

Aryl Hydrocarbon Receptor Antagonist StemRegenin 1 Promotes the Expansion of Human Promyelocytic Leukemia Cell Line, NB4

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Abstract. *Background/Aim:* StemRegenin 1 (SR1), an antagonist of aryl hydrocarbon receptor (AHR), reportedly promotes expansion of hematopoietic stem cells but its effect on leukemia cells is unclear. This study focused on the role of SR1 in leukemia cell proliferation. *Materials and Methods:* AHR expression was compared in the cell lines Jurkat, Kasumi-1, NB4 and K562, using real-time polymerase chain reaction. Highly AHR-expressing NB4 cells were cultured with SR1 for 2 and 4 days, and evaluated for viability and gene expression. DNA microarray was also performed. *Results:* The viability of NB4 cells treated with 1.5 μ M SR1 increased at day 4. Expression of B-cell CLL/lymphoma 2 (BCL2) was up-regulated, while that of BCL2 associated X protein (BAX) was down-regulated at day 2. Increased cyclin D1 (CCND1), CCND2 and v-myc avian myelocytomatosis viral oncogene homolog (MYC) expressions were observed at day 4. Global gene expression profiles showed up-regulation of splice variant-related genes and down-regulation of inflammation-related genes. *Conclusion:* SR1 promotes the expansion of NB4 cells *in vitro*, implying the need for caution regarding *in vivo* use of R1.

Hematopoietic stem cells (HSCs) are the multipotent cells, that can differentiate into various types of functional blood cells, and also have self-renewal capability (1). HSCs are clinically used in HSC transplantation for therapy (2).

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Nowadays, HSCs for transplantation therapy are collected from the bone marrow or peripheral blood of donors, or umbilical-cord blood, however, the supply does not satisfy the demand of the recipients due to the lack of human leukocyte antigen-matched donors and the low number of stem cells (3). Several attempts have been made to develop a technique to expand HSCs *ex vivo* or *in vitro* (3, 4). However, it is not yet known whether chromosomal and genetic abnormalities accumulated through the process of hematopoiesis will occur when attempting to expand HSCs *ex vivo* and lead to abnormal hematopoiesis/leukemia, which increases immature nonfunctional blood-forming cells in the bone marrow (5).

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that is well known for being activated by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) (6). AHR is expressed in almost all human tissues except for skeletal muscle, and has been shown to regulate xenobiotic metabolizing enzymes (7). StemRegenin 1 (SR1), a high-affinity antagonist of AHR, has received special attention because several researches have reported its effects on hematopoietic cell proliferation and differentiation. For example, SR1 promotes the *ex vivo* expansion of human HSCs (4), and also facilitates the *in vivo* generation of hematopoietic progenitor cells (HPCs) from non-human primate-induced pluripotent stem cells (8). Besides HSCs, SR1 improves the development of human plasmacytoid and myeloid dendritic cells or functional natural killer cells from HSCs and HPCs (9, 10). Clinical optimization of SR1 has been attempted using *in vitro* HSC expansion for transplantation therapy (3). However, some studies have reported the cause-effect relation between AHR and cancer (7). Breast, prostate, gastric, small cell lung and liver tumors are reported to exhibit relatively increased levels of AHR expression (7).

Since leukemia cells also express AHR, there is a possibility that SR1 also promotes the proliferation of leukemia cells (11, 12). The effect of SR1 on proliferation of immature acute myeloid leukemia (M0, M1 and M2) cells has been investigated (11, 12), however there is no detailed research targeting mature acute myeloid leukemia (M3) cells. Herein we investigated the effect of SR1 on proliferation of several cell lines, including the human acute promyelocytic leukemia (M3) cell line, NB4.

Materials and Methods

Cell lines. In this study human leukemia cell lines, Jurkat, Kasumi-1 and NB4 were kindly provided by Professor Koichi Akashi, Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan. K562 cell line was obtained from RIKEN BRC Cell Bank, Ibaraki, Japan. The cell source and type of each cell line is referred to in Table I.

Cell culture. Leukemia cell lines Jurkat, Kasumi-1, NB4 and K562 were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS) and 10 U/ml penicillin and 10 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in 5% CO₂. Cells were subcultured every 2-3 days, and maintained at a cell density of 0.5-1.0×10⁶ cells/ml.

Treatment with SR1 and cell counting. SR1 (Abcam, Cambridge, UK) was diluted in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries) to a final concentration of 1.5 mM. NB4 cells were cultured in a 24-well plate at 5.0×10⁵ cells/ml in the presence of three different SR1 concentrations (0.375 µM, 0.75 µM and 1.5 µM, containing 0.1% DMSO), or 0.1% DMSO only (control). Under all conditions, cell culture was performed in triplicate wells. Morphology of the cells was observed under a phase-contrast Olympus CKX41 microscope (Olympus, Tokyo, Japan) and recorded using ZEN 2 (blue edition) software (Carl Zeiss Microscopy GmbH, Jena, Germany). After 2 and 4 days of culture, cells were collected and washed with PBS (-) (Wako Pure Chemical Industries). The cells were mixed with Trypan Blue Solution (Wako Pure Chemical Industries) and the number of live and dead cells was counted by using Bürker Türk blood corpuscle counting chamber (ERMA Inc, Tokyo, Japan).

May-Grünwald Giemsa staining. At day 4 of culture, treated and control cells were suspended in PBS (-) and attached onto glass slides (Matsunami Glass Industry, Osaka, Japan) using a CytoSpinTM4 Cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA), at a speed of 22 × g for 7 min and immediately air-dried. Cells were then stained with May-Grünwald solution (Muto Pure Chemicals, Tokyo, Japan) for 5 minutes, washed with tap water and further stained with diluted Giemsa staining reagents (Muto Pure Chemicals) for 30 min. Slides were then washed with tap water and air-dried thoroughly. Glass coverslips were attached to the slides by using MGK-S mounting solution (Matsunami Glass Industry). Stained cells were observed and recorded using oil immersion lens of an Olympus CKX41 microscope (Olympus) and ZEN 2 software (Carl Zeiss Microscopy GmbH).

Table I. Leukemia cell lines used in this study.

Cell line	Source	Type	Reference
Jurkat	PB	Acute T-cell leukemia	(15)
Kasumi-1	PB	Acute myeloblastic leukemia (M2)	(16)
NB4	BM	Acute promyelocytic leukemia (M3)	(17)
K562	BM	Chronic myelogenous leukemia/erythroleukemia	(18)

PB: Peripheral blood, BM: bone marrow.

Immunocytochemistry (ICC). At day 4 of culture, untreated cells were suspended in PBS (-) and attached onto glass slides using a CytoSpinTM 4 centrifuging at 22 ×g for 7 min and air-dried overnight. Cells were then fixed with PBS (-) containing 1% paraformaldehyde (Wako Pure Chemical Industries), permeabilized with PBS containing 0.05% Triton X-100 (Nacalai Tesque, Kyoto, Japan), and blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich). Cells were then incubated with rabbit polyclonal antibody to human aryl hydrocarbon receptor (Novus Biologicals, Littleton, CO, USA) diluted at 1:1,000 in PBS (-) containing 1% BSA. After overnight incubation at 4°C, cells were stained with AlexaFluor488-conjugated goat antibody to rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted at 1:400 and TOTO-3 iodide (Invitrogen) diluted at 1:1,500 in PBS (-) containing 1% BSA at room temperature for 30 minutes. After washing three times with PBS (-), cells were covered with coverslips and fluorescence mounting medium (Dako, Santa Clara, CA, USA). Fluorescence images were captured using a FluoView 1000 confocal microscope (Olympus).

RNA extraction and real-time PCR. Total RNA was extracted from treated and control cells preserved in RNeasy[®] solutions (Life Technologies, Carlsbad, CA, USA) using RiboPureTM RNA Purification Kit (Thermo Fisher Scientific) or RNA extraction kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. Messenger RNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNATM Kit (Applied Biosystems). Expression of each gene [aryl hydrocarbon receptor (*AHR*), cyclin D1 (*CCND1*), *CCND2*, v-myc avian myelocytomatosis viral oncogene homolog (*MYC*), B-cell CLL/lymphoma 2 (*BCL2*), *BCL2* associated X protein (*BAX*), myeloid cell leukemia 1 (*MCL1*) and actin, beta (*ACTB*)] were evaluated in triplicate wells using StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with Fast SYBR[®] Green Master Mix (Applied Biosystems). The primers sequences are shown in Table II. Relative expression of each gene was normalized with the level of *ACTB* and calculated using a comparative Ct method.

DNA microarray. Global gene expression profiles were evaluated using SurePrint G3 Human GE Microarray 8x60K v2 (Agilent Technologies, Santa Clara, CA, USA). The profiles were functionally analyzed using the online DAVID Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, USA) (13).

Statistical analysis. Student's *t*-test was used to statistically compare two independent samples. *p*-Values less than 0.05 were considered a statistically significant difference.

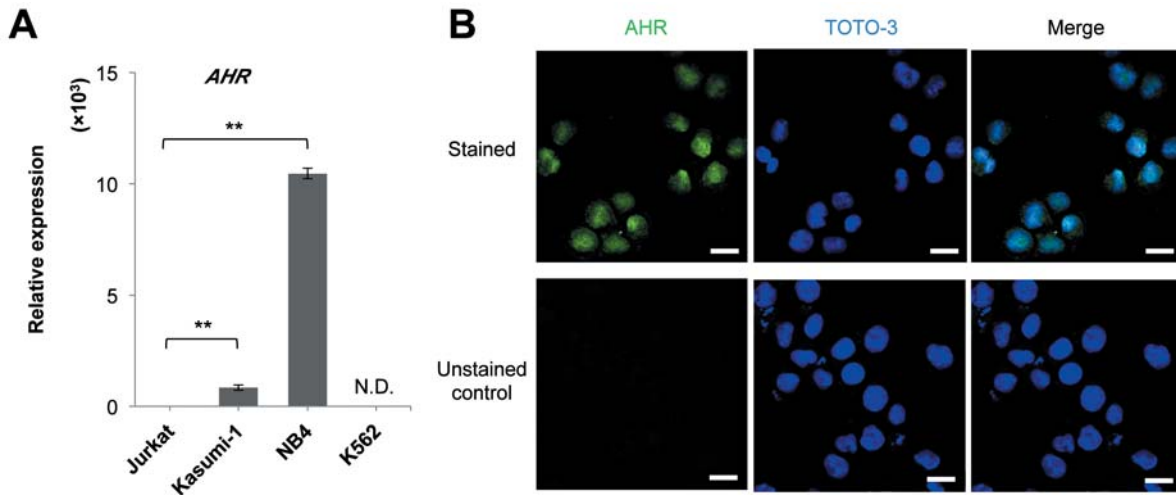


Figure 1. Gene and protein expression of aryl hydrocarbon receptor (AHR) in leukemia cell lines. A: Relative expression of AHR mRNA in the four leukemia cell lines Jurkat, Kasumi-1, NB4, and K562 as measured by real-time polymerase chain reaction. N.D.: Not detected, $**p < 0.01$. B: Immunocytochemical images of AHR protein (green) in NB4 cells. Nucleic acid is stained with TOTO-3 iodide (blue). Scale bar=20 μ m.

Table II. Primer sequences used in this study.

Genes	Encoded protein	Forward primer	Reverse primer
Aryl hydrocarbon receptor (AHR)	Aryl hydrocarbon receptor	5'-GCAGAAAACAGTAAAGCCAATCC-3'	5'-GCTAGCCAAACGGTCCAATC-3'
Cyclin D1 (CCND1)	G ₁ /S-specific cyclin-D1	5'-CCACAGATGTGAAGTTCATTTCCA-3'	5'-AAGCGTGTGAGGCCGGTAGTAG-3'
Cyclin D2 (CCND2)	G ₁ /S-specific cyclin-D2	5'-TTCTTCTTCCAAATGCAGTTCATT-3'	5'-TGCCTCCGTTTCATGTGAGTT-3'
v-myc Avian myelocytomatosis viral oncogene homolog (MYC)	myc Proto-oncogene protein	5'-AAAACCAGCAGCCTCCCG-3'	5'-GCTCTGCTGCTGCTGCTG-3'
B-Cell CLL/lymphoma 2 (BCL2)	Apoptosis regulator BCL2	5'-ATCGCCCTGTGGATGACTGAG-3'	5'-CAGCCAGGAGAAATCAAACAGAGG-3'
BCL2-associated X protein (BAX)	Apoptosis regulator BAX	5'-GGACGAACTGGACAGTAACATGG-3'	5'-GCAAAGTAGAAAAGGGCGACAAC-3'
Myeloid cell leukemia 1 (MCL1)	Induced myeloid leukemia cell differentiation protein Mcl-1	5'-CGGGCAAATCCTCCAAAAG-3'	5'-CCCTGAGAGAAGCGTAAGACAAA-3'
Actin, beta (ACTB)	Actin, cytoplasmic 1	5'-ATTGCCGACAGGATGCAGA-3'	5'-GAGTACTTGCGCTCAGGAGGA-3'

Results

AHR gene and protein are expressed in NB4 cells. To identify leukemia cell lines that express AHR at a high level, we evaluated the expression of AHR mRNA in four leukemia cell lines (Jurkat, Kasumi-1, NB4 and K562) by real-time PCR. As shown in Figure 1A, NB4 cells expressed a significantly higher level of AHR compared to Jurkat (1.04×10^4 -fold, $p = 0.00002$) and Kasumi-1 (12.4-fold, $p = 0.0000096$) cells. We did not detect any amplification in K562 cells. To confirm the AHR expression in the NB4 cell

line at the protein level, immunocytochemical analysis of cultured NB4 cells was performed. In the NB4 cell line, AHR was expressed and localized in the nucleus and cytoplasm (Figure 1B).

SR1 promotes cell proliferation and reduces cell death of NB4 cells. Next, we investigated whether SR1 promotes the proliferation of the NB4 cell line. NB4 cells were treated with three different concentrations of SR1 and seeded at 5.0×10^5 cells/ml of cell density (day 0). After culturing for 48 h (day 2), the numbers of live and dead cells in each condition were

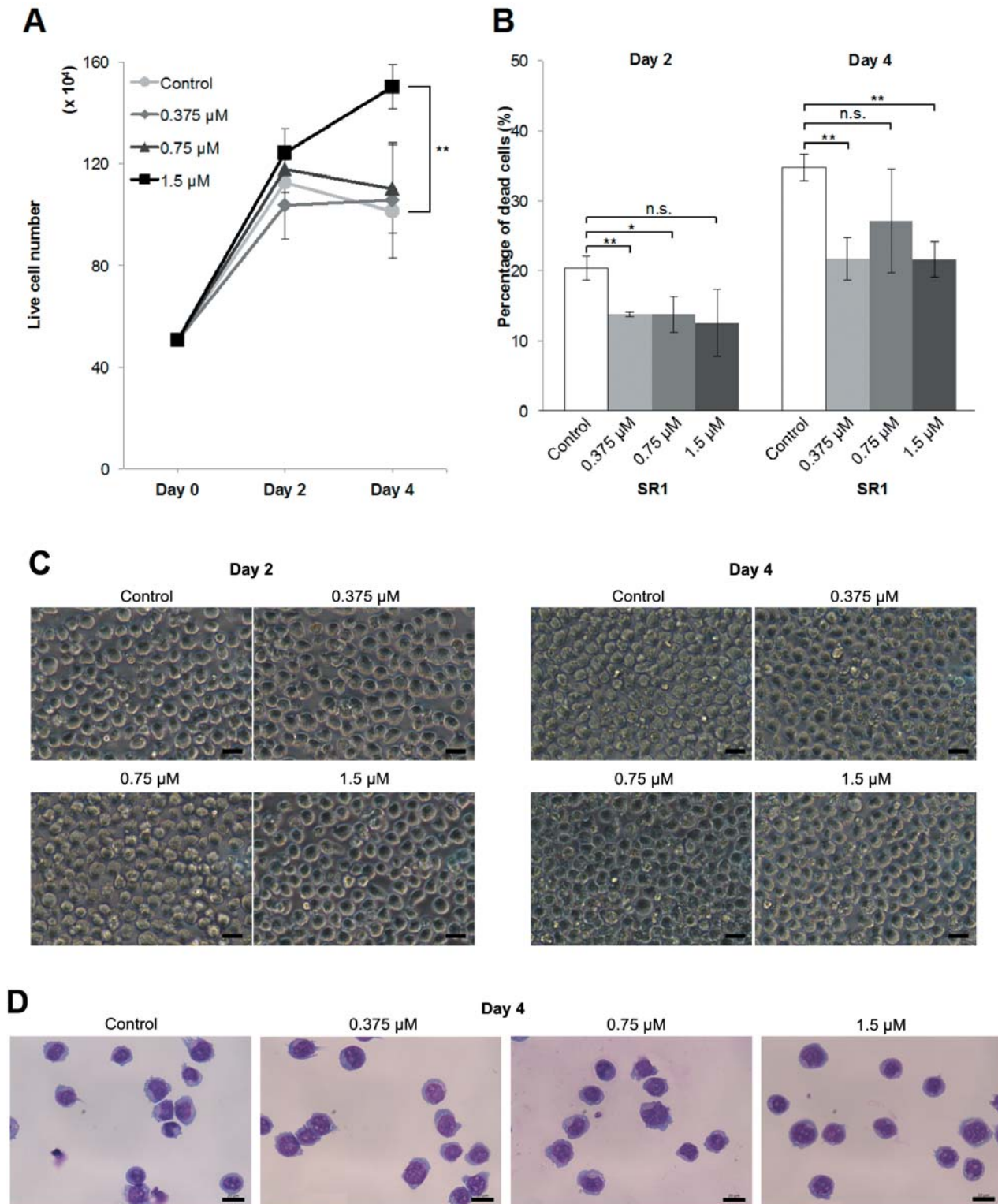


Figure 2. Effect of StemRegenin 1 (SR1) on cell proliferation and death of NB4 cells. NB4 cells were cultured in the presence of three different concentrations of SR1 [0.375 μM, 0.75 μM and 1.5 μM containing 0.1% dimethyl sulfoxide (DMSO)] or 0.1% DMSO only (control) for up to 4 days. A: The number of viable cells was determined at days 0, 2 and 4. $**p < 0.01$. B: The percentage of dead cells at day 2 and 4 of culture was determined. n.s.: Not significant, $*p < 0.05$, $**p < 0.01$. C: Images of the cells under the microscope at day 2 and 4 of culture. D: Images of May-Grünwald Giemsa staining at day 4 of culture. Scale bar=20 μm.

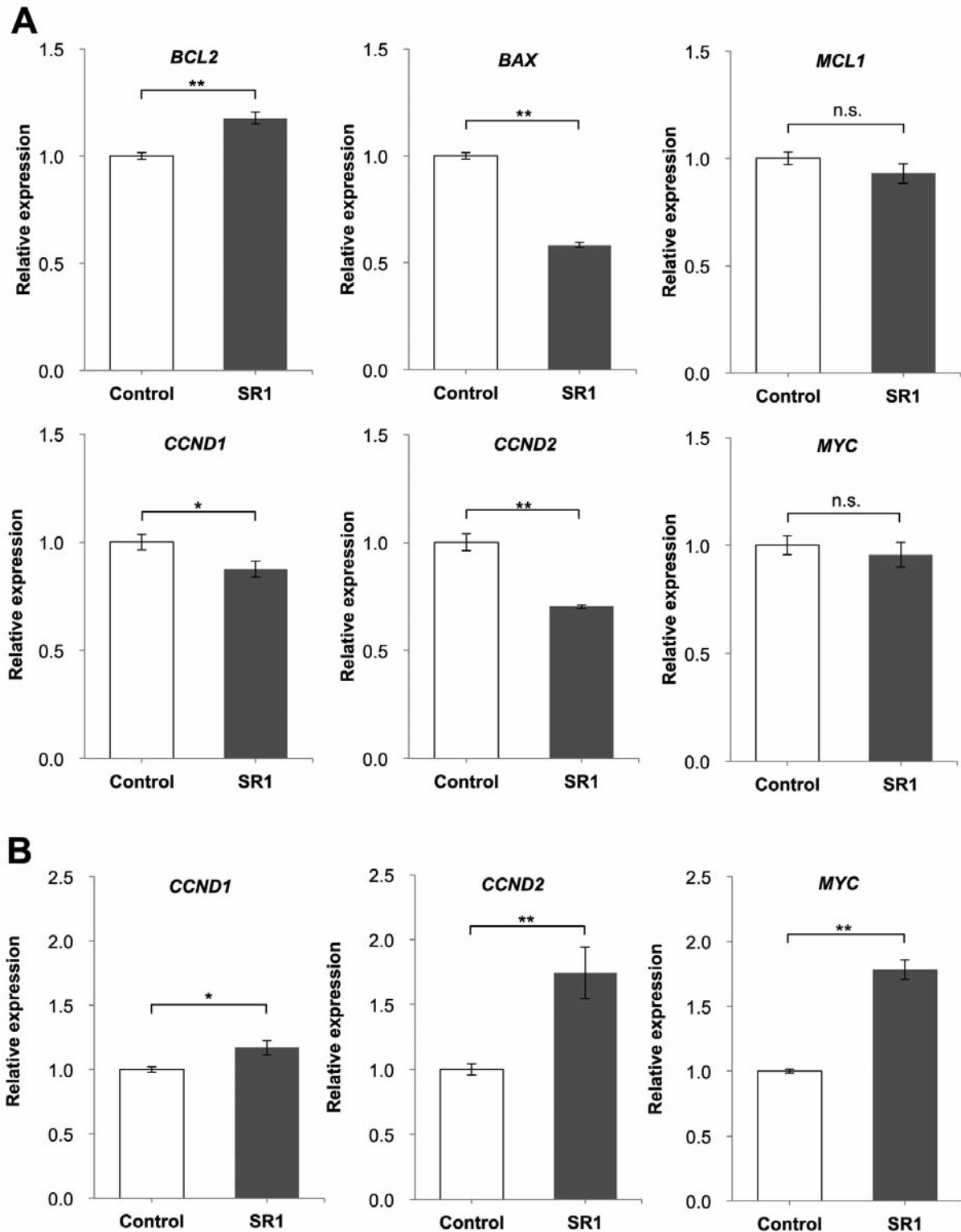


Figure 3. StemRegenin 1 (SR1) up-regulated cell proliferation- and apoptosis-related genes in the NB4 cell line. Relative expression of B-cell CLL/lymphoma 2 (*BCL2*), BCL2 associated X protein (*BAX*), myeloid cell leukemia 1 (*MCL1*), cyclin D1 (*CCND1*), *CCND2* and v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) mRNAs of NB4 cells cultured with 1.5 μ M SR1 containing 0.1% DMSO, or 0.1% DMSO only (control) were measured by real-time polymerase chain reaction. A: Expression of *BCL2*, *BAX*, *MCL1*, *CCND1*, *CCND2* and *MYC* at day 2. B: Expression of *CCND1*, *CCND2* and *MYC* at day 4. n.s.: Not significant, * $p < 0.05$, ** $p < 0.01$.

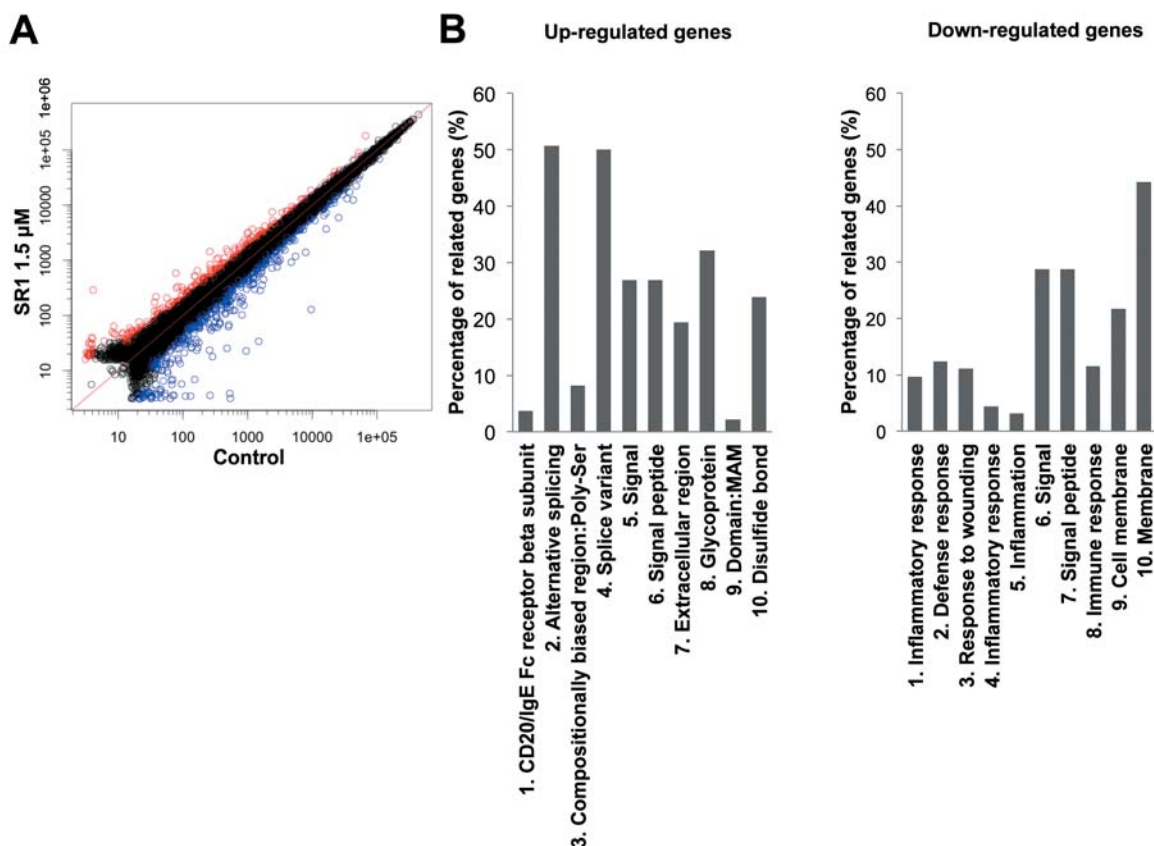


Figure 4. Effect of StemRegenin 1 (SR1) on gene-expression profiles of the NB4 cell line. A: Scatter plot of DNA microarray comparing the signals in control with those in NB4 cells treated with SR1 for 4 days. Significantly up- (red) and down- (blue) regulated genes are indicated. B: Gene functional annotation using the DAVID software. The up- (left) and down- (right) regulated genes functional annotations with the top 10 are shown in order of significant difference from those of the control.

counted. As shown in Figure 2A, no significant difference between treated and control cells was observed, however, the number of live cells cultured with 1.5 μM SR1 tended to be higher compared to the control (1.10-fold, $p=0.186$). Figure 2B indicates that the percentage of dead cells when cultured in the presence of 0.375 μM and 0.75 μM SR1 decreased compared to that of the control, 0.68-fold ($p=0.005$) and 0.75-fold ($p=0.04$) respectively. Under treatment with 1.5 μM SR1 for 2 days, the percentage of dead cells tended to be lower (0.61-fold, $p=0.09$) compared to the control. We further extended the period of culture to 96 h (day 4) to follow the effect of SR1. The number of live cells when treated with 1.5 μM SR1 significantly increased (1.5-fold, $p=0.005$) compared to the control, whereas those treated with 0.375 μM and 0.75 μM did not significantly differ (Figure 2A). As shown in Figure 2B, the percentage of dead cells when cultured with 0.375 μM and 1.5 μM SR1 significantly decreased by 0.62-fold ($p=0.007$) and 0.62-fold ($p=0.004$), respectively. These data imply that *in vitro* culture with SR1 promotes cell proliferation and reduces cell death of NB4 cells. The NB4 cells were also

observed under a microscope (Figure 2C) and there was no obvious difference between control and SR1-treated cells. May-Grünwald Giemsa staining confirmed that there was no obvious difference in size, shape, nuclear/cytoplasm ratio and color of the nucleus and cytoplasm (Figure 2D).

Expression of proliferation- and apoptosis-related genes in NB4 cells. In order to evaluate the effect of SR1 on the expression of proliferation- and apoptosis-related genes, expression of *CCND1*, *CCND2*, *MYC*, *BCL2*, *BAX* and *MCL1* were measured by real-time PCR. The gene expression in NB4 cells cultured for 2 days with 1.5 μM SR1 was compared to those of the control. On day 2, the apoptosis-related gene *BCL2* was up-regulated in cells treated with 1.5 μM SR1 compared to the control (1.18-fold, $p=0.0014$), whereas the expression of *BAX* was down-regulated (0.58-fold, $p=0.00012$) (Figure 3A, upper left and center panels). There was no significant difference in *MCL1* expression (Figure 3A, upper right panel). For the proliferation-related genes, expression of *CCND1* and *CCND2* was down-regulated in the

presence of 1.5 μ M SR1 (0.87-fold, $p=0.027$ and 0.70-fold, $p=0.0022$, respectively), and no significant difference was observed regarding *MYC* expression (Figure 3A, lower panels). Expression of proliferation-related genes *CCND1*, *CCND2*, and *MYC* were also analyzed in cells cultured for 4 days with or without 1.5 μ M SR1 (Figure 3B). On day 4, all of the cell proliferation-related genes were highly expressed in the cells treated with 1.5 μ M SR1 compared to the control: *CCND1*: 1.17-fold, $p=0.015$; *CCND2*: 1.74-fold, $p=0.007$; and *MYC*: 1.78-fold, $p=0.00014$.

Global gene expression profiles in NB4 cells treated with SR1. In order to further investigate the effects of SR1 on expression of cell proliferation-, apoptosis- and AHR pathway-related genes, a DNA microarray analysis was performed on cells cultured for 4 days with 1.5 μ M SR1 or without SR1 as control. In the scatter plot shown in Figure 4A, blue circles indicate significantly down-regulated genes, and red circles indicate up-regulated genes in the SR1 treated cells compared to the control. Among the significantly down-regulated genes, cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) was reduced the most. Functional annotations of these genes were also analyzed using the DAVID software. The functional annotations of up- and down-regulated genes with the top 10 most significantly different are shown in Figure 4B, and the up- and down-regulated genes functional annotation clusters with the highest significant difference are indicated in Table III. Up-regulated genes were mainly annotated as being related to “alternative splicing”, “splicing variants” and “signal”. Down-regulated genes were mainly annotated as being related to “inflammatory response”, “signal” and “membrane”.

Discussion

Here, we reported that the leukemia cell line NB4 proliferates in response to *in vitro* SR1 administration. As we showed, NB4 cells expressed *AHR* at a relatively higher level compared to other leukemia cell lines. An increase in viability and reduction in cell death in the presence of SR1 and a change in expression of proliferation- and apoptosis-related genes was also demonstrated. It was reported that the AHR signaling pathway plays an important role in proliferation and differentiation of cancer cells. For instance, dioxin, an agonist of AHR, induces lung cancer (14). SR1 is also an antagonist of AHR pathway and reportedly inhibits the differentiation of acute myeloid leukemia cells (11).

As we show in Figure 1A, the expression level of *AHR* in different types of leukemia differed greatly. The level of *AHR* was relatively high in both NB4 and Kasumi-1 cells, that were derived from acute myeloid leukemia, compared to Jurkat and K562 cells, which were derived from lymphoid and chronic myeloid leukemia respectively, suggesting *AHR*

Table III. *Functional annotation clusters of the most significantly up- and down-regulated genes.*

Functional clusters	
Up-regulated genes	Down-regulated genes
Enrichment score=2.59	Enrichment score=7.55
Signal	Inflammatory response
Signal peptide	Defense response
Glycoprotein	Response to wounding
Disulfide bond	Immune response
Glycosylation site: N-linked	

expression depends on type and origin of leukemia (15-18). Although both NB4 and Kasumi-1 cell lines are derived from acute myeloid leukemia, the classification of acute leukemia cells also differs between NB4 and Kasumi-1; NB4 is classified as M3 acute promyelocytic leukemia, and Kasumi-1 as M2 acute myeloblastic leukemia with maturation according to the French-American-British classification (16, 17, 19). The differentiation stage of the leukemia cell line likely changes the expression of *AHR*.

Although the expression of *CCND1* and *CCND2* decreased in the presence of 1.5 μ M SR1, the number of live cells did not significantly differ between control and SR1-treated cells at day 2. However, there was an increase in the live cell number at day 4 of culture with SR1, which is supported by up-regulation of the cell proliferation-related genes *CCND1*, *CCND2*, and *MYC* in the same period. It has also been reported that the effect of SR1 on HSC expansion requires longer than 7 days (4). These results imply that the onset of the effect of SR1 on cell proliferation occurs after a relatively long period of incubation.

A reduction in the percentage of dead NB4 cells in the presence of SR1 was also observed (Figure 2B). Considering the effect of SR1 on apoptosis-related genes, the level of *BCL2* gene expression was relatively higher than that of control cells and the *MCL1* gene expression was not significantly different on day 2 (Figure 3A, upper panels), implying that SR1 has little effect on the survival of NB4 cells. Furthermore, the relatively low level of *BAX* expression on day 2 implies that apoptosis of NB4 cells was inhibited in the presence of SR1.

Since AHR is a ligand-regulated transcription factor (7), global gene expression was analyzed and annotated using DAVID software in order to understand the effect of SR1 on the proliferation of leukemia cells. We used NB4 cells cultured with 1.5 μ M SR1 for 4 days and an untreated control. The majority of the genes up-regulated were annotated as “splice variants” (Figure 4B). This finding corresponds to previous reports showing that RNA splicing is increased in acute

myeloid leukemia (20,21). However, it remains unclear how splice variants affect the proliferation status of leukemia cells.

AHR is reported to directly modulate inflammatory signaling (7), and consistent with this, SR1-mediated down-regulated genes were annotated as being related to “inflammatory response”. Moreover, among the down-regulated genes, *CYP1B1*, which encodes cytochrome *P450* *1B1* (one of the enzymes belonging to cytochrome *P450* superfamily metabolizing procarcinogens), was most significantly reduced and is known to be a target gene of AHR (22), thus its down-regulation implies that SR1 antagonized AHR activity in NB4 cells. In addition, among the AHR pathway-related genes, the expressions of *AHRR*, which encodes aryl hydrocarbon receptor repressor, and *HSP90AA1*, which encodes heat-shock protein 90 α (the stress-inducible isoform of molecular chaperon HSP90 forming a cytosolic core complex with AHR), were reduced (23-25). According to microarray and DAVID analysis, genes negatively regulating proliferation were down-regulated, which is compatible with the real-time PCR data shown in Figure 3B.

Our findings confirm the effect of SR1 on proliferation of acute promyelocytic leukemia cells, and additionally reveal that the global gene expression changes especially in proliferation, apoptosis and the AHR pathway. This study could lead to more appropriate use of SR1 in the expansion of HSCs to apply for clinical treatment.

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