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Selenoprotein S Suppression Enhances the Late Stage Differentiation of Proerythrocytes Via SIRT1

Hee-Young Yang[1], Kyoung-Jin Chung[1], Hyang-Rim Park[1], Seong-Jeong Han[2,4], Seung-Rock Lee[2], Kee-Oh Chay[2], Ick Young Kim[3], Byung-Ju Park[1], and Tae-Hoon Lee*[1]

[1]*Department of Oral Biochemistry, Dental Science Research Institute, The 2nd Stage of Brain Korea 21 for Dental School, Chonnam National University, Gwangju 500-757, Korea*
[2]*Department of Biochemistry, Center for Aging and Geriatrics, Chonnam National University Medical School, Gwangju 501-190, Korea*
[3]*Laboratory of Cellular and Molecular Biochemistry, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[4]*School of Biological Science and Technology, Chonnam National University, Gwangju 500-757, Korea*

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Selenoprotein S (SelS) is widely expressed in diverse tissues where it localizes in the plasma membrane and endoplasmic reticulum. We studied the potential function of SelS in erythrocyte differentiation using K562 cells stably over-expressing SelS wild-type (WT) or one of two SelS point mutants, U_{188}S or U_{188}C. We found that in the K562 cells treated with 1 μM Ara-C, SelS gradually declined over five days of treatment. On day 4, intracellular ROS levels were higher in cells expressing SelS-WT than in those expressing a SelS mutant. Moreover, the cell cycle patterns in cells expressing SelS-WT or U_{188}C were similar to the controls. The expression and activation of SIRT1 were also reduced during K562 differentiation. Cells expressing SelS-WT showed elevated SIRT1 expression and activation (phosphorylation), as well as higher levels of FoxO3a expression. SIRT1 activation was diminished slightly in cells expressing SelS-WT after treatment with the ROS scavenger NAC (12 mM), but not in those expressing a SelS mutant. After four days of Ara-C treatment, SelS-WT-expressing cells showed elevated transcription of *β-globin*, *γ-globin*, *ε-globin*, *GATA-1* and *zfpm-1*, whereas cells expressing a SelS mutant did not. These results suggest that the suppression of SelS acts as a trigger for proerythrocyte differentiation via the ROS-mediated downregulation of SIRT1.

*Corresponding author: Tae-Hoon Lee, Ph.D., Department of Biochemistry, School of Dentistry, Chonnam National University, 300 Yongbong-Dong, Buk-Ku, Gwangju 500-757, Korea.
Tel: +82-62-5304842, Fax: +82-62-5304809
E-mail: thlee83@chonnam.ac.kr

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Introduction

Selenium is a well known dietary mineral that is incorporated co-translationally into selenoproteins as the amino acid selenocysteine (Sec), which is encoded by the codon UGA (Windmill *et al*., 2007). Although the physiological functions of selenoproteins are not well characterized, so far 25 selenoproteins have been identified in the mammalian system through SECIS (selenocysteine insertion sequence)-based bioinformatics (Kryukov *et al*., 2003). Among these, selenoprotein S (SelS; VIMP, SEPS1, Tanis) is localized in the plasma membrane and endoplasmic reticulum (ER) in a wide variety of tissues (Walder *et al*., 2002; Kryukov *et al*., 2003; Gao *et al*., 2007; Windmill *et al*., 2007), where it takes part in the removal of misfolded proteins from the ER lumen for degradation and, importantly, acts as an antioxidant (Ye *et al*., 2004). That said, the functions of SelS and its mechanism of action remain mostly undefined.

Oxidative stress, reflected by the levels of reactive oxygen species (ROS), affects the differentiation of many hematopoietic lineage cells and is especially important to the regulation of erythropoiesis (Ghaffari, 2008). ROS appear to exert their effects by inducing activation factors that work as key regulators during the differentiation process in erythrocytes (Chenais, *et al*., 2000; Nakata *et al*., 2004; Marinkovic, *et al*., 2007). For example, we previously described the role of the mitochondrial antioxidant enzyme

peroxiredoxin (Prdx) III during late stage differentiation of K562 erythroid cells *(Yang et al.*, 2007).

SIRT1 (Sirtuin 1; Sir2l1, Sir2α) is an enzyme that catalyzes the deacetylation of a variety of transcription factors, including FoxOs, p53, Ku70 and NFκB, among others. In this way, SIRT1 regulates multiple cellular mechanisms, including cell differentiation, cell survival, tumorigenesis and metabolism (Zschoernig, 2008; Kim *et al.*, 2009). It also plays key roles at specific stages of the differentiation of skeletal muscle cells, adipocytes and pancreatic β cells, in spermatogenesis and in the development of the brain and heart (Sakamoto *et al.*, 2004; Guarente, 2005; Longo, 2006; Coussens *et al.*, 2008). FoxO transcription factors are direct substrates of SIRT1 (Brunet *et al.*, 2004), and the activation of FoxO3a in erythroblasts induces erythrocyte differentiation (Bakker *et al.*, 2004; Bakker *et al.*, 2007), though the direct involvement of SIRT1 in erythrocyte differentiation has not been characterized. In the present study, we characterized the functions of selenocysteine in SelS, and also investigated the SelS dependence of SIRT1-mediated FoxO3 expression during erythrocyte differentiation.

Materials and Methods

Cell culture

K562 cells were derived from *human* chronic myeloid leukemia in blast crisis (Luisi-DeLuca *et al.*, 1984). The cells were grown in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified 5% CO_2 atmosphere at 37°C. To induce erythroid differentiation, K562 cells were seeded at 1×10^5 cells/ml and treated with 1 μM cytosine arabinoside (Ara-C; Sigma). The medium was not changed during the induction period (Yang *et al.*, 2007).

For production of cells stably expressing wild-type (WT) SelS or the $U_{188}S$ or $U_{188}C$ SelS mutant, K562 cells were transfected using FuGENE 6 (Roch, Indianapolis, IN) according to the manufacturer's instructions. After 48 h, the transfectants were selected by culture in the presence of 500 μg/ml geneticine for 2 weeks (Welgene Biotech, Korea). As a control some cells were transfected with empty expression vector (mock). In addition some cells were pretreated for 1 h with the ROS scavenger N-acetyl cysteine (NAC; 12 mM) (Sigma).

Plasmid construction

A full-length SelS cDNA was amplified from a *human* cDNA library (Open Biosystems, Huntsville, AL) by polymerase chain reaction (PCR) using an internal forward primer (5'-CTCGAGATGGAACGCCAAGAGGAGTC-3'), which contains the initiation codon (boldface type) and a *Xho* I site (underlined)), and a reverse primer (5'-AGATCT-AAACCCCATCAACTGTCCAC-3'), which originated at a SECIS region and contains a Sec insertion sequence element from the 3' untranslated region (UTR) and *Bgl* II site (underlined). To improve the ectopic expression of SelS, a matrix attachment region (MAR) was inserted just in front of the *chicken* β-actin promoter. The resulting PCR product was cloned into pCR3.1 vector (Invitrogen, Carlsbad, CA) to produce pCR3.1-MAR-SelS WT. The selenocysteine mutant proteins, $U_{188}C$ and $U_{188}S$, in which selenocysteine 188 was replaced by cysteine or serine, respectively, were generated by PCR-mediated site-directed mutagenesis with complementary primers containing a 1-bp mismatch that converted the codon for selenocysteine to cysteine or serine. The mutated PCR products were ligated into pCR3.1 vector to produce pCR3.1-MAR-SelS $U_{188}S$ or $U_{188}C$, respectively.

Preparation of a specific anti-SelS antibody

Rabbit antiserum raised against SelS was prepared by injection with a hemocyanin-conjugated peptide (C-^{174}SWR-PGRRGPSSGG^{187}-NH_2). Thereafter, polyclonal anti-SelS antibodies were purified from the antiserum using an affinity resin conjugated with the polypeptide.

Western blotting

K562 cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 1 μg/ml leupeptin and 1 mM PMSF. The resolved proteins were transferred onto a PVDF (polyvinylidene fluoride) membrane, after which the membrane was incubated first with anti-SelS, anti-phosphorylated (p)-Sirt1 (Santa Cruz biotechnology, CA), anti-Sirt1 (Santa Cruz), anti-FoxO3a (Cell Signaling Technology, USA) or anti-β-actin (Sigma) and then with HRP-conjugated secondary antibodies (Cell Signaling Technology). Immunoreactive proteins were detected using an ECL system (iNtRON, South Korea).

Intracellular ROS levels

To measure intracellular ROS levels, K562 cells (1×10^6) were washed two times with prewarmed phosphate-buffered saline (PBS). The cells were then loaded with 5 μM 5,6-chloromethyl-2',7'-dichlorodihydroflurescein diacetate (CM-H_2DCFDA) (Molecular Probes, Eugene, OR) for 30 min at 37°C, washed and immediately analyzed by flow cytometry (Beckman Coulter, Miami, FL).

Cell cycle analysis

K562 cells (1×10^6) were serum-starved for 16 h in RPMI 1640 medium containing 0.5% BSA, after which they were incubated for 24 h in RPMI 1640 supplemented with 10% FBS. The cells were then suspended in prechilled 70% ethanol, harvested by centrifugation, washed with PBS, and then incubated with propidium iodide (PI) staining buffer (15 μg/ml PI, 0.2% Triton X-100, 30 μg/ml Ribonuclease A

and PBS (pH 7.5)) for 30 min at 37°C in the dark. The red fluorescence excited at 488 nm was then measured, and the histograms were analyzed using the G1/G2M Only Fit method (Beckman Coulter).

Reverse-Transcription PCR and Real-Time PCR

The specific oligonucleotide primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA). RT-PCR was carried out in a 40- μl reaction mixture using TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was carried out using SYBR GREEN PCR Master Mix (Takara, Japan) and an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). The primer conditions, following sequences, and information about *human β-actin*, *β-globin*, *γ-globin*, *ε-globin*, *GATA-1* and *zfpm-1* genes were as previously reported (Yang *et al*., 2007).

Statistical Analysis

Data are shown as means and standard deviations. *P* values were calculated using unpaired Student's *t*-tests. All statistical analyses were performed using SigmaPlot 9.0.

Results

Expression of SelS during proerythrocyte differentiation

We studied the K562 human erythroleukemia cell line in an effort to better understand the function of SelS in the terminally differentiated erythroid lineage. It was previously shown that expression of *β-globin* gradually increases in K562 cells treated with 1 μM Ara-C (Yang *et al*., 2007). By contrast, we found that expression of SelS protein in K562 cells had declined about 5 fold after 1 day of Ara-C-induced differentiation (Fig. 1A).

We next tested whether overexpression of wild-type (WT) SelS would affect K562 cell differentiation. In addition, we assessed the importance of selenocysteins by substituting active selenocysteine188 with serine ($U_{188}S$) or cysteine ($U_{188}C$) (Fig. 1B). Because selenoproteins are generally considered to be antioxidants, we measured the intracellular ROS levels in cells stably expressing SelS and stained with CM-H_2DCFDA. We found that in the absence of stimulation by Ara-C, there were no differences in ROS levels among the mock (M), SelS-WT and $U_{188}C$ transfectants, whereas ROS levels were higher in the $U_{188}S$ transfectants. Upon stimulation with Ara-C, ROS levels were also increased in SelS-WT- and $U_{188}C$-expressing cells (Fig. 1C), reflecting a corresponding decline in SelS expression (Fig. 2B). Apparently, the ability of SelS to scavenge ROS was retained when selenocysteine (WT) was substituted with cysteine ($U_{188}C$) in undifferentiated cells, but the ability was lost upon Ara-C-induced differentiation.

To examine the effects of ectopic expression of SelS on cell cycle progression in K562 cells, we carried out a FACS analysis after staining with PI. SelS-WT, $U_{188}C$ and mock transfectants showed similar cell cycle patterns; in $U_{188}S$ transfectants, however, the G1 phase fraction was smaller and the G2/M phase fraction was larger than in the other cells (Fig. 1D), which suggests the redox potential-sensitive amino acid contained in SelS-WT and $U_{188}C$ may contribute to cell cycle regulation.

SIRT1 activity in cells stably overexpressing SelS

Recent reports suggest that SIRT1 regulates stimulation-induced differentiation at specific stages (Kim *et al*., 2009; Skokowa *et al*., 2009). To examine SelS-related SIRT1 function during K562 differentiation, we initially assessed

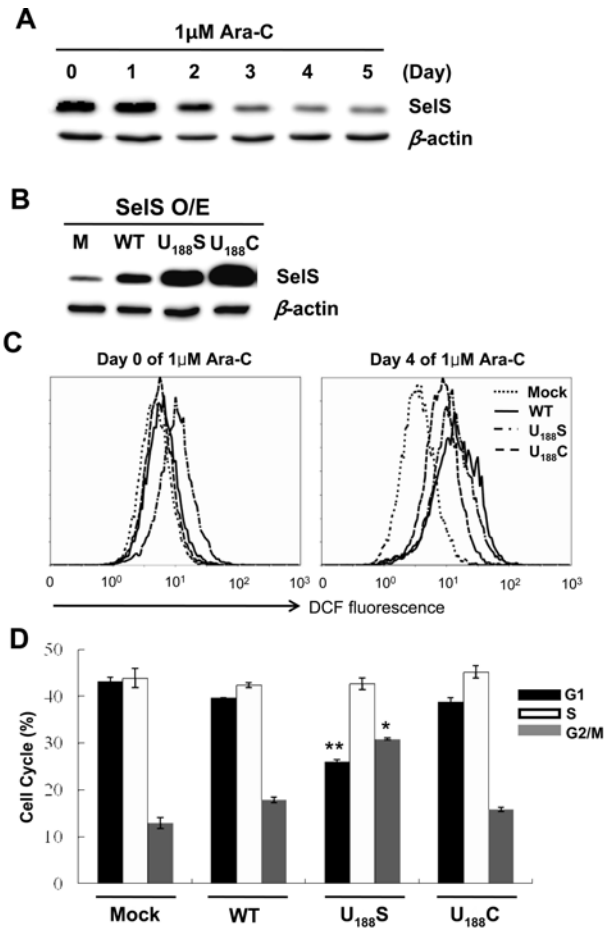

Fig. 1. SelS expression during differentiation of K562 cells. (A) Expression of SelS after treatment with Ara-C (cytosine arabinoside) for the indicated times. (B) Expression of SelS-WT or the indicated SelS mutant following transfection of K562 cells. O/E, SelS overexpression cell lines. (C) ROS levels in K562 cells stably expressing mock (M), Sels-WT, $U_{188}S$ or $U_{188}C$ on day 4 of treatment with 1 μM Ara-C. ROS levels were measured by FACS analysis after staining with 5 μM DCFH-DA (5,6-chloromethyl-2',7'-dichlorodihydroflurescein diacetate). (D) Flow cytometric analysis of cell-cycle progression in cells transfected with the indicated SelS proteins and stained with PI (propidium iodide). The histograms show the percentages of cells in G1-, S- and G2/M- phases. $^{**}P < 0.01$; $^{*}P < 0.05$ vs. WT- and $U_{188}S$-expressing cells.

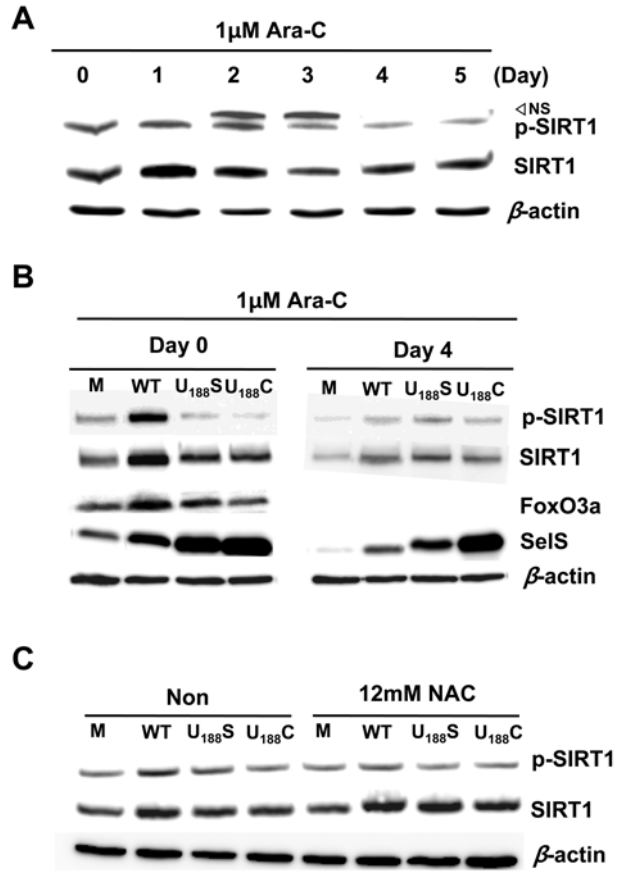

Fig. 2. Expression and activation of SIRT1 in cells transfected with mock (M), SelS-WT, $U_{188}S$ or $U_{188}C$. (A) Expression of SIRT1 and phosphorylated (p)-SIRT1 in K562 cells after treatment with Ara-C for the indicated times. NS, Non-specific band (Yeung *et al.*, 2004). (B) Expression of SIRT1, p-SIRT1 and FoxO3a in K562 cells stably expressing mock (M), Sels-WT, $U_{188}S$ or $U_{188}C$ before and on day 4 of treatment with 1 μM Ara-C. (C) Effect of pretreatment for 1 h with the ROS scavenger NAC (12 mM) on expression of SIRT1 and p-SIRT1 in cells stably expressing mock (M), Sels-WT, $U_{188}S$ or $U_{188}C$. Non, no NAC treatment.

the levels of SIRT1 expression and its activation (phosphorylation) in K562 cells. SIRT1 expression in K562 cells increased dramatically on day 1 of Ara-C-induced differentiation but then declined over the next 4 days (Fig. 2A; the middle line). Activation of SIRT1 deacetylase activity is regulated by phosphorylation (Sasaki *et al.*, 2008; Zschoernig, 2009), and SIRT1 phosphorylation also gradually declined in Ara-C-treated cultures (Fig. 2A; the top line). Thus inactivation of SIRT1 may be a key feature of the terminal erythroid differentiation of Ara-C-treated K562 cells.

Given that SIRT1 activation gradually declined along with SelS levels during Ara-C-induced K562 differentiation (Figs. 1A and 2A), we investigated the pattern of SIRT1 expression in stable SelS transfectants. We found that SelS WT cells showed high levels of both SIRT1 and p-SIRT1 expression, whereas mock, $U_{188}S$ and $U_{188}C$ transfectants did not (Fig. 2B), which suggests that the expression of SelS and SIRT1 are related during Ara-C-induced K562 cell differentiation.

FoxO3a, which is associated with erythropoiesis under diverse experimental conditions (Bakker *et al.*, 2004; Bakker *et al.*, 2007; Marinkovic *et al.*, 2007), is directly regulated by p-SIRT1 (Huang, 2007; Kim *et al.*, 2009; Skokowa *et al.*, 2009). We found that levels of FoxO3a expression were markedly higher in cells expressing SelS-WT than the other cells. Moreover, the pattern of FoxO3a expression was similar to that of SIRT1 in SelS-WT-expressing cells (Fig. 2B). Unexpectedly, however, no FoxO3a expression was detected in any of the transfectants on day 4 of Ara-C treatment (Fig. 2B). Taken together, these findings suggest that the differentiation-related proteins SIRT1 and FoxO3a may be reciprocally associated in cells stably overexpressing SelS-WT.

The low levels of SelS expression seen on differentiation day 4 in SelS-WT-expressing cells were associated with elevated levels of ROS (Fig. 1C). To confirm that observation, we tested the effect of pretreating the cells for 1 h with the ROS scavenger NAC (12 mM) on the expression and activation of SIRT1. We found that p-SIRT1 levels were reduced somewhat in SelS-WT- and $U_{188}S$-expressing cells, with no change in cells expressing mock or $U_{188}C$ (Fig. 2C). At the same time, levels of SIRT1 were unchanged in SelS-WT-expressing cells, but were increased in cells expressing $U_{188}S$ or $U_{188}C$ cells (Fig. 2C). Taken together, these findings suggest that activation of SIRT1, not its expression, is regulated by ROS.

The participation of SelS in terminal erythroid differentiation

To investigate the extent to which SelS participates in Ara-C-induced K562 cell differentiation, we used real-time PCR to assess the expression of *β-, γ-,* and *ε-globin* after 4 days of treatment with 1 μM Ara-C treatment. In the absence of Ara-C stimulation, induction of *β-globin* was slightly increased in SelS-WT-expressing cells, as compared to cells expressing mock or one of the SelS mutants. In the presence of Ara-C, Sels-WT transfectants showed a greater than 5-fold increase of *β-globin* expression (Fig. 3A), while expression of *γ-* and *ε-globin* was increased more than 2.5 fold (Fig. 3B and C). Although there were no significant differences in the expression of *γ-,* and *ε-globin* in mock- and $U_{188}C$-expressing cells, the patterns of *γ-,* and *ε-globin* expression in $U_{188}S$-expressing cells was similar to that in cells expressing SelS-WT.

Finally, we assessed the expression of *GATA-1* and *Zfpm-1*, two transcription factors involved in the terminal erythriod differentiation of K562 cells. In the absence of Ara-C, there were no differences in *GATA-1* and *Zfpm-1* expression among the transfectants. Following Ara-C treatment, expression of both transcription factors was increased in all of the transfectants, but the expression was highest in cells expressing SelS-WT (Fig. 4), lending further support to the idea SelS contributes to proerythrocyte differentiation of K562 cells.

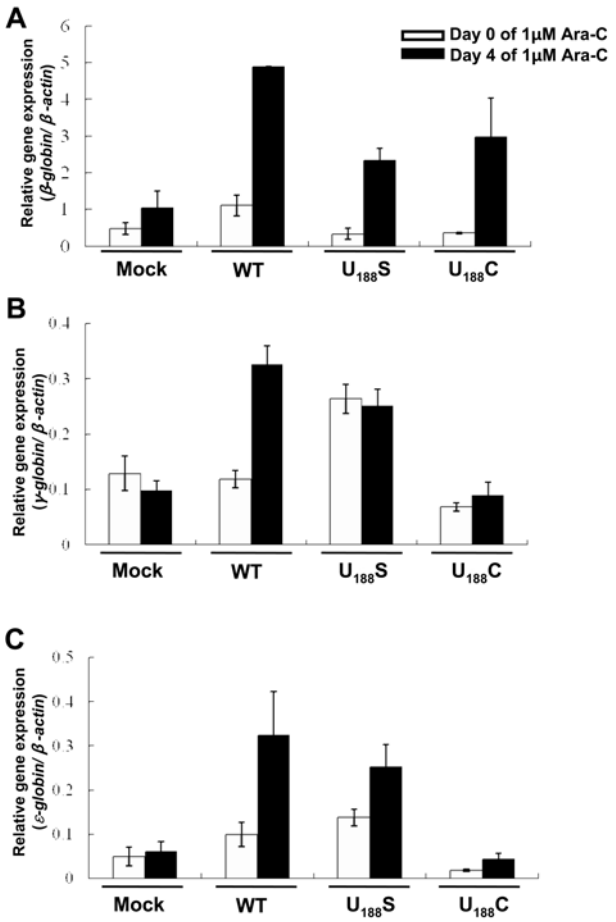

Fig. 3. Transcription of *β-globin*, *γ-globin* and *ε-globin*. Relative levels of *β-globin* (A), *γ-globin* (B), and *ε-globin* (C) mRNAs on day 4 of Ara-C (1 μM) treatment in cells stably expressing mock (M), Sels-WT, U_{188}S or U_{188}C. Levels of *globin* transcription were normalized to that of *β-actin* using real-time PCR analysis.

Discussion

Expression of the selenoprotein SelS was first detected in the liver, skeletal muscle, adipose tissue, hypothalamus, testes, heart and kidney of Psammomys obesus, and its expression in liver was shown to differ depending upon whether the animal was fasted or fed (Walder *et al*., 2002). Human SelS was subsequently identified by searching databases using a SECIS-based method to identify putative selenoproteins, and its mRNA has been detected in a variety of tissues and cell types (Kryukov et al., 2003). Recently, it was reported that SelS is able to influence inflammatory responses induced by lipopolysaccharide in hepatic cells (Gao *et al*., 2004; Curran *et al*., 2005; Gao *et al*., 2007; Kim *et al*., 2007; Windmill *et al*., 2007; Zeng *et al*., 2008). In addition, SelS appears to be localized in the plasma membrane and ER, where it is involved in the elimination of misfolded proteins (Gao *et al*., 2004; Ye *et al*., 2004; Kim *et al*., 2007) and in the response to changes in ROS levels (Hoffmann, 2008; Zeng *et al*., 2008; Steinbrenner, 2009). That said, the functions of SelS remain largely undefined in most cellular environments.

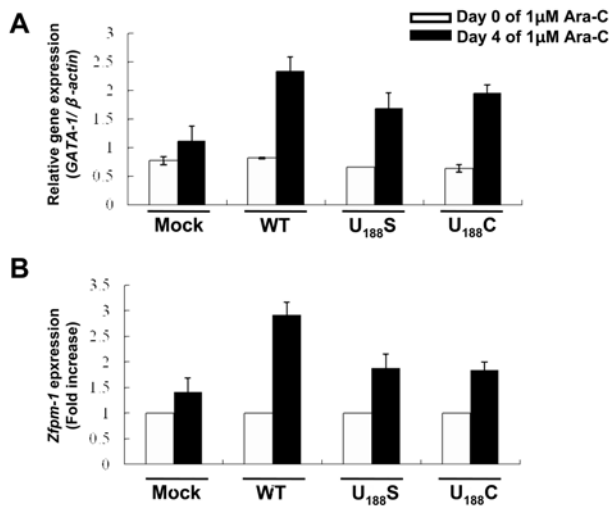

Fig. 4. Transcription of *GATA-1* and *Zfpm-1*. Relative levels of *GATA-1* (A) and *Zfpm-1* (B) mRNAs on day 4 of Ara-C (1 μM) treatment in cells stably expressing mock (M), Sels-WT, U_{188}S or U_{188}C. Levels of *GATA-1* and *Zfpm-1* expression were normalized to that of *β-actin* using real-time PCR analysis.

In the present study, we observed that SelS expression gradually declined during Ara-C-induced K562 cell differentiation, which suggested to us that SelS may be involved in erythrocyte differentiation. To investigate the role played by SelS during that process, we established cultures of K562 cells stably expressing SelS-WT, U_{188}S or U_{188}C and then examined the effects of ROS on proerythrocyte differentiation. In an earlier study we found that in the absence of Ara-C, ROS levels were lower in cells expressing Prdx III O/E than in those expressing Prdx III-D/N (dominant negative) or in mock transfectants, and the Prdx expressing cells arrested at G1 phase. Upon stimulation with 1 μM Ara-C, Prdx III O/E expressing cells exhibited ROS scavenger activity before and after Ara-C treatment, but mock and Prdx III D/N-expressing cells did not (Yang *et al*., 2007). In contrast to Prdx III, expression of SelS was reduced in Ara-C-treated cells (Fig 2B), the ROS levels in SelS-WT transfectants were somewhat higher than in cells expressing mock, U_{188}S or U_{188}C (Fig. 1C). In addition, the cell cycle was unaffected in mock or U_{188}C transfectants, which both expressed SelS containing a redox potential-sensitive amino acid (selenocysteine and cysteine), but it was altered in cells expressing U_{188}S (Fig. 1D). ROS apparently mediates the regulation of erythropoiesis through the action of erythropoiesis-related proteins (Lee *et al*., 2003; Aerbajinai *et al*., 2007; Marinkovic *et al*., 2007; Pinho *et al*., 2008). Although excess ROS impedes erythropoiesis, we suggest that the ROS levels in cells expressing SelS WT are low enough to stimulate K562 erythropoiesis.

SIRT1 deacetylase has been studied under a variety of cellular conditions (Zschoernig, 2008). For instance, SIRT1 expression is dramatically increased in mouse neural progenitor cells exposed to pro-oxidative buthionine sulfoximine

(BSO), an inhibitor of glutathione synthetase that causes intracellular accumulation of ROS (Prozorovski *et al*., 2008). Interestingly, we found that cells transfected with SelS-WT showed high levels of SIRT1 activation (Fig. 2B). And given that we found no direct interaction between SelS and SIRT1 proteins (data not shown), we suggest that SelS acts via ROS to modulate SIRT1 activation.

During the cellular response to oxidative stress, FoxO3a is activated to determine cell fate (Huang, 2007). In the context of oxidative stress-derived erythropoiesis, the activity of FoxO3a is directly regulated by SIRT1 (Brunet *et al*., 2004; Bakker *et al*., 2007; Hattangadi, 2007; Huang, 2007; Marinkovic *et al*., 2007), and our inability to detect FoxO3a on day 4 of Ara-C treatment is consistent with earlier studies (Brunet *et al*., 2004; Giannakou, 2004). Moreover, the low level of SIRT1 expression at that time suggests that, in cells expressing SelS-WT, SIRT1 acts in concert with FoxO3a during early stages of proerythrocyte differentiation. It was recently reported that ROS modulate SIRT1 levels, which in turn affects FoxO3a activation (Hasegawa *et al*., 2008). However, the expression of SIRT1 was unaffected by NAC treatment in human umbilical vein endothelial cells (Ota *et al*., 2008). Similarly, we found that overall SIRT1 expression in SelS-WT transfectants was unaffected by treatment with 12 mM NAC. On the other hand activation (phosphorylation) of SIRT1 was markedly reduced (Fig. 2C).

Collectively then, our findings indicate that SelS may regulate Ara-C-induced proerythrocyte differentiation of K562 cells, and that SelS likely exerts its effects through activation of SIRT1.

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