RepSox Slows Decay of CD34+ Acute Myeloid Leukemia Cells and Decreases T Cell Immunoglobulin Mucin-3 Expression

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ABSTRACT

Despite initial response to therapy, most acute myeloid leukemia (AML) patients relapse. To eliminate relapse-causing leukemic stem/progenitor cells (LPCs), patient-specific immune therapies may be required. In vitro cellular engineering may require increasing the “stemness” or immunogenicity of tumor cells and activating or restoring cancer-impaired immune-effector and antigen-presenting cells. Leukapheresis samples provide the cells needed to engineer therapies: LPCs to be targeted, normal hematopoietic stem cells to be spared, and cancer-impaired immune cells to be repaired and activated. This study sought to advance development of LPC-targeted therapies by exploring nongenetic ways to slow the decay and to increase the immunogenicity of primary CD34+ AML cells. CD34+ AML cells generally displayed more colony-forming and aldehyde dehydrogenase activity than CD34- cells. Along with exposure to bone marrow stromal cells and low (1%) and 5% oxygen, culture with RepSox (a reprogramming tool and inhibitor of transforming growth factor-β receptor 1) consistently slowed decline of CD34+ AML and myelodysplastic syndrome (MDS) cells. RepSox-treated AML cells displayed higher CD34, CXCL12, and MYC mRNA levels than dimethyl sulfoxide-treated controls. RepSox also accelerated loss of T cell immunoglobulin mucin-3 (Tim-3), an immune checkpoint receptor that impairs antitumor immunity, from the surface of AML and MDS cells. Our results suggest RepSox may reduce Tim-3 expression by inhibiting transforming growth factor-β signaling and slow decay of CD34+ AML cells by increasing CXCL12 and MYC, two factors that inhibit AML cell differentiation. By prolonging survival of CD34+ AML cells and reducing Tim-3, RepSox may promote in vitro immune cell activation and advance development of LPC-targeted therapies.

INTRODUCTION

Leukemic stem/progenitor cells (LPCs) are believed to sustain disease, to persist after chemotherapy and radiation, and to contribute to post-treatment relapses. In any given acute myeloid leukemia (AML) patient, these disease-causing cells may encompass a distribution of diverse immunophenotypes that evolves as disease progresses. Over time, leukemia disrupts the bone marrow (BM), suppresses the immune system, and evolves new “immune-escape” and “growth-advantaged” variants [1, 2]. AML LPCs are often distinctive at the molecular level because of high levels of T cell immunoglobulin mucin-3 (Tim-3) [3, 4] and antiapoptotic factors [5] and low immunogenicity [6]. In the setting of minimal residual disease (MRD), immunotherapy may be needed to eliminate quiescent LPCs spared by conventional therapies. Prompt treatment [7] with antibodies, inhibitors of antiapoptotic factors, and/or immune cells that target LPCs while sparing normal hematopoietic stem cells (HSCs) should improve AML patient survival, especially when combined with immune-modulating strategies and chemotherapies that cause immunogenic tumor cell death.

Regarding development of patient-specific LPC-targeted therapies, recent technical advances are encouraging. Notably, immune cell activity, which is impaired by leukemic disease states, can be restored in patients after their immune cells have been manipulated in vitro [8, 9]. When treated with their own custom-engineered T cells, leukemia patients rapidly entered remission and remain disease-free because of the generation of long-lived memory T cells [8, 9]. Because their T cells were genetically engineered against all CD19+ cells, both normal and leukemic B cells were eliminated. Although tumor cells initially trigger an immune response [10], they suppress immune cell activity by engaging co-inhibitory receptors including Tim-3, programmed cell death protein 1 (PD-1), and cytotoxic T-lymphocyte-associated antigen 4.
(CTLA4) and by secreting immunosuppressive factors such as transforming growth factor-β (TGF-β) [11]. Fortunately, cancer-impaired immune cells can be repaired and activated in vitro [12] and in vivo, without genetic engineering, as demonstrated by potent stimulation of tumor-reactive T cells in metastatic melanoma patients treated with ipilimumab to block CTLA4 [13]

In the setting of AML, Glaser and colleagues [14] have shown that in vitro manipulation of AML cells provides relevant insights and predicts promising therapeutic effects in vivo. Furthermore, Noh and colleagues [15] greatly improved survival of mice with colon cancer using a two-pronged immune strategy inspired by the setting of AML. Of clinical relevance, CD34+ AML cells often contain disease-causing LPCs that may trigger relapse [22].

**MATERIALS AND METHODS**

**Human White Blood Cells**

Deidentified leukapheresis specimens were obtained from patients treated at the West Virginia University (WVU) Mary Babb Randolph Cancer Center, in accordance with institutional review board guidelines. Human white blood cells (WBCs) were isolated by Ficoll-Paque Plus (Amersham Biosciences, Upsalla, Sweden, http://www.amersham.com) density gradient separation and cryopreserved (5 × 10^6 cells per ml) in equal volumes of RPMI 1640 medium (Mediatech, Manassas, VA, http://www.celigro.com)/10% fetal bovine serum (FBS) (Hyclone, Logan, UT, http://www.hyclone.com) and freezing solution (80% FBS/20% dimethyl sulfoxide (DMSO); Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). CD34+ cells isolated from the mobilized peripheral blood of healthy donors were purchased from AllCells (Emeryville, CA, http://www.allcells.com).

**Cell Culture**

WBCs were cultured in RPMI 1640 medium (Mediatech, Manassas, VA, http://www.celigro.com) containing 2 mM l-glutamine (Mediatech), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich), and 10% FBS (Hyclone) or serum replacement 1 (Sigma-Aldrich) (also referred to as “serum-free”), as indicated. Cocultures included BM stromal cells, adherent cells isolated from the BM of patients without evidence of hematologic disease. BM stromal cells constitutively express VCAM1, fibronectin, and diverse cytokines that support hematopoietic progenitors, including interleukin-7-dependent pro-B cells [26]. Cells were cultured at 37°C in 6% CO₂ and 1%–5% or 21% O₂, as noted.

**TGF-β Inhibition and Neutralization**

TGF-β inhibitors RepSox (E-616452 or SIN 2511; Sigma-Aldrich), SB 431542 (Sigma-Aldrich), LY 364947, and GW 788388 (Tocris Bioscience, Ellisville, MO, http://www.tocris.com) were dissolved in DMSO and added once at initiation of culture. TGF-β neutralizing antibody (anti-TGF-β1, 2, or 3) or matched mouse IgG1 isotype control antibody (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com) were reconstituted in phosphate-buffered saline (PBS). Antibodies (10 μg/ml) were added at initiation of culture and subsequently every 3 days.

**Surface Immunostaining**

WBCs were suspended in 100 μl PBS/1% bovine serum albumin (BSA). Samples were blocked with 1 μg human IgG (R&D Systems Inc.) for 20 minutes at 4°C and incubated with specific or isotype
control antibodies for 20 minutes at 4°C in the dark. Samples were rinsed three times with PBS/1% BSA and analyzed by flow cytometry within 4 hours of staining. Specific and matched isotype control antibodies directed against the following human antigens were used: CD34-peridinin chlorophyll protein (PerCP) (Becton, Dickinson and Company, San Jose, CA, http://www.bd.com) and mouse IgG1-PerCP (Dako, Carpinteria, CA, http://www.dako.com); human leukocyte antigens A, B, and C (HLA-ABC)-phycocerythrin (PE) and mouse IgG1, κ-PE; CD47-fluorescein and mouse IgG1, κ-FITC, and CD227/MUC1-FITC and mouse IgG1, κ-FITC (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com); and Tim-3-PE and mouse IgG1, κ-PE (BioLegend, San Diego, CA, http://www.biolegend.com). Of note, Aldefluor-stained cells were immunostained in Aldefluor assay buffer, which contains ATP-binding cassette transport inhibitors, to prevent dye efflux.

Isolation of RNA and Reverse Transcription-Polymerase Chain Reaction
RNA was isolated from AML cells using the RNeasy Mini Kit with on-column DNase I digestion (Qiagen, Valencia, CA, http://www.qiagen.com). One-step reverse transcription-polymerase chain reactions (RT-PCRs) were performed in triplicate using 50 ng of RNA per reaction with the QuantiTect SYBR Green RT-PCR kit (Qiagen) on an Applied Biosystems 7500 thermocycler (Foster City, CA, http://www.appliedbiosystems.com). GUSB served as a loading control. Primers for human CD34, CXCL12, MYC, and HACVR2 (encoding Tim-3) were purchased from Qiagen. GUSB primers (forward: AAACGATTGCAGGGTTTCAC, reverse: CTCTCGTGCGTGACTGGTTCA) were synthesized by Invitrogen (Carlsbad, CA, http://www.invitrogen.com). Fold changes in relative gene expression were calculated using the 2^{ΔΔCT} method [27].

Carboxyfluorescein Succinimidyl Ester Staining
WBCs were labeled using the CellTrace carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Molecular Probes, Eugene, OR, http://probes.invitrogen.com), according to the manufacturer’s instructions. Briefly, WBCs were incubated with 1 μM CFSE for 10 minutes at 37°C. Staining was quenched using ice-cold media, and cells were rinsed and placed in culture. After 6 days, cells were analyzed by flow cytometry.

Aldefluor Assay
Aldehyde dehydrogenase (ALDH) activity was measured using the Aldefluor assay, according to manufacturer’s instructions (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). WBCs were suspended in Aldefluor assay buffer and incubated with Aldefluor reagent, a fluorescent substrate of ALDH. An aliquot of each sample was immediately mixed with diethylamino benzaldehyde (DEAB), a specific inhibitor of ALDH, to serve as a negative control. ALDH+ regions were drawn to exclude >99% of DEAB-treated cells. Samples were incubated for 40 minutes at 37°C and analyzed by flow cytometry within 3 hours of labeling.

Flow Cytometric Analysis
Samples were analyzed by fluorescence-activated cell sorting (FACS) using a Becton, Dickinson and Company FACS Calibur. Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA, http://www.dennonsoftware.com). Dead cells, identified by propidium iodide uptake, were excluded from analysis. The Overton histogram subtraction method was used to calculate the percentage of stained (i.e., CD34+) cells relative to matched isotype controls [28]. Normalized median fluorescence intensity (MFI) was calculated by subtracting the MFI of isotype control antibody-stained cells from the MFI of specific antibody-stained cells.

Immunomagnetic Sorting of CD34+ WBCs
WBCs were cocultured with BM stromal cells under 5% O2 for 4–8 hours after thawing. Following recovery, WBCs were labeled with magnetic beads conjugated to anti-CD34 antibody recognizing the QBEND/10 epitope (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.milenyiбиотec.com). WBCs were separated using a Miltenyi Biotec autoMACS, according to the manufacturer’s instructions. Purity of cellular fractions was evaluated by flow cytometry following immunostaining with anti-CD34 antibody recognizing the HPCA-2 epitope (Becton, Dickinson and Company).

FACS of Tim-3+ WBCs
WBCs were cocultured with BM stromal cells under 5% O2 for 4–8 hours after thawing. Following recovery, WBCs were stained with anti-Tim-3-PE antibody (BioLegend) and sorted into Tim-3+ and Tim-3− fractions (>88% pure) using a Becton, Dickinson and Company FACS Aria and FACS Diva 6.2 software. Tim-3+ cells were gated against matched isotype control antibody-stained cells. Purity of sorted fractions was evaluated by flow cytometry.

 Colony-Forming Assays
WBCs were cultured in methylcellulose containing recombinant human stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), and erythropoietin (MethoCult Optimum; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com), according to the manufacturer’s instructions. Colony assays, performed in duplicate, were incubated at 37°C in 6% CO2 and 21% O2. Colonies were counted by light microscopy after 14–18 days of culture.

Diff-Quik Staining
To evaluate morphology, WBCs were stained using the Diff-Quik Stain Set (Siemens, Newark, DE, http://www.medical.siemens.com), according to the manufacturer’s instructions. Briefly, slides were sequentially dipped in methanol fixative, a solution of eosin Y, and a solution containing methylene blue and Azure A. Slides were rinsed with deionized water, allowed to dry, and imaged by light microscopy under oil immersion.

Fluorescence In Situ Hybridization
The WVU Hospital Cytogenetics Laboratory analyzed 100–150 interphase cells for t(8;21), t(15;17), inv(16), del(6q23), and 11q23 chromosomal abnormalities by fluorescence in situ hybridization (FISH). Probes were designed by Abbott Molecular (Des Plaines, IL, https://www.abbottmolecular.com).

Statistical Analysis
Differences between groups were compared using paired Student t tests (two-tailed) and considered statistically significant when p < .05. Values are displayed as the mean ± SE. With
the exception of FISH, all data are representative of two or more independent experiments.

**RESULTS**

**CD34⁺ AML/MDS Cells Have Higher Colony-Forming and ALDH Activity Than CD34⁻ Cells**

Leukapheresis specimens (supplemental online Table 1) from AML/MDS patients (P1–P8) displayed variable CD34 expression (Fig. 1A). To identify LPC-enriched fractions, we compared the morphology, colony-forming potential, and ALDH activity of CD34⁺ and CD34⁻ cells. Following immunomagnetic sorting, CD34⁺ fractions were 75%–99% pure (supplemental online Fig. 1) and shown to be 99%–100% leukemic by FISH (Fig. 1E). CD34⁺ AML cells (P1) exhibited rounder nuclei and a higher nuclear-to-cytoplasmic ratio than CD34⁻ AML cells (Fig. 1B). CD34⁺ AML/MDS cells generated 35- to 65-fold more colonies (Fig. 1C) and generally displayed higher ALDH activity (Fig. 1D; supplemental online Fig. 2) than CD34⁻ cells. Of interest, the relapsed AML patient (P2) had the highest proportion (85%) of CD34⁺ cells with ALDH activity. Colonies generated by CD34⁺ cells were confirmed to be of leukemic origin by FISH (Fig. 1F). In culture, CD34⁺ AML/MDS cells gave rise to CD34⁻ cells, whereas CD34⁻ cells remained CD34⁻ (supplemental online Fig. 3). CD34⁺ cells also expanded by self-renewal on stimulation with SCF, G-CSF, and IL-3 (supplemental online Fig. 4A). In summary, AML/MDS progenitors are enriched within CD34⁺ fractions of leukapheresis specimens.

**RepSox, Low O₂, and Coculture With BM Stromal Cells Maintain CD34⁺ AML Cells**

Serum-free medium (Fig. 2A), coculture with BM stromal cells (Fig. 2B), and low (1%–5%) O₂ (Fig. 2C) more effectively maintained CD34⁺ AML cells than serum-containing medium, culture without BM stromal cells, and high (21%) O₂. These conditions...
mimic in vitro assays designed to support LPCs [19] and the BM niche where LPCs reside in vivo [18, 29]. To generate 3-D spheroids, leukemic cells were cocultured with BM stromal cells or osteoblasts on low-attachment plates (supplemental online Fig. 5). Under all culture conditions examined, RepSox maintained higher proportions of CD34+ cells than DMSO (Fig. 2A–2C). As expected, RepSox inhibited TGF-β-induced phosphorylation of Smad2/3 (supplemental online Fig. 6). Similar proportions of CD34+ cells were maintained in media and DMSO; therefore, only DMSO controls are shown. In summary, optimal conditions for maintaining CD34+ AML cells include 1%–5% O2, coculture with BM stromal cells, and exposure to RepSox.

RepSox Maintains CD34+ AML Cells in a Concentration-Dependent Manner

Increasing concentrations of RepSox maintained higher proportions of CD34+ cells than DMSO (Fig. 2A–2C). As expected, RepSox inhibited TGF-β-induced phosphorylation of Smad2/3 (supplemental online Fig. 6). Similar proportions of CD34+ cells were maintained in media and DMSO; therefore, only DMSO controls are shown. In summary, optimal conditions for maintaining CD34+ AML cells include 1%–5% O2, coculture with BM stromal cells, and exposure to RepSox.

RepSox Slows Decay of CD34+ AML/MDS Cells

Next we investigated the effects of RepSox on diverse AML/MDS specimens. For consistency, all specimens were cultured with 16 μM RepSox, 10% FBS, BM stromal cells, and 5% O2. Inclusion of serum improved viability, although serum-free conditions more effectively maintained the CD34+ subset of cells.

In culture, the proportion of CD34+ AML/MDS cells declined as cells divided asymmetrically and/or differentiated. Culture with RepSox slowed the mean decline in the proportion of CD34+ cells (33% and 54% mean declines with RepSox and DMSO, respectively; P1–P7) (Fig. 4A, 4B); in other words, RepSox maintained 1.2- to 3.0-fold higher proportions of CD34+ cells than DMSO. RepSox-treated CD34+ cells were confirmed to be of leukemic origin by FISH (Fig. 4C). CD34 expression was also 1.9- to 6.5-fold higher on RepSox-treated cells than DMSO-treated controls (CD34 MFIs of 30 and 9 with RepSox and DMSO, respectively; P1–P7) (Fig. 4A). RepSox did not induce CD34 expression on CD34− AML cells (supplemental online Fig. 3). Unlike RepSox, exposure to the TGF-β inhibitors SB 431542, LY 364947, and GW 788388 (supplemental online Fig. 7A) as well as TGF-β neutralizing antibody (data not shown) did not slow decay of CD34+ AML cells. In contrast to AML/MDS cells, RepSox did not slow decay of CD34+ cells from healthy donors (Fig. 4A, 4B).

We investigated whether RepSox slows decay of CD34+ AML/MDS cells by slowing their rate of asymmetric division and/or by inhibiting their differentiation. RepSox-treated AML cells retained greater CFSE fluorescence (Fig. 4D) and incorporated less 5-ethynyl-2′-deoxyuridine (supplemental online Fig. 8) than DMSO-treated controls, consistent with a slower rate of proliferation. On stimulation with IL-3, SCF, and G-CSF, RepSox-treated CD34+ cells expanded as effectively as DMSO-treated controls (supplemental online Fig. 4A). We also evaluated how RepSox may affect differentiation of CD34+ cells into CD34− AML
cells. When cultured with DMSO, the proportion of CD34+ AML cells (P1) decreased as the proportion of CD34-CD33-CD14- cells increased (data not shown). In contrast, RepSox maintained a high proportion of CD34+ cells, and no increase in the proportion of CD34-CD33-CD14- cells was observed (data not shown).

**RepSox-Treated AML/MDS Cells Display Higher CD34, CXCL12, and MYC mRNA Levels and Similar ALDH Activity Compared With DMSO-Treated Controls**

Both c-Myc and CXCL12 (also known as SDF1) may inhibit differentiation of AML cells [30–33]. RepSox-treated AML cells displayed higher CD34, CXCL12, and MYC mRNA levels than DMSO-treated controls (Fig. 5A). CXCL12 and MYC mRNA levels were substantially higher following exposure to RepSox than the TGF-β inhibitors SB 431542 and LY 364947 (supplemental online Fig. 7C).

ALDH is involved in chemotherapy resistance [34] and normal HSC self-renewal [35]. In AML patients, the subset of CD34+ cells with ALDH activity is enriched in long-term culture-initiating cells [36]. Furthermore, persistence of CD34-CD33-CFSE-ALDHhigh AML cells after induction chemotherapy is associated with disease relapse [37]. Given this clinical relevance, we explored whether RepSox-treated CD34+ cells retain ALDH activity. Similar proportions of RepSox- and DMSO-treated CD34+ cells (gated, as shown in supplemental online Fig. 9) displayed ALDH activity (Fig. 5B), demonstrating that RepSox equally slows decay of ALDH+ and ALDH- subsets of CD34+ cells. Overall, a subset of CD34+ cells with ALDH activity is maintained in our culture system. The observed pattern of ALDH activity is characteristic of LPCs rather than normal stem/progenitor cells [38].

**RepSox Reversibly Suppresses AML Colony-Forming Activity**

In addition to CD34 expression and ALDH activity, we compared the colony-forming activity (CFA) of RepSox- and DMSO-treated AML/MDS cells. Normal and AML CD34+ cells generated fewer colonies after 6-day culture with RepSox than with DMSO (Fig. 6A). RepSox did not alter the low CFA of CD34-AML/MDS cells (data not shown). Normal and AML CD34+ cells also generated fewer colonies when RepSox, compared with DMSO, was included within the colony-forming assay (Fig. 6B). In contrast, CD34+ MDS cells generated more colonies when RepSox, compared with DMSO, was included within the colony-forming assay (Fig. 6B).

Potential reasons for the reduced CFA of AML cells after RepSox exposure include death, differentiation, or arrest of colony-forming cells in a quiescent stem-like state. To rule out RepSox-induced death or differentiation of colony-forming cells, we measured CFA following RepSox removal. Removal of RepSox partially rescued AML CFA (Fig. 6C).

**RepSox Decreases Tim-3 Expression on AML/MDS Cells**

Increasing tumor cell immunogenicity is one strategy to improve immune cell activation [6]. Consequently, we investigated
whether RepSox altered expression of immunomodulatory receptors including Tim-3, CD47, human leukocyte antigens, and MUC1 on AML/MDS cells. HAVCR2 (Tim-3) mRNA levels were consistently reduced in RepSox-treated AML cells compared with DMSO-treated controls (Fig. 7A). A smaller proportion of RepSox-treated CD34+ cells expressed Tim-3, a negative regulator of antitumor immunity [39], than DMSO-treated CD34+ cells (means of 27% and 53% Tim-3+ cells with RepSox and DMSO, respectively; P2 and P3) (Fig. 7B). To determine whether RepSox promoted selective survival of Tim-3- cells or decreased Tim-3 expression, Tim-3+ and Tim-3- fractions were separately exposed to RepSox. Tim-3- cells converted to Tim-3+ cells in vitro (Fig. 7C). RepSox accelerated loss of Tim-3 from the surface of FACS-purified Tim-3- AML/MDS cells (mean declines in Tim-3+ cells of 64% and 43% with RepSox and DMSO, respectively; P2, P3, and P8) (Fig. 7C). A greater proportion of MDS cells expressed Tim-3 following removal from RepSox (supplemental online Fig. 10A) compared with continuous culture with RepSox (supplemental online Fig. 10B); this finding suggests that Tim-3 effects are partially reversible. Exposure to both RepSox and the structurally distinct

![Image](image_url)
TGF-β inhibitor SB 431542 [40] accelerated loss of Tim-3 from the surface of AML/MDS cells (mean declines in Tim-3+ cells of 81% and 53% with SB 431542 and DMSO, respectively; in P3 and P8) without altering viability relative to DMSO controls (supplemental online Fig. 11). Expression of CD47 and HLA-ABC was not affected by RepSox, whereas MUC1, an antigen contributing to the poor immunogenicity of AML LPCs [41], was not highly expressed by AML/MDS cells (supplemental online Fig. 12).

**DISCUSSION**

Engineering patient-specific anticancer immune therapies will advance as in vitro techniques improve. Because primitive relapse-causing tumor cells must be targeted, a basic challenge is reducing the in vitro death and differentiation of cancer progenitors. LPCs must be maintained in culture long enough to study their molecular characteristics, to activate immune cells, and to evaluate potential therapies. Currently, key technical obstacles are being identified and addressed. Glettig and Kaplan have explored ways to better maintain CD34+ hematopoietic progenitors in vitro using coculture with adipocytes [42]. Kellner and colleagues report that their “most significant problem” is isolating multiple myeloma stem cells so they can be studied [43]. If relevant stem-like tumor cells can be adequately maintained in vitro, another challenge may be increasing low tumor cell immunogenicity that impedes antigen presentation and immune cell activation. In the setting of AML, abnormal Tim-3 expression on tumor cells and dysfunctional antigen-presenting and immune-effector cells is an especially attractive target because this one receptor impairs antigen presentation and promotes both immune suppression and immune evasion [44–47]. Consequently, Tim-3 should be considered a potentially serious obstacle when engineering immune therapies in vitro.

Considering these technical challenges, the ability of RepSox to extend CD34+ cell survival and decrease Tim-3 expression seems useful for engineering LPC-targeted immune therapies. RepSox is a chemical reprogramming tool with actions linked to maintaining stemness and inhibiting differentiation, two actions that are relevant for maintaining CD34+ AML cells in culture [24, 48]. In mouse embryonic fibroblasts (MEFs), RepSox increased mRNA levels of components of the Wnt, Notch, and Hedgehog signaling pathways that promote pluripotency, self-renewal, and stem cell maintenance [48]. In addition, as a TGF-β inhibitor, RepSox may mitigate immunosuppressive effects induced by TGF-β during cancer progression [49]. Moreover, RepSox may be a valuable tool for manipulating cancer and immune cells because its actions on both normal and cancer cells are potent and consistent [24, 48].

In addition to their poor survival and low immunogenicity, patients’ tumor cells may not be stem-like enough to adequately represent the critical in vivo target: the most recently evolved LPCs capable of triggering relapse. Primary cells may need to

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**Figure 5.** RepSox-treated cells have increased CD34, CXCL12, and MYC mRNA levels and similar ALDH activity compared with DMSO-treated controls. Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) cells were cultured with 16 μM RepSox or DMSO for 6 days (on stroma, 10% fetal bovine serum, 5% O2). (A): Quantitative reverse transcription-polymerase chain reaction analysis of CD34 (p < .05), CXCL12 (p < .05), and MYC (p < .005) mRNA levels within RepSox-treated cells relative to DMSO-treated controls. (B): ALDH activity of RepSox- and DMSO-treated CD34+ AML and MDS cells measured by flow cytometry (mean difference was not significant). ALDH+ regions were drawn to exclude >99% of DEAB-treated (negative control) cells. Abbreviations: ALDH, aldehyde dehydrogenase; DEAB, diethylaminobenzaldehyde; DMSO, dimethyl sulfoxide; P1–P5, patients 1–5.
be reprogrammed toward more stem-like phenotypes to serve as relevant therapeutic targets. Fortunately, technical breakthroughs are encouraging. The same factors that dedifferentiate normal cells may also dedifferentiate tumor cells [15, 23, 50]. Noh and colleagues converted tumor cells into a more stem-like phenotype in vitro after identifying a Nanog-mediated mechanism by which immune-selection pressures drove this conversion in vivo [15]. Furthermore, mouse cells were chemically reprogrammed to pluripotency using seven small molecules [51].

In this study, the TGF-β inhibitors RepSox and SB 431542 reduced Tim-3 expression on AML cells; however, only RepSox-treated AML cells displayed substantially increased levels of CXCL12 and MYC mRNA, and only RepSox slowed decay of CD34+ cells. Slowing decay of CD34+ cells is consistent with prior RepSox findings: RepSox replaces c-Myc during reprogramming [24], RepSox upregulates CXCL12 and MYC in MEFs [48], and both c-Myc and CXCL12 promote tumor cell survival [32, 52–54]. Furthermore, c-Myc is downregulated during differentiation of AML cells [31], and exposing AML cells to a c-Myc inhibitor induces their differentiation [33].

The CXCL12/CXCR4 and CXCL12/CXCR7 axes promote survival of leukemic cells and normal CD34+ cells [55, 56]. Activation of the CXCL12/CXCR4 axis is thought to inhibit AML cell differentiation because inhibition of CXCR4 by AMD 3100 induces AML cells to differentiate [32]. Overall, CXCL12 promotes survival of leukemic progenitors both in vitro and in vivo [57, 58]; however, the implications of cell surface and intracellular receptor levels are still being clarified. In the case of CXCR7, the CXCL12/CXCR7 axis promotes HSC survival even when CXCR7 surface expression is scarce [56]. Conceivably, when used as a potential CXCL12 upregulator, RepSox might help clarify the role of intracellular CXCL12 levels. Of note, the balance of CXCL12- and TGF-β-activated pathways affects CD34+ cell cycle status [59]. Consequently, as both a TGF-β inhibitor and an apparent CXCL12 upregulator, RepSox seems useful for investigating the balance between quiescence and cell cycling.

RepSox may slow decay of CD34+ AML cells by increasing c-Myc, which inhibits AML cell differentiation. c-Myc blocks differentiation of AML cell lines by increasing microRNA-17 and microRNA-20a levels, which, in turn, decrease p21 and STAT3 [60]. RepSox may increase c-Myc by upregulating CXCL12, which signals via CXCR4 to activate nuclear factor-κB (NF-κB) [61]. NF-κB increases expression of microRNA binding protein Lin28 [62], which inhibits maturation of the let-7 family of microRNAs [62, 63]. Because let-7 microRNAs repress translation of MYC mRNA, reduced let-7 may lead to increased c-Myc expression [63, 64]. Furthermore, RepSox-treated MEFs expressed higher mRNA levels of the Wnt signaling components FZD1, FZD4, FZD9, and LEF1 than DMSO-treated controls [48]. Because MYC is a target of Wnt signaling [65], RepSox may upregulate MYC via Wnt activation. Upregulation of MYC by Wnt/β-catenin signaling has been reported independently by Zhang et al. [66] and He et al. [65], and He and colleagues highlighted the T cell factor-4 binding sites in the MYC promoter. CXCL12 exposure also increased mRNA levels of the Wnt target genes CTNNB1 (encoding β-catenin), CCND1 (encoding cyclin-D1), and MYC (encoding c-Myc) in the AML cell line HL60 [67].

In contrast to the “CD34+ effect” unique to RepSox, Tim-3 expression on AML/MDS cells was also reduced by the structurally
distinct TGF-β inhibitor SB 431542. Consequently, TGF-β inhibition may reduce Tim-3 levels, a mechanism supported by reports that TGF-β induces Tim-3 expression on mast cells [68, 69]. Of note, the ALDH activity of RepSox-treated CD34+ AML cells was unaltered, despite reduced Tim-3 expression relative to controls, suggesting these cells remained stem-like. Although Tim-3 is an AML LPC-associated antigen, reduced Tim-3 expression may not diminish the disease-initiating or relapse-causing potential of LPCs. Indeed, RepSox-treated AML cells engrafted the BM of NOD-\textit{scid} IL2Rgamma\textsuperscript{null} mice (data not shown).

Tim-3 may be a critical obstacle when engineering AML immunotherapies because it impairs antitumor immunity [39]. By inducing Tim-3 expression, tumor microenvironments inhibit the antitumor activity of dendritic [70], natural killer [71], T-lymphocytic [39], and monocytic [72] cells. Furthermore, blocking Tim-3 signaling with antibodies restores the cytotoxicity of CD8+ T cells and improves antitumor immunity [12, 73, 74]. Conceivably, reduced Tim-3 expression on AML cells might increase their immunogenicity because Tim-3 binds to and suppresses helper T cells when expressed by endothelial cells [47].

Immunotherapies involving antibodies, activated immune cells, and/or inhibitors of antiapoptotic factors (responsible for tumor cell immune resistance) may be required to eliminate LPCs that trigger AML relapse. Although a cancer patient’s immune-effector and antigen-presenting cells are not malignant, their cancer-induced dysfunctions may need to be reversed. Reprogramming exhausted T cells to pluripotency improves their functions, increases their proliferative capacity, and preserves their antigen-specificity following redifferentiation into T cells [75, 76]. Because immune cell suppression and tumor cell immune-evasion worsen over time, immune therapies may need to be administered shortly after diagnosis to be effective. Thus, the ability to quickly manipulate a patient’s diagnostic cells may be an advantage. Like N-propionylmannosamine [77], resiquimod

Figure 7. RepSox decreases Tim-3 expression on acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) cells. AML and MDS cells were cultured with 16 \textmu M RepSox or DMSO for 6 days (on stroma, 10% fetal bovine serum [FBS], 5% O\textsubscript{2}). (A) Quantitative reverse transcription-polymerase chain reaction analysis of HAVC2 (Tim-3) mRNA levels within RepSox-treated cells relative to DMSO-treated controls (\(p < .005\)). (B): Flow cytometric analysis of Tim-3 surface expression (open histograms) on RepSox- and DMSO-treated CD34+ cells (mean difference was not significant [NS]). Solid histograms display isotype controls. (C): Flow cytometric analysis of Tim-3 surface expression (open histograms) following culture of fluorescence-activated cell sorting-purified Tim-3+ AML and MDS cells with 16 \textmu M RepSox or DMSO for 6 days (on stroma, 10% FBS, 5% O\textsubscript{2}) (mean difference was NS). Solid histograms display isotype controls. Abbreviations: DMSO, dimethyl sulfoxide; MFI, median fluorescence intensity; P1–P8, patients 1–8; Tim-3, T cell immunoglobulin mucin-3.
[78], and the MUC1 inhibitor GO-203 [41], RepSox is a chemical that alters leukemia cells in vitro within a week. Prolonging survival of a patient’s CD34+ cells in culture by days may be sufficient to chemically engineer cells. Exposing cells to small molecules like RepSox is less labor-intensive than genetic engineering, and effects may be reversible. Of note, culture with a leukemic patient’s BM stromal cells, rather than BM stromal cells from healthy donors, may further enhance maintenance of that patient’s CD34+ AML cells because cancer-associated fibroblasts have evolved to support tumor growth in vivo [79, 80]. The ability to quickly and reversibly alter the primary cells of AML patients in vitro may be valuable if sequential cellular manipulations prove useful for therapy development.

**CONCLUSION**

RepSox may be useful for developing immunotherapies as a cell culture additive (to slow decay of CD34+ AML cells, which often contain LPCs) and/or as a cell-engineering tool (to decrease Tim-3 expression). Because Tim-3 expression on the tumor and immune cells of cancer patients may adversely affect tumor cell immunogenicity, immune cell activation, and antigen presentation, RepSox may improve the antitumor actions of antigen-presenting and immune-effector cells that become functionally impaired in cancer patients. Because the effects of RepSox seem potent and predictable, RepSox warrants consideration for manipulating the tumor and immune cells of patients with other cancers. Because molecular tools have recently been shown to chemically reprogram cells, RepSox and other molecules may eventually eliminate the need for genetic engineering when developing patient-specific immune therapies. Characterizing the actions of RepSox and other reprogramming tools may promote development of immunotherapies by simplifying in vitro engineering of tumor and immune cells.

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**AUTHOR CONTRIBUTIONS**

A.N.J.: conception/design, collection and/or assembly of data, processing of patient samples, data analysis and interpretation, manuscript writing; J.E.C. and J.A.V.: collection and/or assembly of data, data analysis and interpretation; K.H.M., J.R.S., and S.L.W.: collection and/or assembly of data; L.F.G.: conception/design, data analysis and interpretation, manuscript writing.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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