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Glycogen Synthase Kinase-3β Inhibition Preserves Hematopoietic Stem Cell Activity and Inhibits Leukemic Cell Growth

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ABSTRACT

Ex vivo expansion of cord blood cells generally results in reduced stem cell activity in vivo. Glycogen synthase kinase-3β (GSK-3β) regulates the degradation of β-catenin, a critical regulator of hematopoietic stem cells (HSCs). Here we show that GSK-3β inhibition activates β-catenin in cord blood CD34$^+$ cells and upregulates β-catenin transcriptional targets c-myc and HoxB4, both known to regulate HSC self-renewal. GSK-3β inhibition resulted in delayed ex vivo expansion of CD34$^+$ cells, yet enhanced the preservation of stem cell activity as tested in long-term culture with bone marrow stroma. Delayed cell cycling, reduced apoptosis, and increased adherence of hematopoietic progenitor cells to bone marrow stroma were observed in these long-term cultures treated with GSK-3β inhibitor. This improved adherence to stroma was mediated via upregulation of CXCR4. In addition, GSK-3β inhibition preserved severe combined immunodeficiency (SCID) repopulating cells as tested in the nonobese diabetic/SCID mouse model. Our data suggest the involvement of GSK-3β inhibition in the preservation of HSC and their interaction with the bone marrow environment. Methods for the inhibition of GSK-3β may be developed for clinical ex vivo expansion of HSC for transplantation. In addition, GSK-3β inhibition suppressed leukemic cell growth via the induction of apoptosis mediated by the downregulation of survivin. Modulators of GSK-3β may increase the range of novel drugs that specifically kill leukemic cells while sparing normal stem cells. STEM CELLS *2008;26:1288–1297*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Ex vivo expansion of hematopoietic progenitor cells generally results in reduced stem cell activity. Wnt signaling plays an important role in the regulation of hematopoiesis [1]. Experimental activation of Wnt was previously demonstrated using recombinant Wnt proteins, bone marrow stroma cells producing Wnt proteins, or by overexpression of the constitutively active *β-catenin* gene, the main downstream effector of Wnt [2–4]. These studies provide evidence that Wnt signaling positively regulates the self-renewal of murine hematopoietic stem cells (HSCs) [5, 6]. Understanding the potential role of Wnt signaling in the provision of self-renewal signals for human HSCs may have potential clinical applications in ex vivo HSC expansion strategies for allogeneic transplantation [7].

Activation of Wnt can be mediated through the inhibition of glycogen synthase kinase-3β (GSK-3β) that prevents the degradation of β-catenin, the main downstream effector of Wnt [8]. In vivo administration of GSK-3β inhibitors was recently shown to improve the regenerative potential of both human and murine HSCs in the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model [9]. These results established GSK-3β as a candidate modulator of stem cell activity and suggested that administration of a GSK-3β inhibitor may provide a clinical means to enhance the ex vivo expansion and in vivo repopulating capacity of transplantable stem cells [9].

Here we show that GSK-3β inhibition activates β-catenin in umbilical cord blood (UCB) CD34$^+$ cells and upregulates its transcriptional targets *c-myc* and *HoxB4*, both known to regulate stem cell renewal. Addition of a GSK-3β inhibitor during ex vivo expansion of UCB cells results in the better preservation of long-term culture-initiating cells as tested in coculture with bone marrow stroma. In addition, GSK-3β inhibition preserves SCID repopulating cells (SRCs) as tested in the NOD/SCID mouse model. Our data suggest the involvement of GSK-3β inhibition in preserving HSCs and their interaction with the bone marrow environment. Finally, when used at a dose that preserves long-term culture-initiating cells, the GSK-3β inhibitor 6-bromoindirubin 3′-oxime (BIO) suppresses leukemic cell growth by inducing apoptosis through the downregulation of survivin. Modulators of GSK-3β may increase the range of novel drugs that specifically kill leukemia cells and spare normal stem cells.

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MATERIALS AND METHODS

Cell Culture

Mononuclear cells were purified from UCB by Ficoll-Hypaque (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) density-gradient centrifugation. $CD34^+$ cells were separated to greater than 95% purity by positive selection using a magnetic cell purification system (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). $CD34^+$ cells were seeded at a density of 1–2 $\times$ 10^5 cells/ml in Iscove's modified Dulbecco's medium (IMDM; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) with 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA, http://www.invitrogen.com); 2 mM L-glutamine; 100 U/ml penicillin/streptomycin; and a cytokine cocktail of Flt3 ligand (FLT3L), stem cell factor (SCF), and thrombopoietin (TPO), all at a concentration of 20 ng/ml (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). $CD34^+$ cells were exposed to 0.1–2 μM BIO (a gift from Dr. L. Meijer, Biologic Station, Roskoff, France) or a matched concentration of dimethylsulfoxide (DMSO). In some experiments cells were treated with two other GSK-3β inhibitors, S3442 and S3567 (Sigma-Aldrich).

Human leukemic TF-1, K562, U937, and HL-60 cells were obtained from American Type Culture Collection (Manassas, VA, http://www.atcc.org). Cells were cultured in Roswell Park Memorial Institute 1640 medium containing 10% FCS. Medium used to grow TF-1 cells was supplemented with 10 ng/ml human recombinant interleukin-3 (IL-3; R&D Systems). Cells were seeded at a density of 2 $\times$ 10^5 cells/ml and exposed to BIO (0.1–5 μM) or to a matched concentration of DMSO. Bone marrow stroma MS5 cells were cultured in α-minimal essential medium (α-MEM; Gibco-BRL, Gaithersburg, MD, http://www.invitrogen.com) supplemented with 10% FCS. UCB $CD34^+$ cells were cultured in either the cytokine cocktail described above or in parallel in the same cytokine cocktail over a monolayer of MS5 cells. Every third day, half-media changes were performed to replenish cytokines and the total volume was adjusted to maintain the cells at 1 $\times$ 10^5 cells/ml. At the termination of the culture, suspension cells were collected for analysis. Conditions for long-term cocultures of $CD34^+$ cells with MS5 cells were IMDM-supplemented with 10% FCS; no exogenous cytokines were added in these assays. Adherent cells were harvested by trypsinization as previously described [10].

Fluorescent-Activated Cell Sorting for $CD34^+$ Cells

Cells to be cultured in long-term culture on MS5 stroma, as described above, were cultured in suspension for 5 days $\pm$ BIO and then sorted for CD34 positivity. Briefly, cells were stained with CD34 PE (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) for 30 minutes at 4°C. Cells were then washed twice with PBS and sorted on a BD FACSVantage SE with Diva option (BD Biosciences). A positive gate was established using an immunoglobulin G (IgG) negative control and $CD34^+$ cells were collected into 2% FCS. Samples of 5 $\times$ 10^4 cells per ml were then seeded onto confluent MS5 cells in triplicate and left to grow for a further 5 weeks. Cells were collected on days 6, 13, 29, and 37 for counting and flow cytometry.

Phenotypic Analysis of Ex Vivo Expanded UCB Cells by Flow Cytometry

A sample of 1 $\times$ 10^6 cells was blocked with mouse IgG and stained with monoclonal antibodies against CD34, CXCR4, CD49d, CD49e, CD14, CD38, and CD19 (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). In all, 1 $\times$ 10^5 events were acquired on a FACS Canto (Becton Dickinson) and data were analyzed with FACSDiva software (Becton Dickinson). Viable cells were gated based on forward versus side scatter (FSC/SSC). An anti-human CD45-specific antibody was used to distinguish human cells from murine cells in cocultures and animal. Propidium iodide was included to assess cell viability.

RNA Extraction and Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was prepared from suspensions of UCB cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany, http://www.qiagen.com) and Trizol (Life Technologies, Rockville, MD, http://www.lifetech.com), following the manufacturers' instructions. Polymerase chain reaction (PCR) was performed using standard techniques (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com). Template cDNA was synthesized using the SuperScriptIII First-Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen). PCR mixtures (25 μl per reaction) contained cDNA, 0.2 μmol/l each of forward and reverse primers, and 12.5 μl iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, http://www.bio-rad.com). The reactions were done in triplicate in 96-well plates using the GeneAmp 5700 Sequence Detection System (Applied Biosystems) and analyzed with the software from the manufacturer. The amount of transcript was determined based on a standard curve specific for each gene and normalized to the amount of β2-microglobulin transcript in the same sample.

Western Analysis

Western analysis was performed using standard techniques. Briefly, 48 μg of total cellular protein was run on a precast 4–15% gradient polyacrylamide gel (Bio-Rad). Samples were then transferred onto a Hybond-C nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) and stained with Ponceau S for visualization of protein bands. Samples were blocked with 5% nonfat dairy milk and probed for human β-actin and β-catenin antibodies. Monoclonal anti-β-catenin (BD Pharmingen, San Diego, http://www.bdbiosciences.com) and anti-actin (Sigma-Aldrich) antibodies were used at a 1:200 dilution. Anti-rabbit conjugated horseradish peroxidase was used as a secondary antibody at a 1:5,000 dilution. Quantification was performed by X-ray film and Versadoc.

Methylcellulose Colony-Forming Unit Assay

UCB-derived cells were plated in Methocult GF H4534 (1% methylcellulose in IMDM, 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 2 mM glutamine, 50 ng/ml SCF, 10 ng/ml granulocyte-macrophage colony-stimulating factor [GM-CSF], 10 ng/ml IL-3 [StemCell Technologies] supplemented with 3 units/ml of erythropoietin [EPO], and 50 ng/ml granulocyte-colony stimulating factor [G-CSF; R&D Systems) as previously described [11]. One thousand cells suspended in 1.1 ml were plated in a 35-mm dish in triplicate and cultured for 10–14 days. Colonies were counted at $\times$4 magnification and scored into three categories: pure erythroid (burst-forming unit [BFU]), myelomonocytic (colony-forming unit, granulocyte-monocyte [CFU-GM]), and mixed (colony-forming unit, granulocytic, erythroid, monocyte-macrophage [CFU-GEMM]). To perform CFC assay with TF-1 cells, Methocult H4230 (Stem-Cell Technologies) supplemented with IL-3 (10 ng/ml) was used. In a number of experiments bulk cultures were collected and cells were washed twice with phosphate-buffered saline (PBS). Single-cell suspensions were prepared and cells were counted. The average cell number per colony was estimated by dividing colony number by cell numbers.

Apoptosis and Cell Cycling

Cell viability was assessed by trypan blue exclusion. For cell cycle and apoptosis analysis, cells were collected by centrifugation and fixed in cold 70% ethanol for 4 hours. Fixed cells were washed with PBS and incubated with 5 μg/ml RNase A (Sigma-Aldrich), followed by staining with 50 μg/ml propidium iodide for 30 minutes at 37°C. The stained cells were then analyzed by flow cytometry. Analysis was performed using CellQuest software (BD Biosciences). Cell cycle profiles were based on data from 10,000 cells and displayed as a frequency histogram of propidium iodide fluorescence. Analysis was performed with the exclusion of cell debris and discrimination of aggregates. Cells in G_1 were identified as those with 2N DNA content, cells in S as those with a DNA content ranging from 2N to 4N, and cells in G_2/M as those with a 4N DNA

content. Apoptotic cells were identified as those with DNA content less than 2N (subgenomic).

Annexin V Staining for the Analysis of Apoptosis. An annexin V staining kit (Becton Dickinson) was used to perform staining according to the manufacturer's instructions. Briefly, cells were washed twice in cold PBS and resuspended in binding buffer.

Annexin V and propidium iodide were added to cells and incubated at room temperature for 15 minutes. Ten thousand cells were acquired and analyzed using CellQuest software. Debris was excluded from the analysis by scatter gating (forward vs. side) and cells that stained positive for both annexin V and propidium iodide were considered apoptotic.

Analysis of β-Catenin Expression in UCB CD34$^+$ Cells by Immunofluorescence Microscopy. Cells were washed twice with cold PBS and then fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 for 10 minutes, blocked with 3% bovine serum albumin (BSA) for 30 minutes, and subsequently incubated at 4°C with primary anti-β-catenin antibody and secondary FITC-conjugated anti-mouse IgG (Pharmingen) for 60 minutes each. For nuclear staining, Hoechst dye was used at a 1:100 dilution of 100 μg/ml stock. Hoechst dye was added to the medium 20 minutes before analysis. Analysis was performed using a fluorescence microscope (Nikon, Tokyo, http://www.nikon.com) fitted with a digital advance camera (Diagnostic Instruments, Sterling Heights, MI, http://www.diaginc.com).

NOD/SCID Mouse Model

NOD/SCID mice were irradiated at 6–8 weeks of age with a sublethal dose of 2.5 Gy from a 60°C source 10–12 hours before being transplanted with UCB cells via intravenous (IV) injection. The UCB CD34$^+$ cells were treated with BIO or DMSO for 5 days prior to IV injection. The cell dose administered to all mice was equivalent to 1×10^5 unexpanded cells. Mice were sacrificed by cervical dislocation at 6 weeks post-transplantation and the bone marrow (equivalent of two femurs) was harvested and assessed for human cell engraftment. The marrow content of both femurs of each mouse was flushed from bones using a 27-gauge needle. All cell suspensions were collected in PBS, subjected to red-cell lysis by treatment with 1 M ammonium chloride, washed once, and then resuspended in PBS. Human and murine cells were distinguished by the species-specific expression of CD45. Bone marrow cells obtained from UCB-transplanted mice were blocked with serum prior to antibody staining to reduce background fluorescence and nonspecific binding. Cells were then stained at 4°C with a combination of anti-human CD45 phycoerythrin (PE) labeled antibody and an anti-mouse CD45 fluorescein isothiocyanate (FITC) labeled antibody (BD Biosciences). The proportion of cells labeled with human CD45 was taken to be the level of engraftment. Cells from bone marrow were analyzed by flow cytometry to assess the contribution of different human hematopoietic cell lineages. Antibodies specific to human CD33-PerCP and CD19-APC were used to examine multilineage reconstitution.

To perform limiting dilution analysis (LDA) of SRCs, 200, 500, 1,000, and 100,000 CD34$^+$ cells were injected IV. To determine the threshold for positive engraftment, an experiment was conducted whereby increasing percentages of human cord blood CD34$^+$ cells were added to murine-irradiated bone marrow cells. The standard protocol for staining cells with fluorescent antibodies for flow cytometry, as described, above was then followed. Using this method, a threshold of 0.3% human CD45$^+$ cells was established as a reliable predictor of positive engraftment. L-Calc-software (StemCell Technologies) was used to determine the frequency of SRC.

Statistical Analysis

Results are expressed as mean ± SD. Differences between groups were examined for statistical significance using a paired *t* test.

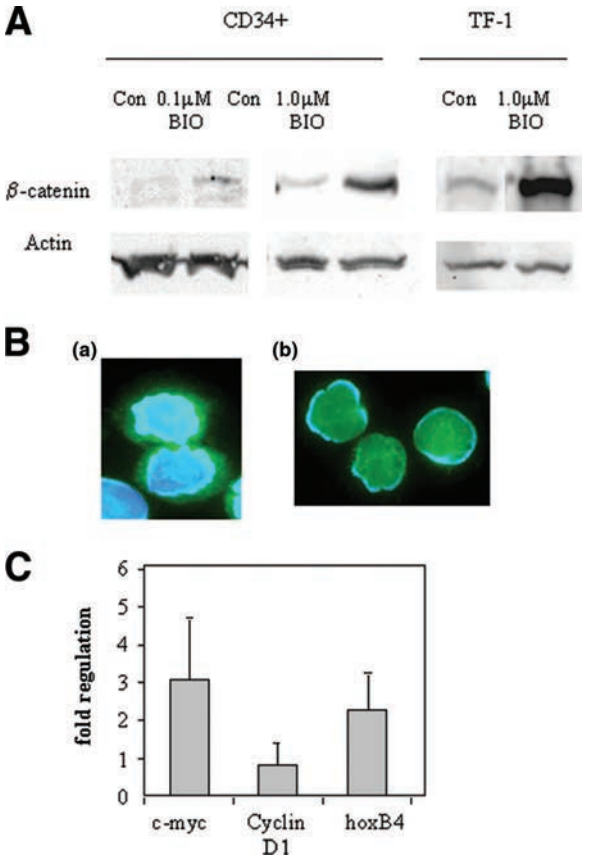

Figure 1. BIO activates β-catenin in UCB and TF-1 CD34$^+$ cells. **(A):** Western blot analysis of protein lysates derived from UCB and TF-1 CD34$^+$ cells. UCB CD34$^+$ cells were incubated with SCF, TPO, FLT3L, all at 20 ng/ml, and 0.1 or 1 μM BIO or DMSO for 24 hours. An average 1×10^6 cultured cells were used for protein analysis. Blots were probed with antibody against β-catenin, stripped, and reprobed with antibody against β-actin. TF-1 cells were growing in the presence of IL-3, 10 ng/ml, and either 1 μM BIO or DMSO. **(B):** β-Catenin translocates from the cytoplasm **(Ba)** to the cell nucleus **(Bb)**. UCB CD34$^+$ cells were incubated with 1 μM BIO for 24 hours. Cytospins were incubated with FITC-conjugated anti-β-catenin antibody. Slides were counterstained with Hoechst. Original magnification ×400. **(C):** Real-time PCR analysis of *c-myc*, *cyclinD1*, and *HoxB4* expression in UCB CD34$^+$ cells treated with BIO (0.1 μM, 5 days). The amount of each mRNA was determined based on a standard curve specific for each gene and normalized to the amount of β2-microglobulin transcript in the same sample. Average of three experiments is presented ± SD. Abbreviations: BIO, 6-bromoindirubin 3′-oxime; Con, Control; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; FLT3L, Flt 3 ligand; IL-3, interleukin-3; PCR, polymerase chain reaction; SCF, stem cell factor; TPO, thrombopoietin; UCB, umbilical cord blood.

RESULTS

BIO Activates β-Catenin in UCB CD34$^+$ Cells

β-Catenin is phosphorylated by active GSK-3β, which targets it for degradation by the proteasome [13]. Inhibition of GSK-3β results in reduced phosphorylation of β-catenin, which leads to its stabilization and accumulation in the cytoplasm with subsequent protein relocation to the cell nucleus [13]. UCB CD34$^+$ cells treated with BIO demonstrated rapid (within 24 hours) upregulation of β-catenin as determined by Western analysis (Fig. 1A). The accumulation of β-catenin increased with BIO dose: a 10-fold increase in β-catenin compared to DMSO-

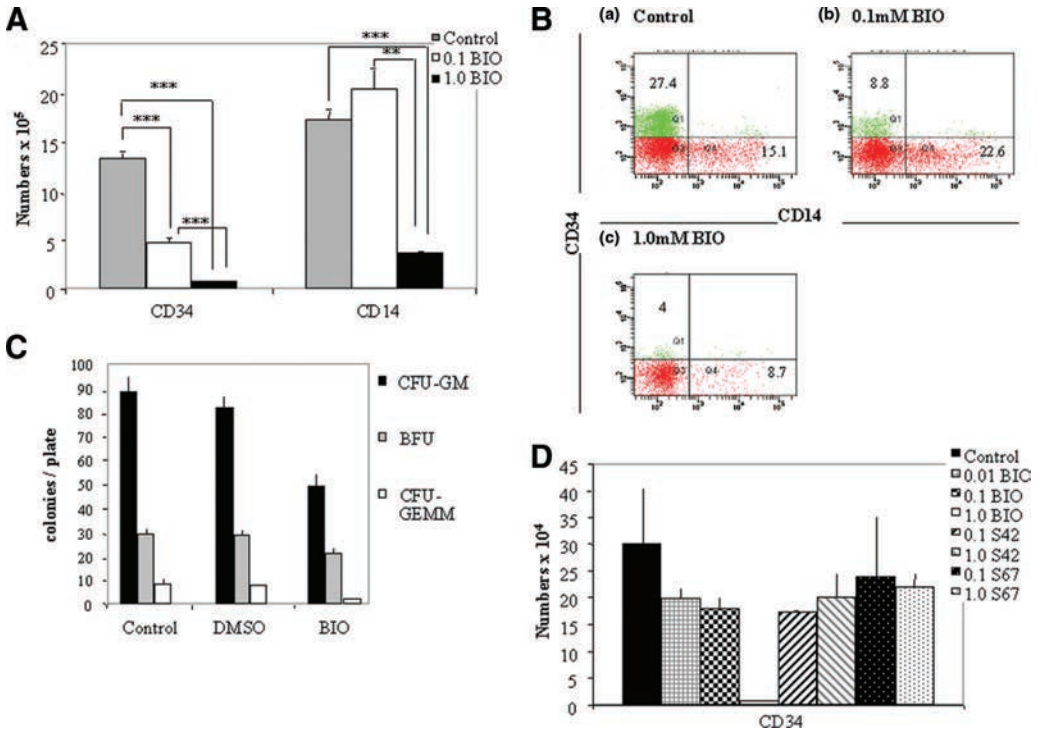

Figure 2. BIO delays CD34$^+$ cell expansion during ex vivo culture of UCB cells. Cells were cultured with SCF, TPO, and FLT3L, all at 20 ng/ml. BIO was added to the cultures at 0.1, 1, and 2 μM doses at the initiation of culture. Control cells were treated with DMSO. Experiments were performed in triplicates. **(A):** CD34$^+$ and CD14$^+$ cells were evaluated on day 5. The results of one representative experiment are shown. Results are expressed as average ± SD (**, $p < .05$; ***, $p < .01$). **(B):** Cells were incubated with antibody against CD34 and CD14. CD34$^+$ and CD14$^+$ cells were measured using two-color flow cytometry in the live gate. The proportion of CD34$^+$ cells was reduced by BIO in a dose-dependent manner from 28% in Control to 9.5% and 4.8% in increasing concentrations of BIO (**Ba–Bc**). Representative dot plots are shown ($n = 10$). **(C):** BIO inhibits growth of CFUs. UCB CD34$^+$ cells were cultured with SCF, TPO, FLT3L, and IL-6 (100 ng/ml) and either BIO (1 μM), DMSO, or neither (Control) for 5 days and then plated in methylcellulose cultures. Total CFU, erythroid (BFU), myeloid (CFU-GM), and mixed (CFU-GEMM) colonies were scored on day 11. Results are presented as averages ± SD ($n = 3$). **(D):** Two other GSK-3β inhibitors S42 and S67 inhibit growth of UCB CD34$^+$ cells compared to control. Each GSK-3β inhibitor was added to UCB at the initiation of the culture at a variety of concentrations as indicated on the graph (μM). Total (data not shown) and CD34$^+$ cell counts were performed on days 5, 7, and 11 ($n = 2$). Day 5 data are presented to reflect earlier effects of GSK-3β inhibition on CD34$^+$ numbers + SD. Abbreviations: BFU, burst-forming unit; BIO, 6-bromoindirubin 3'-oxime; CFU, colony-forming unit; CFU-GM, colony-forming unit, granulocyte-monocyte; CFU-GEMM, colony-forming unit, granulocytic, erythroid, monocyte-macrophage; DMSO, dimethylsulfoxide; GSK-3β, glycogen synthase kinase-3β; IL-6, interleukin-6.

control was seen at 0.1 μM BIO and 40-fold at 1 μM BIO (Fig. 1A). In addition to the accumulation of β-catenin seen in total UCB cell lysates, relocation of β-catenin from the cell cytoplasm to the nucleus was observed 24 hours following addition of BIO (Fig. 1B).

It was previously shown that when β-catenin relocates to the nucleus it binds with T-cell factor (TCF)/lymphocyte enhancer factor (LEF) to induce transcription of target genes such as *c-myc* and *HoxB4*, both involved in the regulation of stem cell activity [14]. Real-time RT-PCR analysis revealed upregulation of *c-myc* and *HoxB4* in UCB CD34$^+$ cells treated with BIO (Fig. 1C). Thus GSK-3β inhibition in UCB CD34$^+$ cells activates β-catenin and modulates the expression of genes involved in the regulation of stem cell activity.

GSK-3β Inhibition Delays the Ex Vivo Expansion of CD34$^+$ Cells but Preserves Long-Term Culture Initiating Cells

β-Catenin activation was previously shown to promote stem cell activity [5, 6]. Contrary to our expectations, GSK-3β inhibition did not further promote, but rather delayed, the expansion of CD34$^+$ cells induced by cytokines: a reduction in percentage and numbers of CD34$^+$ cells, but not total cell counts, was observed in BIO-treated cultures compared with control (Fig. 2A, 2B). Cell cycle analysis performed on days 1 and 5 following treatment did not reveal growth arrest or apoptosis in BIO-treated cells (data not shown). A small increase in the proportion of myeloid progenitor CD34$^-$CD14$^+$ cells was seen at 0.1 μM of BIO (Fig. 2A, 2B). A further increase in the BIO dose suppressed both CD34$^+$ and CD14$^+$ cells (Fig. 2A, 2B). A reduction in CFU numbers—including myeloid (CFU-GM), erythroid (BFU), and mixed (CFU-GEMM) colonies—was observed in BIO cultures compared with control (Fig. 2C). Importantly, CFU activity measured per input cell was not affected by BIO (data not shown). No lineage bias was seen in this assay: there was no significant difference in the proportion of colony types represented in either control or BIO samples (data not shown). These effects were seen at BIO concentrations as low as 0.1 μM, a dose that is 10- to 50-fold lower than the dose shown to expand embryonic stem cells (1–5 μM) [15]. Two other synthetic GSK-3β inhibitors, S3442 and S3567, also suppressed CD34$^+$ cell expansion in a similar pattern (Fig. 2D). Therefore, the delayed expansion of UCB CD34$^+$ cells seen in BIO cultures is not specific for this compound but is common for all pharmacological inhibitors of GSK-3β used in this study.

Notably, reduced CD34$^+$ expansion is not necessarily indicative of depletion of the most primitive progenitor cells. This idea was recently supported in a study showing that delayed expansion of CD34$^+$ cells seen in thrombopoietin-depleted conditions did affect SRCs, the most primitive population [16]. To

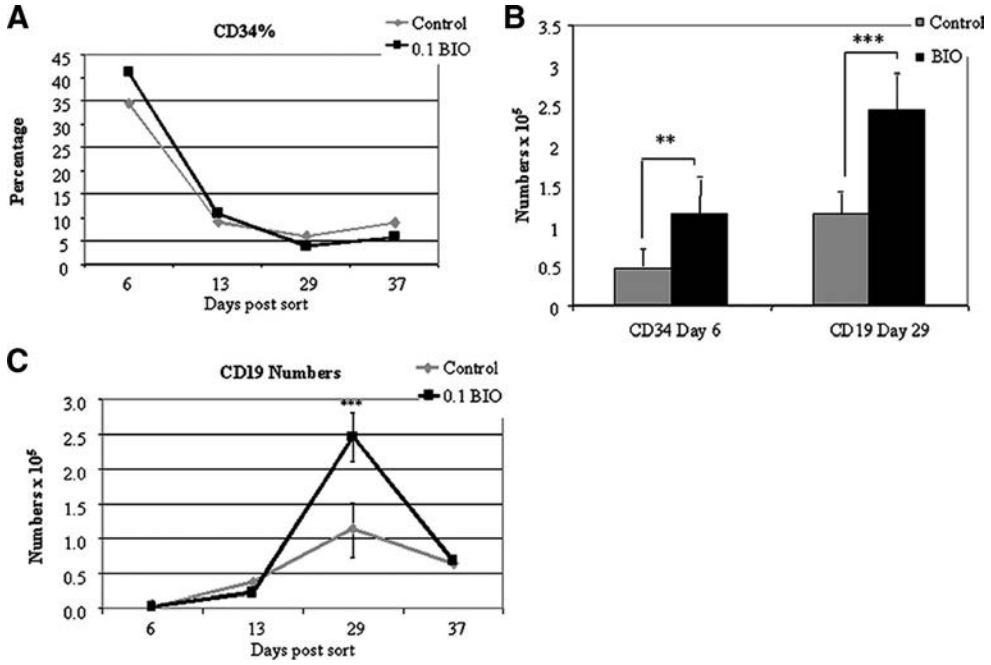

Figure 3. GSK-3β inhibition preserves long-term culture initiating progenitor cells as tested in coculture with murine stroma MS5 cells. UCB $CD34^+$ cells were cultured with SCF, TPO, FLT3L, and IL-6 (100 ng/ml) ± BIO (0.1 μM) for 5 days, fluorescence-activated cell sorted, and then placed on a preformed monolayer of MS5 cells. Flow cytometry analysis of $CD34^+$ and $CD19^+$ cells in the nonadherent fraction of UCB cells cocultured with MS5 cells was performed weekly. The result of one representative experiment performed in triplicate is shown ($n = 3$) (***, $p < .01$). **(A):** CD34% decreased over the time course of the cultures. The percentage was at its peak 6 days post sort with BIO-pretreated cells producing significantly more $CD34^+$ cells at this time point **(B)**. **(C):** $CD19^+$ B-progenitor production peaked much later in the cocultures (day 29) with a similar enhancement of production seen in cultures pretreated with BIO **(B)** Abbreviation: BIO, 6-bromoindirubin 3'-oxime.

analyze stem cell activity, $CD34^+$ cells were cocultured with bone marrow stroma, which has previously been shown to maintain the growth of primitive progenitor/stem cells [17, 18]. Equivalent numbers of unexpanded UCB cells were treated with BIO or DMSO for 5 days in suspension culture, sorted for $CD34^+$ cells by flow cytometry, and subsequently plated on bone marrow stroma MS5 cells and grown for a further 5 weeks without the addition of exogenous growth factors. As was previously shown, gradual reduction in percentages of $CD34^+$ cells was seen over the course of the culture (Fig. 3A). BIO-treated cells produced significantly more $CD34^+$ cells on day 6 (Fig. 3B). The production of $CD19^+$ B-lymphoid progenitor cells supported by MS5 stroma peaked on day 29 in both cultures (Fig. 3B). $CD34^+$ cells treated with BIO produced significantly more $CD19^+$ B-progenitor cells compared with control (Fig. 3C).

Outgrowth of cells at later time points have been previously shown to represent the progeny of long-term culture-initiating cells [17, 18]. When cultures were terminated on day 37, the numbers of cells adherent to the stroma were found to be significantly higher in BIO-treated cultures (Fig. 4A). Moreover, the numbers of the most primitive $CD34^+CD38^-$ cells recovered from the adherent fraction of BIO-treated cultures were also significantly higher than those in control (Fig. 4B). Additionally, the numbers of $CD34^+$ cells and $CD33^+$ myeloid progenitor cells also were higher in BIO-treated cultures, although this did not reach statistical significance (Fig. 4C). Also of note was that $CD34^+CD14^+$ myeloid progenitor cell numbers were similar in both cultures (Fig. 4B). This result indicates that treatment of $CD34^+$ cells with GSK-3β inhibitor prior to coculture with stroma enhances the production of primitive $CD34^+CD38^-$ cells, although this is not at the expense of progenitor development.

Propidium iodide-DNA staining of permeabilized progenitor cells (recovered from the nonadherent fraction of cultures on days 8 and 12) revealed a reduction in the proportion of cells with subgenomic DNA content previously identified as apoptotic, in BIO-treated samples (Fig. 4D). The effect of BIO on UCB cell survival was dose-dependent (Fig. 4Dii). Thus the progeny of BIO-treated cultures demonstrates delayed apoptosis. This may account for the superior preservation of primitive progenitor cells seen in cocultures of BIO-treated UCB cells with MS5 stroma.

Interestingly, an increase in the expression of the chemokine receptor CXCR4 was observed in the adherent fraction of BIO-treated cocultures (Fig. 4E). The expression of CXCR4, VLA-4 (CD49d), and VLA-5 (CD49e) was measured in BIO-treated $CD34^+$ cells 5 days following treatment and a similar level of expression was observed (data not shown). In addition, we did not observe modulation of adherence of these cells to the fibronectin fragment retronectin (ligand for VLA-4 and -5) or to collagen immediately following treatment with the inhibitor (data not shown). It is apparent that other molecular mechanisms are involved in the promotion of the interaction of BIO-treated cells with stroma.

GSK-3β Inhibition Preserves SCID Repopulating Cells

When equivalent numbers of ex vivo expanded UCB cells were transplanted into sublethally irradiated NOD/SCID mice, there were no differences in the mortality between control mice and mice that were injected with BIO-treated UCB cells (data not shown). The percentage of human $CD45^+$ cells recovered from murine bone marrow was similar in control and BIO groups (Fig. 5A, 5B). Despite this, the percentage and total human $CD34^+$ cell numbers recovered from the recipient's bone marrow were significantly higher in mice that received a BIO-treated graft compared with controls. This substantial improvement in $CD34^+$ reconstitution in the BIO group was seen

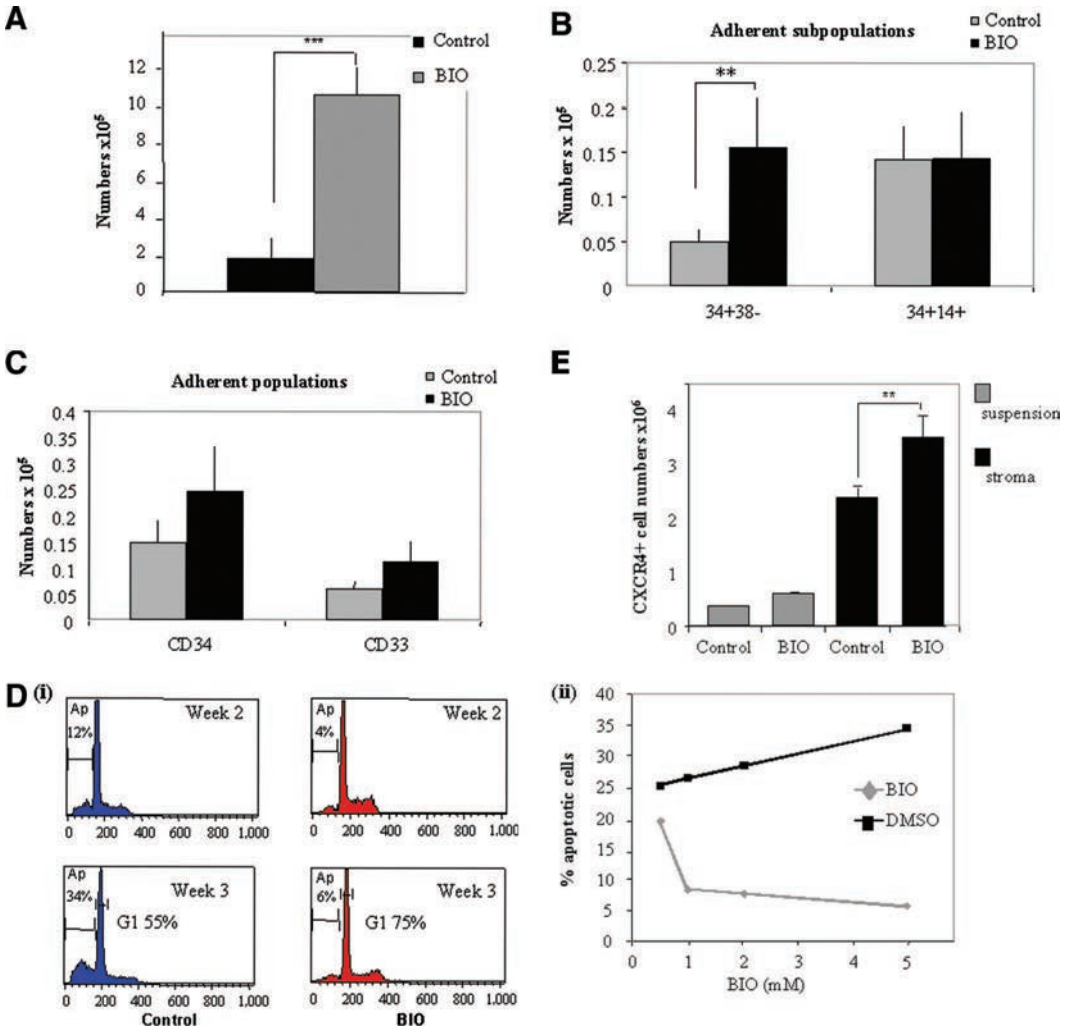

Figure 4. GSK-3β inhibition in UCB cells modifies their interaction with MS5 stroma cells. **(A):** A significant increase was seen in the total numbers of human hematopoietic progenitor cells that became adherent to MS5 stroma cells in BIO-treated cultures (***, $p < .01$) and all lineages were expanded after treatment with 0.1 μM BIO ($n = 4$). Results are presented as averages with SD. **(B):** The numbers of primitive $CD34^+$ $CD38^-$ progenitor cells in the adherent fraction of cocultures initiated with BIO-treated cells were expanded over control. This boost in the primitive compartment was not at the expense of committed $CD14^+$ progenitors, with their numbers unchanged (**, $p < .05$). **(C):** Similar to $CD34^+CD14^+$ progenitor numbers, production of total $CD34^+$ and $CD33^+$ myeloid cells was not affected by BIO treatment in the adherent fraction of cocultures. **(D):** Cell cycle analysis of UCB $CD34^+$ cells cocultured with MS5 cells was performed on day 8 and day 12 following plating. A reduced proportion of cells with subgenomic DNA content (apoptotic cells) was seen in BIO-treated UCB cells cocultured with MS5 stroma **(i)**. This reduction in the proportion of cells undergoing apoptosis was shown to be BIO-dose-dependent **(ii)**. **(E):** Increased CXCR4 expression on $CD34^+$ cells cultured with BIO (0.1 μM), both in suspension and cocultures (**, $p < .05$), was revealed by flow cytometry analysis. Cells were gated on CD34 expression and consequently CXCR4 ($n = 4$). Data shows average with SD. Abbreviations: BIO, 6-bromoindirubin 3'-oxime; DMSO, dimethylsulfoxide.

despite these mice receiving a lower dose of human $CD34^+$ cells as a consequence of delayed ex vivo expansion of BIO-treated cells (Fig. 5C). In addition, the extent of multilineage reconstitution seen in BIO mice was similar to controls, again in spite of the BIO mice receiving a lower dose of human $CD34^+$ cells (Fig. 5D). From these findings, we can suggest that GSK-3β inhibition during ex vivo expansion of UCB cells acts to improve the functional capacity of $CD34^+$ cells and sustains the in vivo expansion of the most primitive SRCs.

LDA was performed to examine the frequency of SRC in ex vivo expanded UCB $CD34^+$ cell samples: 200, 500, 1,000, and 100,000 $CD34^+$ cells derived either from control or BIO-treated cultures were injected into NOD/SCID mice and human $CD45^+$ cell engraftment was examined 6 weeks post-transplant. With the threshold 0.3% established using different doses of human cells mixed with murine bone marrow cells (see Materials and Methods), the percentage of nonengrafted mice in all groups was significantly lower in mice that received BIO-treated cells compared with control mice that received control cells (Fig. 5E). A higher frequency of SRC in BIO-treated $CD34^+$ samples was observed in comparison with control as measured by L-Calc software (Fig. 5E). The total SRC pool was approximately 2.5-fold higher in BIO-treated UCB cultures when normalized to total $CD34^+$ cell numbers per culture. Evidently the sustained in vivo expansion of BIO-treated $CD34^+$ cells results from the higher numbers of SRCs as seen using LDA. Collectively, these results show that GSK-3β inhibition during ex vivo expansion of UCB cells results in better preservation of SRC.

GSK-3β Inhibitors Suppress Leukemic Cell Growth

$CD34^+$ growth suppression induced by BIO prompted us to examine the effect of GSK-3β inhibition on leukemic cell growth. Upregulation of β-catenin was observed in human

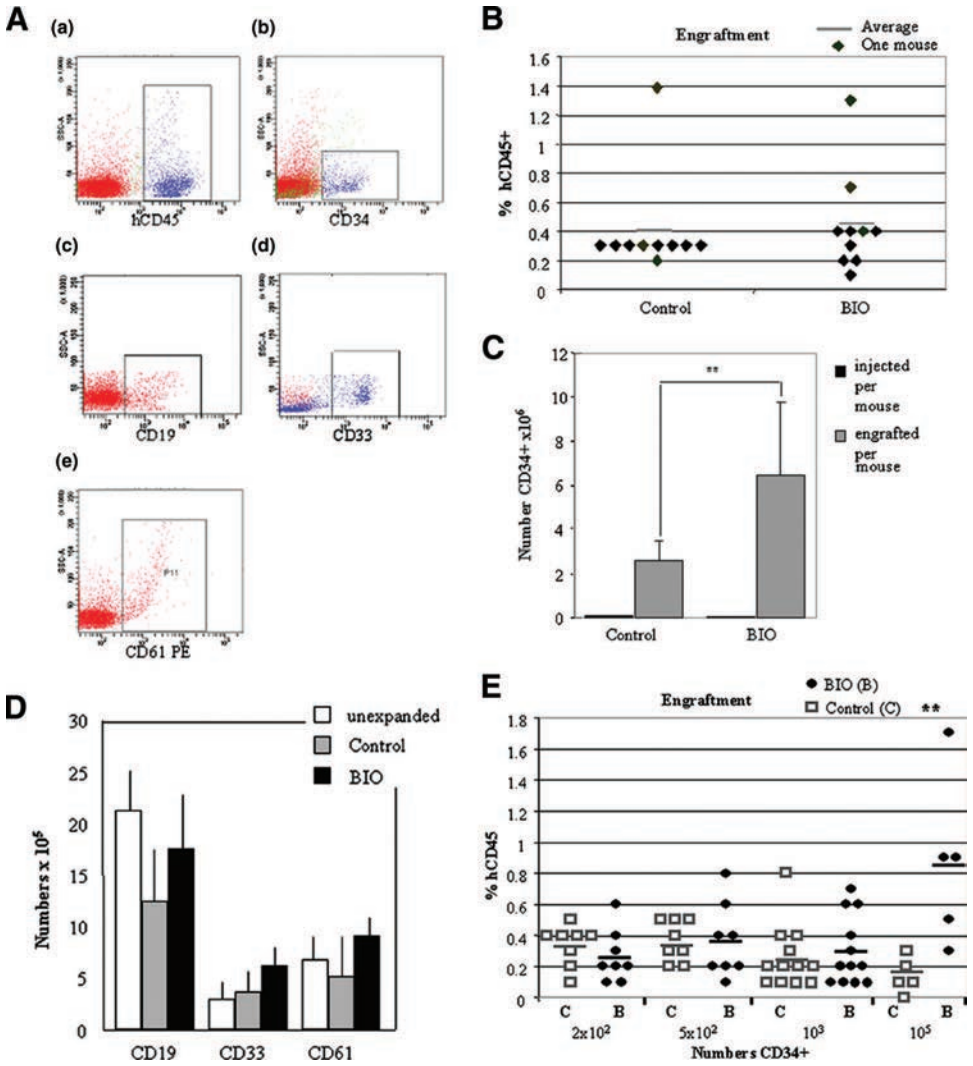

Figure 5. UCB CD34$^+$ cell engraftment in the NOD/SCID mouse model. UCB CD34$^+$ cells were treated with BIO or DMSO (0.1 μM) for 5 days and cell equivalents of unexpanded UCB cells (numbers in culture on day 5 split between five mice per group) were injected intravenously in sublethally irradiated NOD/SCID mice ($n = 2$). **(A):** Representative dot plots demonstrating expression of human CD45$^+$, CD34$^+$, CD19$^+$, CD33$^+$, and CD61$^+$ on cells taken from murine bone marrow. **(B):** Human cell engraftment was measured as the proportion of human CD45$^+$ cells in the recipient bone marrow. No significant difference was seen in the level of human CD45$^+$ engraftment between BIO and Control groups. **(C):** A significant increase in the in vivo expansion of injected human CD34$^+$ cells was seen in the bone marrow of BIO mice compared with control mice (**, $p < .05$). **(D):** Markers specific for B-cells, myelomonocytic cells, and megakaryocytes were evaluated by flow cytometry to show successful multilineage reconstitution in recipient mice from all groups. Graph represents average values with SD. None of these values reached statistical significance. **(E):** LDA of ex vivo expanded UCB CD34$^+$ cells: 200, 500, 1,000, and 100,000 CD34$^+$ cells derived from control or 0.1 μM BIO-treated cultures were injected into each mouse following 5 days ex vivo culture. Human CD45$^+$ cell engraftment in the bone marrow of the recipient mice was analyzed 6 weeks following injection. Mice were considered as engrafted when the percentage of hCD45$^+$ cells in the bone marrow was equal to or higher than 0.3%. Horizontal bars represent the mean values for each group. L-Calc-software (StemCell Technologies) was used to determine the frequency of SRC ($n = 3$). Abbreviations: BIO, 6-bromoindirubin 3'-oxime; LDA, limiting dilution analysis; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; PE, phycoerythrin; SRC, SCID repopulating cell.

CD34$^+$ leukemic TF-1 cells treated with BIO (Fig. 1A). The basal level of β-catenin in TF-1 cells was sixfold higher than that in UCB CD34$^+$ cells (Fig. 1A).

Four acute myeloid leukemia (AML) cell lines were used to evaluate the effect of BIO on leukemic cells. When BIO was added to human leukemic TF-1, K-562, U937, and HL-60 cells, suppression of cell growth was observed that was associated with the induction of apoptosis: both the numbers of subG1 cells and annexin-positive cells were much higher in BIO-treated cultures compared with control, whereas the proportions of actively cycling cells remained comparatively unchanged (Fig. 6A–6D). Growth inhibition was BIO-dose-dependent and was seen as early as 24 hours following BIO administration (Fig. 6A, 6B).

TF-1 cells treated with BIO for as short a time as 24 hours exhibited reduced CFU activity when plated in clonogenic assay: both the numbers of CFUs per culture and the number of cells collected from pooled CFUs were dramatically reduced following treatment with BIO (Fig. 6Ea). It is relevant that the decline in the numbers of clonogenic cells was significantly higher compared with the number of apoptotic cells detected following treatment with BIO (Fig. 6B, 6Ea). Thus BIO acts not only to induce apoptosis but also to suppress the clonogenic proliferation of surviving cells.

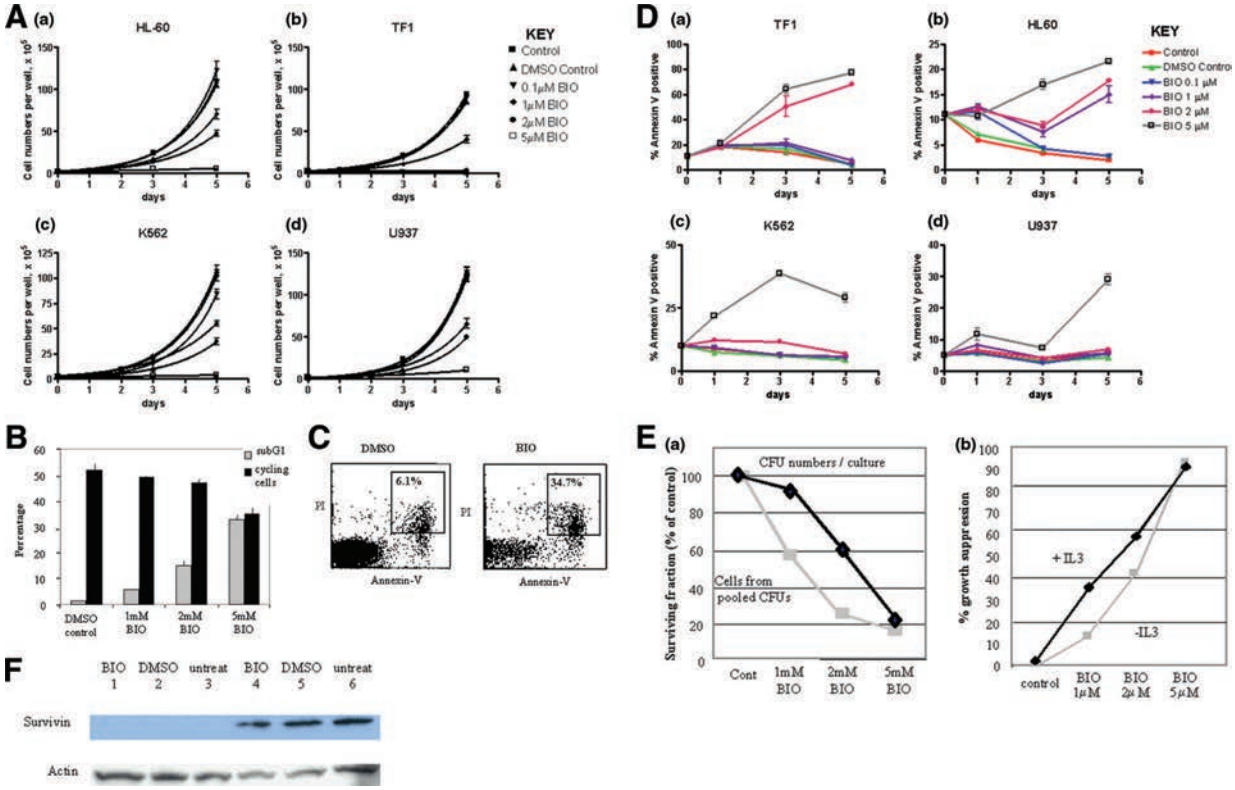

Figure 6. BIO inhibits leukemia cell growth. **(A):** Growth curves of four acute myeloid leukemia leukemic cell lines treated with different concentrations of BIO shows a BIO-dose-dependent suppression of cell numbers compared to control over 5 days of culture. Results show one representative experiment performed in triplicate ($n = 2$). **(B):** Cell cycle analysis of TF-1 cells treated with BIO. The percentage of cells with subgenomic DNA content (subG1) and cycling (S + G2/M) cells was analyzed by flow cytometry 24 hours following addition of BIO. Results show one representative experiment performed in triplicate ($n = 2$). **(C):** Flow cytometry analysis of annexin/PI staining of TF-1 cell treated with BIO. Double-positive cells were considered as apoptotic (gated). The proportion of apoptotic cells in the control group was 6.1% compared with BIO-treated cells that contained almost sixfold more than this at 34.7%. Staining was performed at 24 hours following addition of 1 μM BIO. **(D):** The cytotoxic effect of BIO was seen in human leukemia TF-1, HL-60, K562, and U937 cells measured by annexin staining for apoptosis over 5 days of culture. Results show one representative experiment performed in triplicate ($n = 2$). **(E):** BIO inhibits clonogenic proliferation of leukemic TF-1 blasts. TF-1 cells were incubated with BIO for 24 hours, washed twice with phosphate-buffered saline, and plated in clonogenic assay. The surviving fraction of clonogenic blasts is presented as percentage of control cells treated with DMSO only **(Ea)**. The presence of IL-3 in cultures of TF-1 cells modified the effect of BIO on leukemic cell death. Depletion of IL-3 from TF-1 cultures resulted in resistance of cells to BIO-induced cytotoxicity **(Eb)**. Results show one representative experiment performed in triplicate ($n = 2$). **(F):** Expression of survivin in TF-1 cells treated with 1 μM BIO at 24 hours. Lanes 1–3: UCB $CD34^+$ cells. Lanes 4–6: TF-1 cells. Treatment with BIO resulted in a fourfold reduction in survivin protein expression in TF-1 cells after short-term exposure. Abbreviations: BIO, 6-bromoindirubin 3'-oxime; CFU, colony-forming unit; DMSO, dimethylsulfoxide.

In vitro, TF-1 cells are reliant on IL-3 for proliferation, so we investigated the interaction between IL-3 and BIO-induced growth suppression. In cultures of TF-1 cells that were prestimulated for 2 days in IL-3-depleted conditions prior to the addition of BIO, a decrease in the extent to which BIO was able to induce cell death was observed compared to IL-3-replete cultures (Fig. 6Eb). It is of interest that IL-3 withdrawal resulted in a 40% reduction in cell numbers, although no apoptosis was observed (data not shown). Thus IL-3 withdrawal appears to hinder cell proliferation and also makes TF-1 cells less susceptible to apoptosis induced by BIO. Apoptosis induced by BIO in TF-1 cells was accompanied by reduced expression of the inhibitor of apoptosis protein survivin (Fig. 6F). It is relevant that TF-1 cells express abnormally high levels of survivin message and protein compared with those of UCB $CD34^+$ cells (Fig. 6F). It was previously shown that survivin expression in leukemic cells is, at least in part, regulated by NF-κB [20]. Expression analysis revealed a twofold higher expression of DNA binding and p100 subunits of NF-κB in leukemic TF-1 cells compared with UCB $CD34^+$ cells (data not shown). We hypothesize that BIO-mediated downregulation of NF-κB and survivin acts to deregulate the balance of pro- and anti-apoptotic signals toward apoptosis in TF-1 cells.

DISCUSSION

Here we show that GSK-3β inhibition in UCB $CD34^+$ cells results in the accumulation of β-catenin and its relocation from the cytoplasm to the cell nucleus [21]. In addition, we observed upregulation of *c-myc* and *HoxB4*. We chose to focus on these genes because both are transcriptional targets of β-catenin previously shown to regulate stem cell activity [22, 23]. Activation of β-catenin and downstream target genes *c-myc* and *HoxB4* correlates with the preservation of long-term culture-initiating cells observed in coculture with bone marrow stroma cells. In addition, the increased frequency of SRC and the sustained in vivo expansion of human $CD34^+$ cells were seen in the bone marrow of NOD/SCID mice transplanted with BIO-treated UCB cells. We hypothesize that GSK-3β inhibition maintains the

pool of HSCs through the activation of β-catenin and its target genes *c-myc* and *HoxB4*.

Reduced apoptosis was observed in BIO-treated UCB $CD34^+$ when cocultured with stroma. This reduction in cell death may contribute to the preservation of HSC activity as previously suggested [24]. GSK-3β inhibition also upregulated CXCR4 and increased adherence of UCB $CD34^+$ cells to bone marrow stroma MS5 cells. It was previously shown that bone marrow stroma cells secrete CXCR4-specific ligand SDF-1 [25]. The interaction of UCB $CD34^+$ cells with bone marrow stroma cells promoted by GSK-3β inhibition may contribute to the improved regeneration produced by BIO-treated UCB cells in the NOD/SCID mouse model. The latter is in line with recently published data showing that in vivo administration of GSK-3β inhibitors improves the engraftment and multilineage reconstitution in the NOD/SCID mouse model [9].

Here we also show that GSK-3β inhibition acts to suppress leukemic cell growth. Remarkably, apoptosis was seen in leukemic TF-1, U937, K562, and HL-60 cells treated with the same dose of BIO. It was recently reported that GSK-3β inhibition increases the sensitivity of AML cells to chemotherapy [27]. The induction of AML cell death by GSK-3β inhibitors alone has not been reported previously. Paradoxically, upregulation of β-catenin in TF-1 cells after treatment with BIO correlated with apoptosis, whereas in normal $CD34^+$ cells, upregulation of β-catenin was associated with a decline in cell death. Accordingly, the susceptibility of HPCs to apoptosis does not correlate with the level of β-catenin. Likewise, it was recently shown that the sensitivity of AML cells to chemotherapy does not correlate with the expression of β-catenin but rather depends on the activity of NK-κB, which is regulated by GSK-3β inhibition in AML [27]. Recent data demonstrated that inhibition of GSK-3β activity leads to epigenetic silencing of NF-κB target genes and induction apoptosis in chronic lymphocytic leukemia B cells [28]. In addition, it was shown that NF-κB controls the expression of survivin in TF-1 cells [27, 29]. Apoptosis induced by BIO in TF-1 cells correlates with downregulation of survivin. It was recently shown that inhibition of GSK-3β interferes with the transcriptional activity of NF-κB and affected the expression of survivin in TF-1 cells [29]. It is relevant that normal $CD34^+$ cells do not depend on the NF-κB survival pathway to the extent that leukemic cells do [29].

CONCLUSION

Collectively, our results show that GSK-3β inhibition plays an essential role in restricting the activation of normal hematopoietic stem cells ex vivo, thereby preserving their in vivo potential while inhibiting leukemogenesis. Consequently, inhibitors of GSK-3β may increase the range of novel anticancer tools.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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