteins were loaded in each lane and blotted for PU.1 and  $\beta$ -actin. 2-10, 3-10 and 3-12 cells are K562PU.1KD cells, whereas vec 4, vec 5 and vec 6 cells are their controls. (B) Expression levels of PU.1 in K562PU.1KD cells. Results of real-time quantitative PCR. PU.1 gene transcript level was adjusted by the expression of GAPDH and the relative levels are shown.

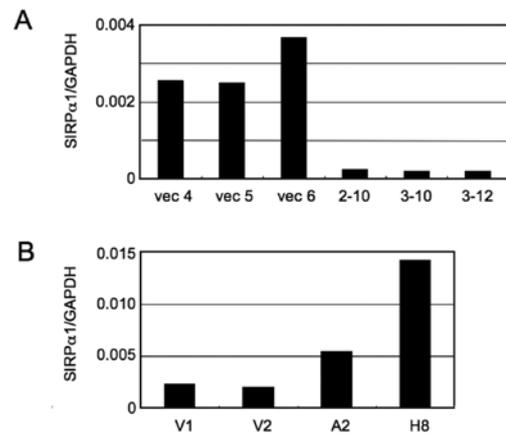


Figure 2. mRNA expression levels of SIRP $\alpha$ 1 in PU.1 transgenic K562 cells. Results of real time quantitative PCR in (A) K562PU.1KD cells and their controls, and (B) K562PU.1OE cells (A2,H8) and their controls (V1,V2). SIRP $\alpha$ 1 gene transcript level was adjusted by the expression of GAPDH and the relative levels are shown. The data presented were obtained from 3 independent PCR reactions and reproducibility was confirmed by independent real-time PCR from different batches of cDNA.

for 15 min and supernatants were collected, representing the nuclear extract to examine the expression of PU.1. Proteins (20-30  $\mu$ g) were separated by SDS-PAGE, transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, NJ) and immunoblotted as described previously (16). To examine the expression of PU.1, rabbit polyclonal anti-PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti- $\beta$  actin (Sigma, St. Louis, MO) antibodies were used. To examine the signaling, anti-phospho-ERK rabbit polyclonal, anti-phospho-Akt rabbit monoclonal and anti-total-Akt rabbit monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-total-ERK mouse monoclonal was obtained from BD Biosciences (San Jose, CA).

## Results

**Establishment of PU.1-knockdown K562 cells.** We previously established K562PU.1KD cells (2-10 and 3-10), stably

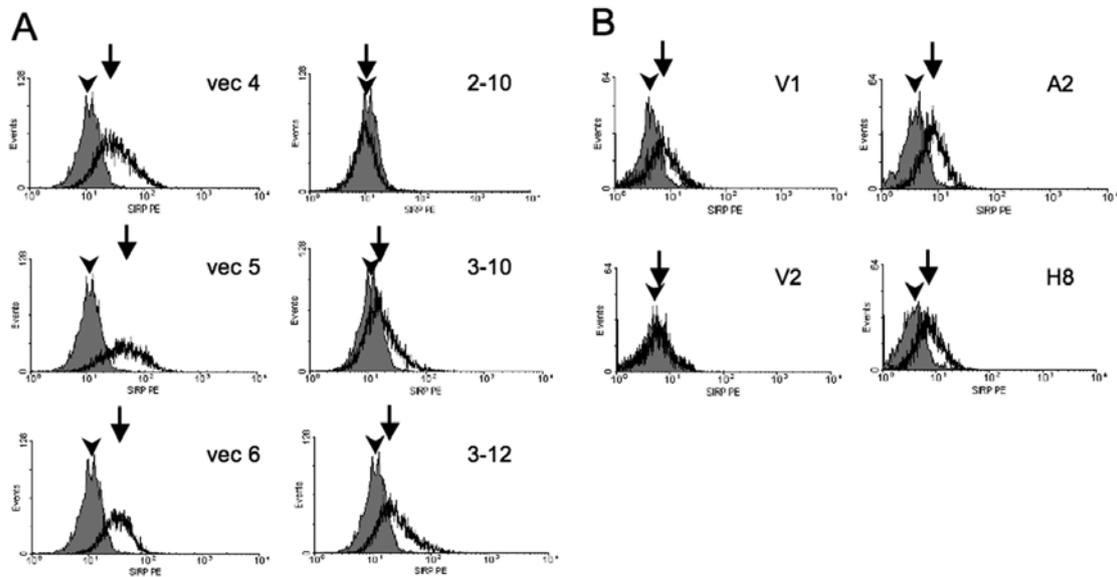


Figure 3. Surface expression levels of SIRP $\alpha$ 1 in PU.1 transgenic K562 cells. Flow cytometry analysis of SIRP $\alpha$ 1 expression on (A) K562PU.1KD, (B) K562PU.1 OE and their control cells. Arrows indicate the SIRP $\alpha$ 1 expression while arrowheads indicate the control PE-IgG.

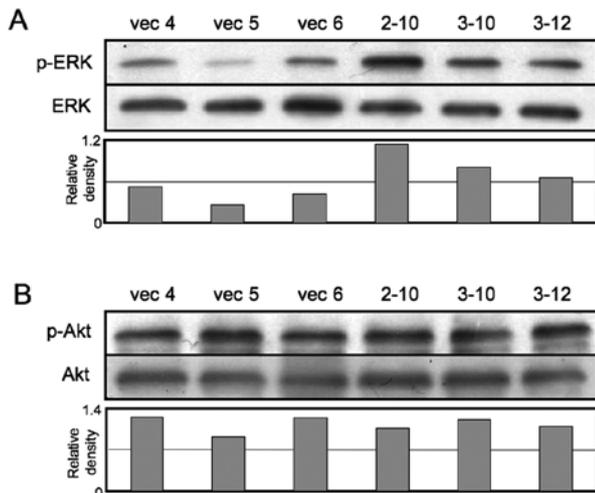


Figure 4. Down-regulation of PU.1 results in the constitutive phosphorylation of ERK. Phosphorylation levels of (A) ERK, and (B) Akt examined by Western blotting. The membranes were first probed with (A) an anti-phospho-ERK and (B) an anti-phospho-Akt rabbit polyclonal antibody. Next, the membranes were stripped and reprobed with (A) anti-total-ERK and (B) anti-total Akt mouse monoclonal antibody to verify equal protein loading. Relative density (lower panels) was obtained from the density of the p-ERK or p-Akt band divided by that of total-ERK or total Akt-band, respectively, measured and quantified by Image J1.36b software (<http://rsb.info.nih.gov/ij/>).

expressing PU.1 siRNAs, and their control, scrambled siRNA vector transfected vec 5 and vec 6 cells (8,12). To elucidate the relations between PU.1 and SIRP $\alpha$ 1, we first attempted to isolate more K562PU.1KD cells and their control cells. Fig. 1 demonstrates the protein (Fig. 1A) and mRNA (Fig. 1B) expressions of PU.1 in K562PU.1KD cells including newly established K562PU.1KD, 3-12 cells. We observed the lowest expression of PU.1 in 2-10 cells and modest suppression in 3-10, and 3-12 cells, compared to their controls, vec 5, vec 6 cells and newly isolated vec 4 cells.

*Expression of SIRP $\alpha$ 1 in K562PU.1KD and OE cells.* The mRNA expression of SIRP $\alpha$ 1 in these cells was examined by real-time PCR (Fig. 2). Compared to their control cells (vec 4, vec 5, vec 6), expression of SIRP $\alpha$ 1 decreased in all of the PU.1KD cells (2-10, 3-10, 3-12) examined (Fig. 2A). In contrast, SIRP $\alpha$ 1 expression significantly increased in K562PU.1OE (A2,H8) cells (Fig. 2B). We then examined the surface expression of SIRP $\alpha$ 1 in these PU.1 transgenic K562 cells by flow cytometry analysis. SIRP $\alpha$ 1 expression was significantly suppressed in all K562PU.1KD cells (Fig. 3A), whereas it was induced in K562PU.1OE cells (Fig. 3B). These results suggest that the expression of SIRP $\alpha$ 1 is positively correlated with the expression of PU.1 in various PU.1 transgenic K562 cells.

*Constitutive activation of ERK in K562PU.1 KD cells.* The down-regulation of SIRP $\alpha$ 1 leads to the activation of downstream signaling pathways (9,15). We previously demonstrated that, by using fewer lines of K562PU.1KD cells, ERK is constitutively activated in these cells (12). To ascertain the activation of downstream signaling pathways by the down-regulation of PU.1, in this study, we employed 3 different K562PU.1KD cells for Western blot analysis. ERK was constitutively phosphorylated in all of the K562PU.1KD cells examined (Fig. 4A). Additionally, there were no differences in Akt phosphorylation in K562PU.1KD compared to control cells (Fig. 4B). Taken together, the down-regulation of PU.1 expression suppresses the expression of SIRP $\alpha$ 1, which may play a role in the aberrant activation of ERK in these cells.

## Discussion

It was reported that SIRP $\alpha$ 1 expression is significantly reduced in myeloid cells from different leukemias. Seiffert *et al* (10) reported that all tested myeloid chronic myeloid leukemia blasts (4 of 4) and almost half of the analyzed AML samples (26 of 59) were negative for SIRP $\alpha$ 1. In addition, they reported

that the blasts of 15 of 59 AML samples expressed reduced SIRP $\alpha$ 1 levels. We recently identified that the suppression of SIRP $\alpha$ 1, by the stable transfection of SIRP $\alpha$ 1 siRNA into K562 cells, resulted in the significant phosphorylation of ERK and activation of several downstream signaling pathways (15). Together with these, reduced expression of SIRP $\alpha$ 1 may play a role in the mechanisms of leukemogenesis through the aberrant activation of downstream signaling pathways.

SIRPs comprise a family of cell surface receptors that regulate signal transduction through tyrosine kinase receptors by interacting with SHP-1 and SHP-2 (11). SHP-1 tyrosine phosphatase is the key negative regulator for numerous signaling pathways. SHP-1 is expressed primarily in the hematopoietic and epithelial cells. SHP-1 acts in the immune and other hematopoietic cells by inhibiting signaling through receptors for cytokines, growth factors, and chemokines as well as receptors directly involved in the immune responses and programmed cell death (17,18). SHP-1 acts as tumor suppressor and loss of its expression has been identified in the whole spectrum of lymphoid and myeloid cell malignancies (19-21). Recently, it was reported that SHP-1 is directly regulated by PU.1 and suppressed by the PU.1 down-regulation (22). They also revealed that the transient siRNA transfection of PU.1 into Burkitt's lymphoma-derived BJAB cells resulted in the increment of the total intracellular phosphotyrosine (22). In addition, we previously identified that Annexin 1 is induced by the down-regulation of PU.1 (12). Annexin 1 is a calcium- and phospholipids-binding protein and increased expression leads to the constitutive activation of ERK (12,23). Collectively, the reduced expression of PU.1 plays a role in the mechanisms of leukemogenesis thorough the activation of downstream signaling pathways, by modulating the expressions of various genes including SIRP $\alpha$ 1, SHP-1 and Annexin 1.

Although genetic alterations affecting the functions of transcription factors that regulate myeloid maturation play important roles in leukemogenesis, inappropriate mitogen-activated protein kinase (MAPK) activation may also play a role in leukemic transformation (24). The Ras-ERK-MAPK pathways and phosphoinositide 3-kinase (PI3K)-Akt pathways are frequently activated in hematological malignancies (25-27). Up-regulation of the MAPK network in AML has been reported to arise through several mechanisms, including the Fms-like tyrosine 3 kinase receptor, c-kit and Ras mutations frequently detected in AML (14,28-30). Our current study sheds light to the fact that the down-regulation of PU.1 transcription factor plays a role in the aberrant induction of ERK-MAPK pathway. Further clarifying the molecular pathways related to PU.1 lead to better understanding of pathogenesis of AML and other hematological malignancies.

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