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Inhibition of all-*trans* retinoic acid-induced granulocytic differentiation of WEHI-3B D⁺ cells by forced expression of SCL (TAL1) and GATA-1

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Abstract

All-*trans* retinoic acid (ATRA) induces granulocytic maturation of WEHI-3B D⁺ leukemia cells and LiCl enhances this maturation, while WEHI-3B D[−] cells are non-responsive to ATRA. Transfection of SCL, expressed in D[−] but absent in D⁺ cells, into D⁺ cells, caused resistance to ATRA, while transfection of GATA-1 into D⁺ cells produced resistance to the combination of ATRA and LiCl. SCL expression in D⁺ cells did not induce the expression of *c-Kit*, a putative target gene for SCL. LiCl, known to inhibit some kinases by displacing Mg²⁺, did not affect tyrosine kinase activity of the cytoplasmic domain of c-Kit.

Keywords

WEHI-3B D⁺; WEHI-3B D[−]; SCL (TAL1); GATA-1; All-*trans* retinoic acid (ATRA); Lithium chloride (LiCl); c-Kit

1. Introduction

The WEHI-3B cell line, established from the myelomonocytic leukemia developed in a mineral oil injected BALB/c mouse [1], exhibited differentiation-responsiveness to granulocyte-colony stimulating factor (G-CSF). Subsequently, Metcalf and Nicola [2] isolated a variant of WEHI-3B cells that was hyperdiploid and no longer responsive to G-CSF; the original and the variant thereof were designated as WEHI-3B D⁺ and WEHI-3B D[−], respectively. Further studies in our laboratory demonstrated that WEHI-3B D⁺ cells were responsive to ATRA induced granulocytic maturation [3] and that ATRA with either G-CSF or LiCl produced synergistic differentiation [4–6]. In contrast, WEHI-3B D[−] cells were non-responsive to ATRA [7].

The induction of differentiation as a therapeutic intervention was conceived upon the premise that a maturation block imposed in tumor cells by the carcinogenic event is not completely irreversible and that conversion of malignant cells to mature end-stage forms lessens their proliferative and invasive capacity [8,9]. This antineoplastic strategy has culminated in the

successful use of ATRA as a primary treatment for patients with acute promyelocytic leukemia (M3 AML) [10]. Although application of ATRA-based differentiation therapy is currently limited to M3 AML, a recent phase I clinical trial with the retinoic $\times$ receptor agonist bexarotene has shown that this retinoid exhibits antileukemic activity in patients with a certain subtype of non-M3 AML, suggesting the utility of retinoid-based differentiation therapy in non-M3 AML as well [11]. Thus, murine WEHI-3B D⁺ myelomonocytic leukemia cells responsive to ATRA and WEHI-3B D⁻ cells non-responsive to the retinoid represent a useful non-M3 AML model to study the molecular mechanism(s) of sensitivity/resistance to the retinoids.

WEHI-3B D⁻ cells differ from D⁺ cells with respect to DNA ploidy with D⁻ cells being near-tetraploid and D⁺ cells near-diploid [2,12]. Experiments with fused D⁺ and D⁺ cells have indicated that polyploidy *per se* is not responsible for causing resistance to ATRA, while experiments using fused D⁺ and D⁻ cells have suggested the presence of a repressor(s) of ATRA induced differentiation in D⁻ cells [12]. We have identified SCL, a transcription factor expressed in D⁻ but absent in D⁺ cells [13], as one such repressor [12]. Although expression of SCL in D⁺ cells produces resistance to ATRA-induced differentiation, the resistance to ATRA is overcome by co-exposure to LiCl in these cells [14], whereas D⁻ cells are resistant to both ATRA alone and in combination with LiCl. Therefore, D⁻ cells would appear to contain an additional repressor(s) to express resistance to the combination of both agents. In this report, GATA-1, another transcription factor detected in D⁻ cells at the level of mRNA but not in D⁺ cells [13], was transfected into D⁺ cells and the effects on responsiveness to ATRA and LiCl were compared to those of SCL.

The *SCL* gene, discovered because of recurrent involvement in chromosomal translocations in human T-cell ALL [15], encodes a basic helix–loop–helix transcription factor essential for the generation of all types of hematopoietic lineages [16,17]. Despite numerous reports on SCL, questions as to whether (a) the chromosomal translocation *t*(1:14) leads to activation of the *SCL* gene and (b) *SCL* is an oncogene in transgenic mice remain incompletely answered. Furthermore, the mechanism by which SCL exerts indispensable roles in hematopoiesis are largely unknown. This is in part due to technical difficulties in accurately detecting SCL expression. In this report, we have conducted comprehensive analyses on SCL expression in a variety of cell lines at the levels of both mRNA and protein.

In addition, based upon the observations that the tyrosine kinase receptor *c-Kit* is a target gene for SCL [18,19] and that LiCl inhibits several kinases, including glycogen synthase kinase-3 β by displacing Mg²⁺ [20], we have explored the possibilities that c-Kit acts as an inhibitor of ATRA-induced differentiation downstream of SCL and that LiCl circumvents SCL-induced resistance to ATRA through inhibition of c-Kit.

2. Materials and methods

2.1. Cell lines and differentiation assay

The WEHI-3B D⁺ and WEHI-3B D⁻ cell lines were gifts of Dr. Malcolm A. Moore; they were maintained in McCoy's 5A medium containing 10% fetal bovine serum (FBS). Suspension cell lines, including K-562, U-937, HL-60, NB4, Raji, F-MEL, L1210 and P388, were maintained in RPMI-1640 medium containing 10% FBS. The maintenance of Ba/F3 cells was previously described [21]. Attached cell lines, including HeLa S3, HCT 116, A549, DU 145, EMT6, NRK and CHO, were maintained in McCoy's 5A medium containing 10% FBS. For induction of differentiation, WEHI-3B cells at an initial density of 7×10^4 cells/ml were incubated with 5 μ M ATRA, 5 mM LiCl, or the combination of these agents for 3 days. ATRA was dissolved in absolute anhydrous ethanol at a concentration of 10 mM and 2 μ l of this stock solution was directly added to 4 ml of cell suspension. Ethanol at the final concentration of 0.05% caused neither cytotoxicity nor differentiation in WEHI-3B D⁺ and D⁻ cells. At the end

of treatment, adherent cells were scraped from the surface of culture flasks using a cell scraper. Differentiation was assessed using nitroblue tetrazolium (NBT) assay [22]; we have previously established a direct relationship between the ability to reduce NBT and the capacity to express neutrophil cell surface markers such as Mac-1 antigen and FcγII/III receptor in differentiated WEHI-3B D⁺ cells [4,6,23]. Statistical significance was analyzed by an unpaired two-tailed *t*-test using Prism software.

2.2. SCL and GATA-1 expression plasmids and transfection

Murine *SCL* cDNA was the gift of Dr. C. Glenn Begley and the 990 bp coding region was inserted into pCRII-TOPO by TA cloning (Invitrogen, Carlsbad, CA). The 1242 bp coding region of murine GATA-1 cDNA was generated from F-MEL RNA by reverse transcription (RT)-PCR using the Titan One-Tube RT-PCR system (Roche Diagnostics, Indianapolis, IN) and inserted into pCRII-TOPO by TA cloning. The coding sequences of *SCL* and GATA-1 were subcloned into mammalian expression vectors, p75/15 containing the human metallothionein IIA (MT) promoter [23] and pcDNA3.1 (+) containing the CMV promoter (Invitrogen) using *Bam*HI (5') and *Xba*I (3') sites. All fragments generated by PCR were verified by DNA sequencing. Transient transfection of *SCL* and GATA-1 expression plasmids into NRK and CHO cells was carried out in 6-well plates as described previously [24]. Following transfection, cells were incubated for 20 h to allow expression in the absence or presence of the gene activator, i.e., 10 nM trichostatin A for the CMV promoter [24] and 80 μM ZnSO₄ for the human metallothionein IIA promoter [25]. WEHI-3B D⁺ cells were transfected with p75/15 containing the coding sequence of *SCL* or GATA-1 by electroporation as described previously [26]. Transfectants were selected with 0.8 mg/ml of G418 either in semi-solid medium containing 0.8% methylcellulose or liquid medium.

2.3. Western blot analyses

Whole cell extracts were prepared by washing cells with Hanks' balanced salt solution (HBSS) and solubilizing 5×10^6 cells in 0.25 ml of 2× Laemmli's sample buffer at 100°C for 5 min [6]. Cytoplasmic extracts were prepared by washing cells with HBSS and solubilizing 5×10^6 cells in 50 μl of a lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 0.5% (for attached cells) or 0.1% (for suspension cells) Triton X-100, 10 mM MgCl₂, 30 mM KCl and 100 μg/ml of phenylmethanesulfonylfluoride. Cell lysates were cleared by centrifugation at 16,000 × *g* for 2 min. Twenty micro-liters per lane of the whole cell extract (equivalent to 4×10^5 cells) or the cytoplasmic extract (equivalent to 10^6 cells) was subjected to 0.1% SDS/7.5% polyacrylamide gel electrophoresis. Anti-*SCL* antibody (AF3360) was from R&D Systems (Minneapolis, MN). Antibodies for GATA-1 (sc-266), GATA-2 (sc-9008), c-Kit (sc-1494) and HSC 70 (sc-1059) were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. In vitro assays using purified c-Kit and Lyn kinases

The effects of LiCl and imatinib on tyrosine kinase activities of the cytosolic domain of c-Kit (Calbiochem, La Jolla, CA) and Lyn B (Sigma, St. Louis, MO) were determined using the SignaTECT assay kit (Promega, Madison, WI). Briefly, c-Kit (0.28 units/ml) or Lyn (0.39 units/ml) were equilibrated at 25 °C in 13.3 mM imidazole-HCl (pH 7.3) buffer containing 10 mM MgCl₂, 0.5 mM MnCl₂, 13.3 mM glycerophosphate, 0.2 mM EGTA, and 0.1 mg/ml of BSA in the presence of 100 μM ATP with 50 μCi/ml of [γ -³²P]ATP and 0–20 mM inhibitor. The reaction for c-Kit (30 min) and Lyn (5 min) were initiated with 500 μM biotinylated peptide substrate #1 and 125 μM biotinylated peptide substrate #2, respectively. Activities were normalized to positive and negative controls and expressed as fractional activity. IC₅₀ values were calculated by fitting the data to the following equation: $1/(1 + (x/a)^b)$, where 'x' refers to the fractional activity and 'a' is solved as the IC₅₀ value.

2.5. Analyses of mRNAs for SCL and GATA-1 by northern hybridization and RT-PCR

Total cellular RNA was extracted from 7×10^6 attached cells or 10^7 suspension cells using a Trizol reagent (Invitrogen). Poly A⁺ RNA was purified from total cellular RNA using an Oligotex mRNA Spin-column (QIAGEN, Valencia, CA). Total cellular RNA and poly A⁺ RNA (equivalent to 1.4×10^6 attached cells/lane or 2×10^6 suspension cells/lane) were subjected to non-radioactive northern analyses using digoxigenin-labeled antisense RNA probes made from the 990 bp SCL and 1242 bp GATA-1 coding regions [24]. Semi-quantitative RT-PCR was carried out using 1 μ g of total cellular RNA and the Titan One-Tube RT-PCR system (Roche). Forward (F) and reverse (R) primers were designed to include an exon/intron boundary indicated by a slash (/) except for murine GAPDH R-primer; (F) 5'-CTCTCGGCAG/CGGGTTCT-3' and (R) 5'-GTGGGGAC/CATCAGTAATC-3' for human SCL 113 bp product, gene ID 6886; (F) 5'-CACTAGGCAG/TGGGTTCT-3' and (R) 5'-GTGAGGAC/CATCAGAAATC-3' for murine SCL 113 bp product, gene ID 21349; (F) 5'-GATTCAACGG/ATTTGGTTCG-3' and (R) 5'-GGATCTCGC/TCCTGGAAG-3' for human GAPDH 226 bp product, gene ID 2597; (F) 5'-GTGTGAACGG/ATTTGGCCG-3' and (R) 5'-GCTTCCCGTTGATGACAAGC-3' for murine GAPDH 198 bp product, gene ID 14433. RT-PCR products were analyzed by 3% MetaPhor agarose (Cambrex Bio Science, Rockland, ME) gel electrophoresis using a low molecular weight DNA ladder (New England BioLabs, Beverly, MA) as a molecular weight marker.

3. Results

3.1. Transient transfection of SCL and GATA-1 expression plasmids into NRK and CHO cells

To enable western blot analyses for SCL and GATA-1, the specificity of the antibodies for SCL (AF3360, R&D Systems) and GATA-1 (sc-266, Santa Cruz) was determined by transfecting the expression plasmids for SCL and GATA-1 controlled by the CMV promoter (Fig. 1A) or by the human metallothionein IIA promoter (Fig. 1B) into non-hematopoietic normal rat kidney (NRK) and Chinese hamster ovary (CHO) cells. The expression of SCL and GATA-1 was dependent upon transfection of the expression plasmids and stimulated by activators of the respective promoter, i.e., the histone deacetylase inhibitor trichostatin A (TSA) for the CMV promoter [24] and the heavy metal ion Zn²⁺ for the metallothionein promoter [25]. These results verified both the specificity of the anti-SCL and anti-GATA-1 antibodies and the integrity of the expression plasmids for SCL and GATA-1.

GATA-1 (the molecular weight of 42,543 Da deduced from the murine cDNA) exhibited amigration of the expected molecular size, while SCL (the molecular weight of 34,279 Da deduced from the murine cDNA) migrated as a polypeptide of ~42 kDa, consistent with previous observations [27,28].

3.2. Stable transfection of SCL and GATA-1 expression plasmids into WEHI-3B D⁺ cells

To determine whether the expression of SCL and GATA-1 is involved in the resistance of WEHI-3B D⁻ cells to ATRA-induced differentiation, plasmid DNA harboring these genes controlled by the human metallothionein IIA promoter was transfected into ATRA sensitive WEHI-3B D⁺ cells, and G418-resistant clones and mixed populations of G418-resistant cells were propagated in semi-solid medium and in liquid medium, respectively (Fig. 2). SCL protein was detected in WEHI-3B D⁻ cells but not in WEHI-3B D⁺ cells and transfection allowed the expression of SCL in D⁺ cells at levels equal to or higher than that in D⁻ cells (Fig. 2A). GATA-1 protein was not detected in D⁻ or D⁺ cells and GATA-1 expression was achieved in D⁺ cells after transfection (Fig. 2B). As discussed subsequently, it was necessary to purify poly A⁺ RNA to perform northern analyses for SCL. The northern analyses demonstrated that the levels of mRNAs for SCL and GATA-1 were roughly related to the levels of the respective proteins. Contrary to a previous report [13], GATA-1 mRNA was not detected in WEHI-3B

D⁻ cells. Variability in the expression of SCL and GATA-1 in clonal transfectants (S1–S5 and G1–G5) presumably reflects heterogeneity in the integration of the plasmid DNA into genomic DNA.

Table 1 summarizes the effects of transfection of SCL and GATA-1 into D⁺ cells on their responsiveness to ATRA, LiCl, and the combination thereof. ATRA at a concentration of 5 μ M produced a moderate level of differentiation in non-transfected D⁺ cells, whereas LiCl at a concentration of 5 mM, which was ineffective in producing differentiation by itself, significantly enhanced the sensitivity to ATRA. In contrast, non-transfected D⁻ cells were non-responsive to ATRA alone, as well as in combination with LiCl. Empty vector transfected D⁺ cells (V1 and Vm) were comparable to D⁺ cells in their response to these agents, indicating that transfection *per se* did not alter the responsiveness to these agents. In general, SCL transfectants S1 to S5 displayed reduced responses to ATRA except for S1, with the highest SCL expressor S3 displaying the lowest response to ATRA. Consistent with our previous report [14], addition of LiCl to the ATRA treatment resulted in a degree of differentiation of all of the SCL transfectants equivalent to that of non-transfected D⁺ cells. In contrast, the highest GATA-1 expressors (G2 and G3) were not only considerably less sensitive to ATRA-induced differentiation, but also resistant to the combination of ATRA and LiCl (Table 1).

3.3. Relationship between the expression of SCL and c-Kit

If *c-Kit* were a target gene for SCL, transfection of SCL might be expected to induce the expression of this kinase in WEHI-3B D⁺ cells. The levels of c-Kit in cytoplasmic extracts from non-transfected D⁺, non-transfected D⁻, and SCL-transfected D⁺ cells measured by western analyses were negligible, regardless of the status of SCL expression (data not shown). To further analyze the relationship between the expression of SCL and c-Kit, the levels of the two proteins were measured in a variety of non-transfected cell lines (Fig. 3). K-562 cells overexpressing SCL was positive for the expression of c-Kit, while Raji cells lacking SCL expressed c-Kit. In addition, five out of six murine hematopoietic cell lines (F-MEL, Ba/F3, WEHI-3B D⁻, L1210 and P388) were positive for SCL expression and none expressed c-Kit (Fig. 3A). Moreover, c-Kit was expressed in human carcinoma cell lines such as HeLa S3, HCT 116 and DU 145 cells, which did not express SCL (Fig. 3B). The levels of GATA-1 and GATA-2, the potential transcriptional complex partners of SCL [19], were also determined in hematopoietic cell lines and the findings are summarized in Fig. 3A (bottom panel). The spectra of expression of SCL, GATA-1, GATA-2 and c-Kit in various human and murine hematopoietic cell lines did not correlate with each other.

3.4. Effects of LiCl and imatinib on tyrosine kinase activities of c-Kit and Lyn *in vitro*

Since Li⁺ competes with Mg⁺ in the active site of glycogen synthase kinase-3 [29], the effects of LiCl on the catalytic activities of c-Kit and Lyn, the Src family member of tyrosine kinase downstream of c-Kit [30], were determined *in vitro* using imatinib as a reference inhibitor. LiCl at a concentration of 20 mM did not significantly inhibit phosphorylation of the peptide substrates of c-Kit and Lyn under conditions where imatinib inhibited c-Kit and Lyn with IC₅₀ values of 0.32 and 5 μ M, respectively. The latter value was unexpected, since the reported IC₅₀ value of imatinib for Lyn was greater than 100 μ M [31]. LiCl at concentrations as high as 100 mM did not exert significant inhibition of c-Kit and Lyn even at a sub-saturating concentration (6.7 mM) of MgCl₂.

3.5. Levels of mRNA for SCL and GATA-1 analyzed by northern hybridization and by RT-PCR

Total cellular RNA was extracted from various cell lines and northern hybridization was carried out using the coding regions of SCL and GATA-1 as probes. The SCL probe non-specifically hybridized with 28S and 18S ribosomal RNA (Fig. 4A, top panel), whereas the GATA-1 probe employed under comparable conditions produced specific signals, the levels of which

corresponded to the levels of GATA-1 protein determined by western analyses. The SCL probe failed to produce specific signals for SCL mRNA, even when poly A⁺ RNA was used as a source of RNA in northern analyses (Fig. 4A, bottom panel). Finally, semi-quantitative RT-PCR yielded signals of the expected length for human (Fig. 4B, top panel) and murine (Fig. 4B, bottom panel) SCL mRNA. The cell lines positive for SCL mRNA determined by RT-PCR, i.e., K-562, Ba/F3, WEHI-3B D⁻, F-MEL, L1210 and P388 were completely matched with those expressing positivity for SCL protein determined by western analyses (Fig. 3A). A faint expression of SCL was detected in WEHI-3B D⁺ cells by RT-PCR (Fig. 4B, bottom panel). Treatment of untransfected WEHI-3B D⁺ cells with ATRA (5 μ M) or ATRA (5 μ M)/LiCl (5 mM) produced a slight down-regulation of SCL expression (data not shown).

4. Discussion

The generation of a diverse blood-cell repertoire from hematopoietic stem cells is at least in part coordinated by lineage-specific transcription factors [32]. Inappropriate expression of these factors caused by mutations therein or chromosomal translocations is frequently observed in the leukemias [33]. In this report, we have evaluated the effects of the expression of two important hematopoietic transcription factors SCL and GATA-1 on granulocytic differentiation, in a non-M3 AML model consisting of murine WEHI-3B D⁺ myelomonocytic leukemia cells responsive to ATRA and WEHI-3B D⁻ cells non-responsive to the retinoid. The forced expression of SCL and GATA-1 in ATRA sensitive WEHI-3B D⁺ cells interfered with the induction of differentiation by ATRA. The addition of LiCl restored the sensitivity of SCL⁺/D⁺ cells to ATRA, while GATA-1⁺/D⁺ cells not only exhibited resistance to ATRA alone, but also in combination with LiCl. Two GATA binding motifs are present in the proximal promoter of the *SCL* gene [34]. To test the possibility as to whether the stronger inhibition of ATRA-induced differentiation exerted by GATA-1 than by SCL was due to induction of SCL expression in GATA-1⁺/D⁺ cells, SCL levels were determined by RT-PCR. In the higher GATA-1 expressors such as G2, G3 and G4, in which the stronger inhibition of ATRA-induced differentiation occurred (Table 1), SCL expression was somewhat down-regulated (data not shown), making the proposed mechanism unlikely. In addition, although we were not able to confirm the existence of GATA-1 mRNA in WEHI-3B D⁻ cells, these results collectively imply that the composition of transcription factors such as GATA-1 and SCL has a direct impact on susceptibility of leukemic cells to retinoid-induced granulocytic differentiation. Exogenous expression of SCL diminishes responsiveness to ATRA in HL-60 human promyelocytic leukemia cells [14], indicating that the phenomenon is not limited to murine WEHI-3B D⁺ cells.

While gene targeting studies have unequivocally demonstrated that the *SCL* gene is essential for the generation of all blood cell lineages [15,16], the mechanism(s) by which SCL plays indispensable roles in hematopoiesis, including its exact transcription complex partners and its exact target genes, are yet to be uncovered. These studies require methods to accurately detect SCL. In earlier studies, SCL expression has often been analyzed at the level of mRNA by northern hybridization [35–38] and data analyzing SCL at the protein level are scarce, presumably due to the lack of availability of antibodies specific for SCL. In this report, the specificity of the anti-SCL antibody (AF3360, R&D Systems) was confirmed by transiently transfecting SCL expression plasmids into non-hematopoietic NRK and CHO cells that lack the expression of SCL.

In northern hybridization analyses, an SCL probe consisting of the 990 bp coding sequence produced non-specific binding to 28S and 18S rRNA, regardless of whether total cellular RNA or poly A⁺ RNA was used as the source of RNA, presumably due to the high GC content (66%) of the coding region of the *SCL* gene. Therefore, analysis of SCL expression solely by northern hybridization [35–38] could be misleading. Since nascent transcripts for the *SCL* gene have

been shown to be heterogeneous at the 5' end [39], this may explain the lack of discrete signals for SCL mRNA in northern analyses. The problem of detecting SCL mRNA by northern hybridization was overcome by the use of RT-PCR that produced a discrete signal of the expected size for human and murine SCL mRNAs.

K-562 cells over-expressed SCL protein, while other human leukemia cell lines including HL-60, NB4, Raji and U937 cells were negative in SCL expression. In contrast, SCL was expressed in five out of six murine hematopoietic cell lines including Ba/F3 (IL-3-dependent), F-MEL (erythroid), L1210 (lymphoid), P388 (lymphoid) and WEHI-3B D⁻ (myeloid), suggesting that the two species differ in SCL expression. Since semi-quantitative RT-PCR analyses showed that the levels of SCL mRNA are similar in all SCL positive cell lines, SCL may be post-transcriptionally stabilized in K-562 cells. In contrast, F-MEL cells overexpressed GATA-1 at the levels of both mRNA and protein, suggesting that GATA-1 expression is determined at the transcriptional level.

Based upon previous observations by others that *c-Kit* is a target gene for SCL [18,19] and that Li⁺ inhibits some kinases by displacing Mg²⁺ in the catalytic site of the enzymes due to similarity in the ionic radii of the two ions [29], we have postulated that (a) *c-Kit* acts as an inhibitor of differentiation downstream of SCL, and (b) LiCl overcomes SCL-induced resistance to ATRA by inhibiting the tyrosine kinase activity of *c-Kit*. If prediction (a) is correct, co-expression of SCL and *c-Kit* could be expected. Transfection of SCL into WEHI-3B D⁺ cells, however, did not induce *c-Kit* expression. Moreover, SCL positive cell lines, such as WEHI-3B D⁻, Ba/F3, F-MEL, L1210 and P388, did not express *c-Kit*, while SCL negative cell lines, such as Raji, HeLa S3, HCT 116 and DU145, expressed this kinase. Although Krosi et al. [18] reported that forced expression of SCL in Ba/F3 cells induced *c-Kit* ligand-dependent cell growth, non-transfected Ba/F3 cells were positive for SCL expression in our studies. Elefanti et al. [40] have also reported that SCL^{-/-}, SCL^{+/-} and SCL^{+/+} ES cells express comparable amounts of *c-Kit*. Thus, these observations collectively do not support the proposal that *c-Kit* is a target gene for SCL. Since LiCl at concentrations as high as 100 mM did not cause inhibition of tyrosine kinase activity of the cytoplasmic domain of *c-Kit* *in vitro*, these results also rule out the possibilities that SCL interferes with the myeloid differentiation of WEHI-3B cells through induction of *c-Kit* expression and that the reversal of SCL-induced resistance to ATRA by LiCl is mediated through inhibition of *c-Kit*.

Because the success of ATRA-based differentiation therapy is tempered by obstacles such as the development of resistance to ATRA and the inability of ATRA alone to convert the entire leukemic cell population to mature end-stage cells [41], an understanding of the molecular mechanisms by which (a) WEHI-3B D⁻ cells exhibit resistance to ATRA and (b) LiCl enhances differentiation in combination with ATRA may provide information to improve differentiation therapy by ATRA. Determination of the target site(s) for LiCl warrants further investigation, since the addition of LiCl to ATRA increased the differentiation response over that of ATRA alone in leukemic blast cells from patients with not only M3 but also non-M3 AML in *ex vivo* studies [42].

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Fig. 1.

Transient transfection of SCL and GATA-1 expression plasmids into NRK and CHO cells. (A) NRK (normal rat kidney) cells were transfected with expression plasmids under the control of the CMV promoter, and cultured for 20 h in the absence or presence of 10 nM trichostatin A (TSA) for expression. (B) CHO (Chinese hamster ovary) cells were transfected with expression plasmids under the control of the human metallothionein IIA (MT) promoter and cultured for 20 h in the absence or presence of 80 μ M ZnSO₄. Whole cell extracts were then subjected to western analysis. HSC 70 is a heat shock protein used as a loading control because of constant expression.

Fig. 2.

Stable transfection of human metallothionein IIA-dependent expression plasmids for SCL and GATA-1 into WEHI-3B D⁺ cells. The expression plasmids for SCL (A) and GATA-1 (B) along with an empty vector were transfected into WEHI-3B D⁺ cells by electroporation. The transfectants were selected either in semi-solid medium (V1, S1–S5 and G1–G5), or in liquid medium (Vm, Sm and Gm) containing 0.8 mg/ml of G418. The expression at the level of protein was analyzed by western analyses (top panels) and the expression at the level of mRNA was measured by northern analyses (bottom panels). WCE, whole cell extracts; AO stain, acridine orange stain.

Fig. 3.
The expression of SCL, GATA-1, GATA-2 and c-Kit in a variety of human and rodent cell lines. (A) Western analyses of whole cell extracts and cytoplasmic extracts from human and murine hematopoietic cells (top panels) and a summary of the data (bottompanel). (B) Western analyses of cytoplasmic extracts from human and rodent attached cell lines.

Fig. 4. Northern and RT-PCR analyses of SCL and GATA-1 mRNA levels from a variety of cell lines. (A) Northern analyses using total cellular RNA or poly A⁺ RNA. AO stain; acridine orange stain. (B) Semi-quantitative RT-PCR analyses for human (top panel) and murine (bottom panel) SCL mRNAs. Bone marrow cells were collected from the femurs of two 6-week-old C57BL/6 mice.

Table 1Response of SCL or GATA-1 transfected WEHI-3B D⁺ cells to ATRA, LiCl, and the combination thereof.

Cell type	NBT positive cells (%)	
	ATRA (5 μ M)LiCl (5 mM)	ATRA + LiCl
D ⁻	00	7 $\pm$ 1
D ⁺	40 $\pm$ 94	85 $\pm$ 3
V1	43 $\pm$ 43	72 $\pm$ 10
Vm	39 $\pm$ 34	83 $\pm$ 9
S1	47 $\pm$ 155	84 $\pm$ 9
S2	27 $\pm$ 8 [*] 3	84 $\pm$ 14
S3	7 $\pm$ 5 ^{**} 4	72 $\pm$ 10
S4	18 $\pm$ 8 [*] 3	78 $\pm$ 8
S5	24 $\pm$ 12 [*] 4	85 $\pm$ 4
Sm	32 $\pm$ 101	72 $\pm$ 12
G1	25 $\pm$ 5 [*] 1	40 $\pm$ 9 [*]
G2	9 $\pm$ 3 ^{**} 0	10 $\pm$ 2 ^{**}
G3	12 $\pm$ 6 [*] 0	24 $\pm$ 8 [*]
G4	21 $\pm$ 9 [*] 1	37 $\pm$ 12 [*]
G5	38 $\pm$ 113	81 $\pm$ 10
Gm	24 $\pm$ 121	36 $\pm$ 15 [*]

D⁺ and D⁻ are non-transfected WEHI-3B cells. V1–Vm, S1–Sm and G1–Gm are empty vector, SCL and GATA-1 transfectants, respectively, described in Fig. 2. The values of NBT positivities represent the means $\pm$ S.D. from three independent experiments with *P* values compared to V1.

* *P* < 0.05.

** *P* < 0.001.