GSK-3 β inhibition promotes early engraftment of *ex vivo*-expanded haematopoietic stem cells

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Abstract

Objectives: Umbilical cord blood (UCB) is a source of stem cells used for allogeneic transplantation, in addition to bone marrow and peripheral blood. Limited numbers of stem cells in a single UCB unit is associated with slow haematopoietic recovery and high risk of graft failure, particularly in adult patients. UCB stem cells can be expanded *ex vivo*; however, rapid differentiation reduces their regenerative potential. We have recently shown that Wnt/ β -catenin signalling is down-regulated in *ex vivo*-expanded stem cells; therefore, we propose that re-activation of Wnt signalling using GSK-3 β inhibition may act to improve regenerative potential of these *ex vivo*-expanded stem cells.

Materials and methods: Immunocompromised mice were employed in transplantation studies to determine stem-cell engraftment. Flow cytometry was used to phenotype the engrafted human cells. Retroviral gene transfer was used to examine the role of *Myc* gene up-regulated by GSK-3 β inhibition, in *ex vivo*-expanded stem cells.

Results: Treatment with GSK-3 β inhibitor, 6-bromindirubin 3'-oxime (BIO) improved early human cell engraftment in the mice and elevated the numbers of myeloid progenitor cells in cytokine-stimulated culture. BIO up-regulated β -catenin and c-myc in *ex vivo*-expanded stem cells. Ectopic expression of *Myc* acted to increase clonogenic potential and to delay differentiation of haematopoietic progenitor cells, suggesting the potential

mechanism to improve regenerative potential of *ex vivo*-expanded grafts.

Conclusions: Pharmacological inhibition of GSK-3 β provided a novel approach to improve early engraftment of *ex vivo*-expanded haematopoietic progenitor cells.

Introduction

Umbilical cord blood (UCB) is an attractive stem-cell graft option for patients who need allogeneic haematopoietic stem-cell transplantation (allo-HSCT). UCB has several advantages over bone marrow (BM) or peripheral blood (PB) stem-cell transplantation, such as reduced incidence and severity of graft-versus-host disease, which allows partial matching between donor and recipient (1). However, the limited low number of haematopoietic progenitor cells (HPC) in a single UCB unit can lead to increased risk of graft failure, delayed haematological recovery and prolonged immunosuppression, particularly in adult patients (1,2). *Ex vivo* expansion of HPC with stimulatory cytokines and growth factors, mesenchymal stem cells, Notch ligand, copper chelators, prostaglandins, complement components, nicotinamide and CD26/DPPIV inhibitors have all been used to expand HPC *ex vivo* [see (1) and (2) for reviews]. Clinical data demonstrate that transplantation of *ex vivo*-expanded HPC, in most cases, does not reduce early haematopoietic engraftment (3,4). In line with these results, we have recently demonstrated a defect in early engraftment of *ex vivo*-expanded HPC using the 'humanized' NOD-SCIDIL2R $\gamma^{-/-}$ (NSG) mouse model (5). Using this model, we have shown that early engraftment was delayed in mice that received *ex vivo*-expanded HPC (5). *Ex vivo*-cultured HPC had reduced rate of expansion and self-renewal. Rates of short-term engraftment in PB, *de novo* T-cell

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generation and multi-lineage reconstitution of BM in mouse models was lower despite transplantation of expanded numbers of HPC. High-resolution division tracking revealed that CD34⁺ CD38⁺ HPC, that we would expect to promote short-term engraftment in the expanded product, had reduced rates of expansion and self-renewal probability and increased rate of differentiation, compared to non-expanded cells. Gene expression analysis showed complex modulation of multiple genes and signal cascades, consistent with reduced self-renewal and increased differentiation rate in *ex vivo*-expanded CD34⁺ HPC. In addition, decline in short-term engraftment in mice transplanted with expanded cells was associated with reduction in numbers of CD34⁺ CD38⁻ cells with short-term and long-term SCID repopulating ability. Thus, reduced regenerative capability and increased propensity for differentiation is likely to account for reduced engraftment of *ex vivo*-expanded HPC (4).

Wnt/ β -catenin signalling plays an important role in regulation of stem cell regenerative capacity during homeostatic haematopoiesis (6). Wnt ligands are provided to HPC by BM stromal cells (7); Wnt signalling in the BM niche enforces haematopoietic stem cell quiescence, and is necessary to preserve self-renewal *in vivo* (8). UCB-derived HPCs do not have stromal support and therefore are depleted of exogenous Wnt stimulation. UCB-derived CD34⁺ HPC exhibit low β -catenin expression (5). β -catenin is up-regulated by cytokine stimulation *in vitro*, and then rapidly declines in differentiating HPC (5). We proposed that induction of Wnt signalling via glycogen synthase kinase-3 β (GSK-3 β) inhibition, using the small molecule inhibitor 6-bromindirubin 3'-oxime (BIO), may improve regenerative potential of *ex vivo*-expanded HPC. Indeed, *ex vivo*-expanded HPC treated with BIO for the last 24 h of *ex vivo* culture have demonstrated improved stem cell engraftment, assessed in a NOD/SCID mouse model (9). The effect of GSK-3 β inhibition on early haematopoietic engraftment, however, was not investigated in this study, as early engrafting lineage committed HPC are rejected by residual natural killer (NK) cells in NOD/SCID mice (10). NOD-SCID IL2R γ -/- (NSG) mice however, lacking NK cells, provide an excellent model that allows monitoring of both early and late haematopoietic cell engraftment following human stem cell transplantation (11). Here, we demonstrate that treatment with BIO improved early engraftment of *ex vivo*-expanded human HPC. Limiting dilution analysis demonstrated increased frequency of early engrafting cells in BIO-treated cultures. BIO-treatment promoted attachment of *ex vivo*-expanded CD34⁺ cells to retronectin, a specific fragment of fibronectin, which is a major

component of extracellular matrix in the BM. Thus, we speculated that BIO-treatment may act to promote early engrafting stem cell homing to the BM. In addition, our *in vitro* analysis revealed that BIO-treatment promoted myeloid progenitor cell expansion in cytokine-stimulated suspension culture and increased clonogenic growth in methylcellulose culture. Mechanistically, BIO acted to up-regulate β -catenin and c-myc proteins. It is relevant that c-Myc gene expression is regulated by β -catenin (12). Similar to BIO treatment, forced expression of v-Myc, viral homolog of c-Myc, promoted myeloid progenitor cell expansion and clonogenic growth, suggesting a potential mechanism triggered by BIO through activation of Myc. Together with our previous data, this study demonstrates that transient pharmacological inhibition of GSK-3 β provides a novel approach to improve early and late engraftment of *ex vivo*-expanded HPC.

Materials and methods

Cell culture

Umbilical cord blood was provided by Sydney Cord Blood Bank. All experiments were approved by the Sydney Children's Hospital Human Research Ethics Committee. Mononuclear cells were purified from UCB by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA) density-gradient centrifugation. CD34⁺ cells were separated to >95% purity by positive selection, using a magnetic cell purification system (autoMACS Miltenyi Biotec, Auburn, CA, USA). CD34⁺ cells were seeded at $0.5-1 \times 10^5$ cells/ml in serum-free medium Stemline II (Sigma Aldrich, Castle Hill, NSW, Australia), 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 100 ng/ml concentration (R&D Systems, Minneapolis, MN, USA). CD34⁺ cells were expanded for 5 days then treated with small molecule inhibitor of GSK-3 β , BIO at 0.5 μ M for 24 h, or DMSO. Cells were washed to remove the inhibitor and either injected into mice or further cultured *in vitro* in the presence of the same cytokines.

Flow cytometry

Flow cytometric analysis was performed using CD34 antibody (Becton Dickinson, San Jose, CA, USA). 1×10^5 events were acquired on a FACS Canto, and data were analysed using FACSDiva software (Becton Dickinson). Viable cells were gated based on forward vs. side scatter (FSC/SSC). Cell cycle analysis was performed as previously reported (13).

Colony-forming unit (CFU) assay

Umbilical cord blood-derived CD34⁺ cells were plated in MethoCult GF H4434 (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, 10 ng/ml IL-3, supplemented with 3 U/ml of erythropoietin (Stem Cell Technologies, Vancouver, BC, Canada). One thousand cells suspended in 1.0 ml were plated in a 35-mm dish in triplicate, and cultured for 10–14 days. CFU were scored into three categories: burst forming unit erythroid (BFU-E), granulo-myelomonocytic (CFU-GM) and mixed CFUs – producing granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM). CFU analysis was performed at time point indicated in figures/figure legends.

Adhesion assay

Adhesion of CD34⁺ cells was assayed as previously described, with minor modifications (14). Briefly, 96-well tissue culture plates were coated with 50 μ l/well PBS containing 10 μ g/ml retronectin, and incubated overnight at 4 °C. Culture plates were washed three times in PBS and blocked with IMDM containing 2% BSA for 2 h at 37 °C. Control or BIO-treated re-selected CD34⁺ cells were added to the wells (1 $\times$ 10⁴ cells/well in 100 μ l IMDM in the presence of 10% FCS and SCF, TPO and Flt3L, all at 20 ng/ml), allowed to adhere for 15, 30 and 60 min at 37 °C, then washed three times carefully in PBS to remove non-adherent cells. Subsequently, adherent cells were isolated using cell dissociation solution (Invitrogen, Life Technologies, Mulgrave, Vic., Australia). All measurements were performed in triplicate, percentages of total plated cells bound to retronectin being determined. Mean and SD were calculated. In a separate experiment, VLA4 and VLA5 expressions were measured in suspended and adherent cells, 24 h post-plating, using anti-VLA4 and anti-VLA5 antibody (Becton Dickinson; product numbers 5559881 and 555617, respectively) by flow cytometry.

Bone marrow transplantation model

Approval from the UNSW Animal Care and Ethics Committee was obtained for animal experiments. NOD-SCID Il2rg^{-/-} (NSG) mice were obtained from the Jackson Laboratory and bred in the animal facility at Children's Cancer Institute Australia for Medical Research. Mice 6–8 weeks old were irradiated with a sub-lethal dose of 2.5 Gy from a cobalt⁶⁰ source, 12–24 h before being transplanted with UCB cells by intravenous (IV) injection. *Ex vivo*-expanded CD34⁺ cells were administered to

all mice at cell dose equivalent to 1 $\times$ 10⁵ unexpanded cells. Weekly bleeding was performed from the tail vein to monitor human cell engraftment in PB of transplant recipient mice. Human and murine cells were distinguished by species-specific expression of CD45 and proportion of cells labelled with human CD45 (hCD45) only was taken to be the level of engraftment. Antibodies specific to human CD33 and CD19 were used to examine multilineage reconstitution in PB. Human platelets and T-cells in PB were examined using anti human CD41a and CD3 antibody, respectively.

Limiting dilution analysis was conducted to determine frequency of early engrafting cells per sample. 1 $\times$ 10⁵, 0.5 $\times$ 10⁵ and 0.1 $\times$ 10⁵ of *ex vivo*-expanded BIO- or DMSO-treated cells per mouse were transplanted, *n* = 4 for each cell dose, both for control and BIO-groups. Human CD45⁺ cell engraftment in PB was measured at week 3 post-transplantation. To determine the threshold for positive engraftment, experimentation was conducted whereby increasing percentages of human CD45⁺ cells were added to murine irradiated BM CD45⁺ cells. Using this method, a threshold of 0.5% human CD45⁺ cells was established as a reliable predictor of positive engraftment. The proportion of non-engrafted mice was then determined. L-Calc software (Stem Cell Technologies) was used to determine frequency of early engrafting cells.

Retroviral gene transfer

Umbilical cord blood-derived CD34⁺ cells were infected with AV virus carrying v-Myc gene (myelocytomatosis viral homologue of c-Myc), and Neo gene, or control LNL6 virus carrying only Neo gene, as previously described (15). Briefly, CD34⁺ cells (0.5 $\times$ 10⁵ cells/ml) were pre-stimulated with SCF, Flt3L, TPO and IL6, all at 100 μ g/ml, for 2 days, then plated on RenroNectin (25 μ g/ml)-covered plates and incubated with virus-containing supernatant collected from AV- or LNL6-virus producing cells. Two rounds of infection were performed. G418-selection was used to determine transduction efficiency in CFU-assay. G418 (800 μ g/ml) was added to methylcellulose, and transduction efficiency was determined as ratio of CFUs generated in G418-containing cultures, to CFUs generated in cultures without addition of G418. Transduction efficiency was determined on days 11, 19 and 34 post infection. Each CFU analysis was performed in triplicate.

Western blot analysis

Protein lysates were prepared as previously described (13). Antibodies used were: rabbit-derived anti-human

actin (A2006; Sigma Aldrich), mouse-derived anti- β -catenin, anti-human c-myc (610154; BD Pharmingen, North Ryde, NSW, Australia) and anti-mouse HRP (NA931V; GE Healthcare, Rydalmere, NSW, Australia). Chemiluminescence was detected with SuperSignal West Femto Maximum Sensitivity Substrate kit (34096; Thermo Scientific, Hudson, NH, USA). QuantityOne (Bio-Rad, Hercules, CA, USA) software was used to quantify protein expression.

Statistical analysis

All *in vitro* experiments were performed at least twice. Differences between conditions were examined for statistical significance using Student's *t*-test. In animal experiments, number of mice is indicated on the figure or in the legend. Student's *t*-test was used to determine significance of differences between two groups. *P* values of <0.05 were considered statistically significant.

Results

BIO treatment promoted early engraftment of ex vivo-expanded HPC in NSG mice

The NSG mice were transplanted with 6-day expanded CD34⁺ HPC, treated with BIO or with DMSO, for the previous 24 h. Each mouse was transplanted with the equivalent of 10^5 unexpanded CD34⁺ cells. It is relevant that pre-treatment with BIO did not modulate total or CD34⁺ cell numbers in the graft, thus, mice from both groups received similar numbers of cells. Human CD45⁺ cells were detectable in PB of all transplanted mice as early as 4 weeks post-transplant in both groups (Fig. 1a). Mice were considered positive for human cell engraftment if the proportion of hCD45⁺ cells was more than 0.5%. Proportions of hCD45⁺ cells gradually increased, reaching a plateau of approximately 70% by week 14 (Fig. 1a). Proportions of hCD45⁺ cells determined at weeks 4, 7 and 8 were significantly higher in the BIO-group compared to the control group ($P = 0.0424$, 0.0179 and 0.0157 , respectively (Fig. 1a). No significant differences were observed between control and BIO-group at later stages post-transplant (Fig. 1a). Human CD45⁺ cells were predominantly of the B-lineage (CD19⁺), with a small proportion of hCD33⁺ myeloid cells detectable in PB at week 9 post-transplantation (Fig. 1b,c). This is consistent with poor myeloid cell differentiation reported in NSG mice that can be improved by providing human GM-CSF and IL3 (16). CD19/CD33 ratio was not affected by BIO-treatment (Fig. 1c), suggesting that GSK-3 β inhibition did not modulate commitment of CD34⁺ HPC to differenti-

ate into B cell lymphoid and myeloid cells. Human CD3⁺ T-cells were detectable in PB of transplant recipient mice beginning from week 8, and their proportions gradually increased by week 20 (Fig. 1d). Proportions of hCD3⁺ cells were similar in control and BIO-groups (Fig. 1d). Thus, the results show that transient inhibition of GSK-3 β prior to transplantation acted to improve early engraftment of *ex vivo*-expanded stem cells and did not affect their commitment to differentiate into B-cell lymphoid, myeloid and T cells.

Limiting dilution transplants were performed to determine whether early increase in human cell engraftment reflected an increase in early engrafting cell (EEC) numbers. *Ex vivo*-expanded cells derived either from control or BIO-treated cultures were injected into mice, at doses equivalent to 1×10^5 , 0.5×10^5 and 0.1×10^5 unexpanded cells per mouse ($n = 4$ per cell dose), and hCD45⁺ cell engraftment was examined 3 weeks post-transplantation. With the threshold 0.5% established using different doses of hCD45⁺ cells mixed with mCD45⁺ cells (see Materials and methods sections), percentage of non-engrafted mice in groups injected with 1×10^5 and 0.5×10^5 cells/per mouse was lower in mice that received BIO-treated cells compared to control mice (Fig. 2a). Frequency of EECs in control and BIO-treated samples was calculated using L-Calc software. Frequency of EEC was 1 in 5.99×10^5 in BIO-cells and 1 in 3.3×10^6 , $P = 0.0429$ (Fig. 2b). Thus BIO-treatment appeared to increase frequency of EEC.

BIO-treatment increased cell attachment to retronectin

Stem cell homing to BM is a critical step in post-transplantation haematopoiesis and integrins VLA4 and VLA5 play an important role in stem cell homing to the BM (17). *Ex vivo* expansion of HPC was associated with down-regulation of VLA4 integrin-mediated engraftment in NOD/SCID β 2-microglobulin-null mice (18). Here, we show that more than 80% CD34⁺ cells, including CD38⁺ and CD38⁻ fractions, expressed VLA4 in control and BIO-treated cells (Fig. 3a, left panels). Expression of VLA5 was seen in $<30\%$ CD34⁺ cells in both samples (Fig. 3b). Control and BIO-treated cells were plated on retronectin-covered plates, and proportions of adherent cells were analysed 15, 30 and 60 min after plating. BIO treatment increased cell attachment to retronectin – statistically significant differences between control and BIO-treated cells being seen 15 and 30 min after plating [$P = 0.003$ and 0.02 , respectively (Fig. 3c)]. By 60 min, more than 80% CD34⁺ cells in each sample type were adherent to retronectin (Fig. 3c). BIO effects were shown in 2 independent experiments performed using CD34⁺ cells from 2 different UCB

Figure 1. BIO treatment promotes early engraftment in mice transplanted with *ex vivo*-expanded haematopoietic progenitor cells (HPC). (a) Ten mice were transplanted with 6-day expanded CD34⁺ HPC treated with BIO (5 mice) or DMSO (5 mice) for the previous 24 h. Mice transplanted with BIO-treated cells exhibited significantly higher proportion of hCD45⁺ and hCD19⁺ cells in peripheral blood (PB), by weeks 4, 7 and 8. At later time points, there were no significant differences between control and BIO-groups. (b) Human CD33 and CD19 analysis was performed with hCD45⁺ gating (shown in circle). One representative analysis performed at week 20 post-transplant is shown. (c) Proportions of hCD19⁺ and hCD33⁺ cells in the hCD45⁺ gate were analysed at weeks 9, 11, 14 and 17 post-transplantation. There were no significant differences between control and BIO-groups. (d) Human CD3⁺ T cells were detected in PB of mice beginning from week 8 post-transplantation. Proportion of hCD3⁺ T cells gradually increased in PB of mice from both groups, and no difference in the percentage of hCD3⁺ cells between the two groups was demonstrated at any time point.

Figure 2. Limiting dilution analysis (LDA) to determine frequency of early engrafting cells (EEC) in control and BIO-treated cells. 1×10^5 , 0.5×10^5 and 0.1×10^5 *ex vivo*-expanded cells from control or BIO-treated cultures were injected into each mouse, 4 mice per cell dose. (a) Human CD45⁺ cell engraftment in peripheral blood (PB) of the recipient mice was analysed 3 weeks following injection. Mice were considered engrafted when percentage of hCD45⁺ cells in PB was equal to, or higher than 0.5%. (b) L-Cal software (Stem Cell Technologies) was used to determine frequency of EEC. Frequency of week 3 engrafting cells was 1 in 5.99×10^5 in BIO-cells and 1 in 3.3×10^6 , $P = 0.0429$. Of note, one mouse from BIO-group injected with 0.5×10^5 cells, died.

Figure 3. BIO-treatment promoted attachment of *ex vivo*-expanded CD34⁺ cells to retronectin. CD34⁺ cells were expanded for 5 days, treated with BIO for an additional 24 h and then plated on retronectin covered plates or cultured in suspension. (a) Control and BIO-treated CD34⁺ haematopoietic progenitor cells (HPC) cultured in suspension exhibited similar VLA4 expression (left panel for control and BIO, respectively). Reduced VLA4 expression was registered in both control and BIO-treated cells recovered from the adherent fraction 24 h following plating (right panel for control and BIO, respectively). VLA4 expression in CD38[−] fraction of CD34⁺ HPC is shown, circled. One representative flow image is shown. VLA4 expression was measured in triplicate. Reduced VLA4 expression in retronectin-attached cells confirms VLA4 engagement in HPC/retronectin interaction. P values for VLA4 expression in control and BIO-groups were >0.05 and therefore are not significant. (b) VLA5 expression in CD34⁺ cells (top-right quadrants, circled) was not affected by BIO treatment in suspension cells (left panels for control and BIO); VLA5 expression was reduced in adherent cells (right panels for control and BIO, top-right quadrants, circled). Reduced VLA5 expression in retronectin-attached cells confirms VLA5 engagement in HPC/retronectin interaction. One representative flow cytometric analysis is shown. VLA5 expression was performed in triplicate. There were no significant differences in VLA5 expression between control and BIO-cells ($P > 0.05$). (c) BIO-treatment promoted attachment of *ex vivo*-expanded CD34⁺ HPC to retronectin. CD34⁺ cell attachment was analysed 15, 30 and 60 min after plating as described in the Materials and methods section. Average of three measurements and SD were determined. Proportion of retronectin attached cells analysed 15 and 30 min after plating was significantly higher in BIO-treated cultures compared to controls, $P = 0.003$ and 0.02 , respectively. At 60 min, approximately 90% in both cultures were adherent to retronectin with no significant differences between control and BIO-treated cells observed.

donors. Integrin expression in cells recovered from the adherent fraction, 24 h post-plating, was significantly lower than in cells cultured in suspension (Fig. 3a,b), which is consistent with internalization of integrins

following binding to retronectin, as previously reported (19). Thus, both VLA4 and VLA5 integrins appeared to be engaged in CD34⁺ cell attachment to retronectin. There were no difference in VLA4 or VLA5 expression

between control and BIO-cells, suggesting that the effect of BIO on rapid attachment of CD34⁺ cells to retronectin was not mediated through modulation of integrin expression (Fig. 3a,b). Apparently, an alternative mechanism is triggered by BIO to promote attachment of *ex vivo*-expanded CD34⁺ HPC to retronectin. We propose that increased attachment of BIO-treated stem cells to the extracellular matrix may act to improve stem cells binding to the BM.

BIO-treatment improved HPC regeneration in cytokine-supported culture

Early neutrophil and platelet engraftment is critical for the outcome of HSCT. *In vivo* characterization of human HPC with myeloid restricted activity remains a challenge in immunocompromised mouse models due to poor differentiation of human myeloid progenitor cells (16). Thus, the effect of BIO-treatment on regenerative potential of myeloid progenitor cells was examined in long-term culture supporting expansion of myeloid progenitor cells, as previously reported (13). CD34⁺ cells cultured with SCF, TPO and FLT3L were rapidly losing CD34 expression, as a result of their differentiation (Fig. 4a). BIO treatment (0.5 μ M, for 24 h following 5-day expansion) did not modulate the proportion of CD34⁺ cells; however, it markedly elevated generation of both CD34⁺ and CD34⁻ cells (Fig. 4b,c). Expansion of CD34⁺ HPC declined after day 8 in control cultures, while a steady-state level of CD34⁺ HPC was maintained in BIO-treated cultures for as long as up to day 22. Duration of differentiating CD34⁻ cell expansion was also significantly longer in BIO-treated cells (to day 45) compared to control (to day 22). Proportion of CD34⁻ differentiating HPCs expressed CD33 and CD14, myeloid and immature monocytic cell markers, respectively, and BIO-treatment did not modulate their expression (Fig. 4d). Morphological analysis confirmed granulomonocytic nature of differentiating HPC analysed on day 8 and day 20 (Fig. 4e). Proportion of apoptotic cells with sub-genomic DNA content and proportion of cells in G1 (M2 gate), S (M3 gate) and G2/M (M4 gate) were similar in both cultures when analysed 24 h after BIO or DMSO-treatment (Fig. 4f).

Colony-forming unit analysis performed immediately after withdrawal of BIO (day 6 after initiation of cultures) revealed that total CFU and BFU-E numbers were significantly higher in BIO-treated cells compared to controls (Fig. 5a). Clonogenic potential of *ex vivo*-cultured differentiating CD34⁺ cells declined at later stages of culture – control cells produced on average 2 CFU per 10⁴ cell input by day 39 compared to 65 CFU per 10³ cell input on day 6 (Fig. 5a,b). CFU-GM was the

predominant CFU type and no BFU-E or CFU-GEMM was detectable at this stage of culture. BIO-treated cells formed more CFU per input cells and CFU normalized to total cell numbers by day 39 of culture (Fig. 5b,c). Thus, BIO treatment appeared to preserve clonogenic activity of *ex vivo*-expanded progenitor cells. Collectively, these results demonstrate that pre-treatment with BIO acted to improve regenerative potential of *ex vivo*-expanded HPC.

Molecular mechanisms triggered by BIO in ex vivo-expanded HPC

BIO treatment induced accumulation of β -catenin, the major intracellular effector of Wnt signalling, in *ex vivo*-expanded CD34⁺ cells (Fig. 6a). In addition, c-myc protein accumulation was registered following BIO treatment (Fig. 6a). c-myc protein degradation is regulated by GSK-3 β , and accumulation of c-myc was demonstrated in cardiomyoblasts treated with GSK-3 β inhibitor (20). In addition, *Myc* gene transcription is regulated by β -catenin; therefore, accumulation of c-myc protein in BIO-treated cells could result from up-regulated β -catenin (12). Deregulated expression of c-myc in murine myeloid progenitor cells was shown to promote cell population growth, delay terminal growth arrest and differentiation (21). Thus, we hypothesized that BIO-induced up-regulation of β -catenin, and its target c-myc could trigger the cascade of events promoting progenitor cell expansion. To test this hypothesis, CD34⁺ cells were infected with a retrovirus expressing mutant version of c-Myc, v-Myc and gene-encoding neomycin resistance *Neo* (15). Control cells were infected with the LNL6 virus expressing *Neo* only (15). Transduction efficiency was determined as proportion of CFU surviving 10-day G418 selection, by comparing CFU numbers formed in the presence of G418, and in the absence of G418, on days 11, 19 and 34 post transduction (Fig. 6b). Proportions of G418-resistant CFU in v-Myc-culture increased from 30% on day 11 to 50 and 80% on days 19 and 34, respectively, while proportion of G418-resistant CFU in control culture declined (Fig. 6b). Thus v-Myc expression appeared to confer selective advantage to CFU in the presence of G418 selection. Decline in proportion of G418-resistant cells in control culture apparently was due to reduced LTR-driven transgene expression in differentiating HPC, as a result of methylation (22).

G418-selected v-Myc-transduced cells produced more CFU per input cells and per culture (Fig. 6c,d). This is similar to the effect of BIO treatment on CFU activity of *ex vivo*-expanded CD34⁺ HPC (Fig. 5a). Thus, both BIO-treatment and v-Myc expression in CD34⁺ cells acted to expand numbers of clonogenic

Figure 4. Expansion of myeloid progenitor cells following treatment with BIO. CD34⁺ cells were cultured with SCF, TPO and Flt3L, all at 100 ng/ml, for 5 days, and treated with BIO, 24 h. On day 6, culture medium was replaced with BIO-free medium containing the same cytokines. CD34 expression and cell numbers were measured at time points as indicated in the figure. Long-term culture experimentation was performed twice using CD34⁺ cells from two cord blood donors. One representative experiment is shown. (a) Percentage of CD34⁺ cells rapidly declined in control and BIO-treated cells. (b) BIO-treated cells generated significantly more CD34⁺ progenitor cells as determined on days 22 and 29 (*P* = 0.0257 and 0.019, respectively). (c) BIO-treated cells generated significantly more CD34⁺ progenitor cells as determined on days 21, 29, 39 and 46. Values of *P* are shown in the figure. (d) Differentiating HPC expressed myelomonocytic cell markers CD33 and CD14. One representative flow cytometry analysis performed on day 20 demonstrates no significant difference in expression of CD33 and CD14 in control and BIO-cultures. (e) Morphological analysis of differentiating HPC confirmed myelomonocytic nature of differentiating progenitor cells. The whole content including adherent and non-adherent fractions was collected on day 8 and day 20, left and right panels, respectively. Cyto-centrifuge preparations were stained with May-Grunwald and Giemsa stains. View shows representative cells from control cultures only. Arrows indicate macrophage-like cells (magnification ×200). Proportion of macrophage-like cells (20 fields analysed per condition) was similar in control and BIO-treated cells (data not shown). (f) Proportion of apoptotic cells with sub-genomic DNA content (M1 gate), G1-cells (M2 gate), S-phase cells (M3 gate) and G2/M-cells (M4 gate) was similar in both cultures. Cell cycle analysis was performed 24 h after BIO-treatment.

precursor cells. It is relevant that proportions of CFU-GM/BFU-E were higher in experiments shown in Fig. 6c and 6d compared to Fig. 5a, since CFU analysis of BIO-treated cells was performed using 6-day cultured CD34⁺ cells (Fig. 5a) while CFU analysis of *v-Myc*-transduced cells was performed using 16-day cultured cells (6 days stimulation and infection, plus 10 days G418 selection) (Fig. 6b). In addition, CFU-GM/BFU-E

ratio changed in favour of CFU-GMs due to CD34⁺ cell differentiation in those *v-Myc*-transduced (Fig. 6b).

Collectively, our results show that *v-Myc* expression alone mimicked certain aspects of BIO-induced effects on human HPC such as CFU expansion. Apparently, other factors, in addition to myc up-regulation, may account for effects of BIO on regenerative potential of *ex vivo*-expanded HPC.

Figure 5. BIO-treated cells outperformed control DMSO cells when analysed in CFU assay. (a) CFU analysis was performed immediately after withdrawal of BIO (on day 6 after initiation of the culture). Total CFU and BFU-E numbers were significantly higher in BIO-treated cells compared to controls. (b) BIO-treated cells formed more CFU per input cells in assay performed on day 39 of culture. (c) Overall CFU numbers/per culture (CFU/input normalized to total cell numbers in culture, day 39) were significantly higher in cells treated with BIO.

Discussion

Wnt signalling provided by BM stromal cells maintains stem cell homeostasis (8). UCB-derived HPC are depleted of Wnt signals, and we have earlier shown that pharmacological inhibition of GSK-3 β can re-activate Wnt (5). This prompted us to examine whether re-activation of Wnt, using small molecule inhibitor of GSK-3 β , BIO, would modulate regenerative potential of *ex vivo*-expanded HPC. Pre-treatment with BIO induced Wnt-mediated gene re-programming in *ex vivo*-expanded HPC and improved stem cell function assessed in the NOD/SCID mouse model (9). This model, however, did not permit analysis of early engrafting HPC as they are rejected by residual NK (10). Here, we show that pre-treatment with BIO promoted donor cell engraftment in the NSG mouse model, permissive for early engrafting HPC. Engraftment of hCD45⁺ cells was higher in the BIO-group compared to controls. The effect was seen by weeks 4, 7 and 8. Similar hCD45⁺ cell engraftment was observed in control and BIO-mice at later stages post-transplantation. Apparently, different stem cell subsets contribute to engraftment at early and late stages post-transplant. In the early stages post-transplantation in NSG mice, almost all hCD45⁺ cells co-express hCD19 B cell lymphoid marker and do not express myelomonocytic marker CD33. Myeloid reconstitution is delayed by limited human-mouse cross-reactivity of cytokines promoting myeloid differentiation (16). At later stages post-transplantation, however, significant numbers of myelomonocytic CD33⁺ cells are generated in the BM and PB. Limiting dilution transplants determined that early increase in human cell engraftment in

NSG mice transplanted with BIO-treated cells may reflect an increase in frequency of early engrafting cells in BIO-treated cell culture. In addition, our adhesion assay revealed that BIO-treatment promoted attachment of *ex vivo*-expanded HPC to retronectin mimicking stem cell attachment to fibronectin, one of the important components of extracellular matrix in the BM. Thus, we propose that BIO may act to improve homing of *ex vivo*-expanded HPC impaired by *ex vivo* expansion (18). Our data presented in Fig. 3a–c show that VLA4 and VLA5 did not appear to be involved in enhanced attachment of BIO-treated cells to retronectin. The results do show, however, that both VLA4 and VLA5 integrins were involved in CD34⁺ cell-adherent interaction to retronectin. There were no differences in VLA4 or VLA5 expression between control and BIO-cells, suggesting that effects of BIO on rapid attachment of CD34⁺ cells to retronectin were not mediated through modulation of integrin expression. Apparently, alternative mechanism(s) may be triggered by BIO to promote rapid integrin-mediated adherence of *ex vivo*-expanded CD34⁺ HPC to retronectin. Integrins can be modified by agonists (such as growth factors) that bind to non-integrin cell receptors and in turn modify integrin activation/function, a process termed 'inside-out' signalling. Therefore, BIO may modulate one of these agonists directly or indirectly. We have earlier shown that BIO up-regulates CXCR4 expression of CD34⁺ cells (13). Interaction of integrins and CXCR4 will be further examined in the future. In addition, there may be further integrins such as α 2 β 1 and α V β 3 that bind to retronectin and are modulated by BIO. No matter the mechanism triggered by BIO, improved

Figure 6. Molecular mechanisms triggered by BIO in *ex vivo*-expanded HPC. (a) *Ex vivo*-expanded HPC treated with BIO accumulated β -catenin and c-myc proteins. Western blot analysis of β -catenin, c-myc and β -actin expression in *ex vivo*-expanded CD34⁺ cells treated with DMSO or BIO, shown in the left panel. Expression of β -catenin and c-myc was normalized to β -actin (middle and right panels, respectively). (b) CD34⁺ cells were infected with retrovirus AV expressing v-Myc and Neo. Control cells were infected with LNL6 virus expressing Neo only. Proportions of transduced CFU were determined as percentage of CFU formed in the presence of G418 of the CFU formed in the absence of G418. CFU analysis was performed on days 11, 19 and 34 post-infection. Each CFU analysis was performed in triplicate. Proportion of transduced CFU increased from approx. 30–80% from day 11 to day 39. (c) CFU analysis of v-Myc-transduced HPC was performed after 10 days G418 selection of 6-day cultured cells. v-Myc-transduced HPC produced more CFU than control cells. P values were <0.05 for BFU, CFU-GM and total CFU. CFU/input cells are shown. (d) v-Myc-transduced HPC formed more CFU per culture. CFU numbers per input cells were normalized to the total cell numbers per 10-day culture. P values were <0.05 for BFU, CFU-GM and total CFU.

attachment of BIO-treated stem cells to extracellular matrix may act to improve stem cell binding to the BM.

BIO-treatment promoted CD34⁺ and CD34[−] HPC expansion in long-term cytokine-supported culture. Expansion of CD34⁺ HPC rapidly declined in controls after day 8 in culture, while a steady-state level of CD34⁺ HPC was maintained in BIO-treated cultures for as long as up to day 22. Duration of differentiating CD34[−] HPC expansion was also significantly longer in BIO-treated cells (day 45) compared to controls (day 22). Importantly, BIO-treated HPC produced more CFU than did control HPC.

BIO-treated cells exhibited higher expression of β -catenin, the main intracellular effector of Wnt, and c-myc previously shown to be regulated by β -catenin at the transcriptional level (12). In addition, c-myc protein degradation was shown to be directly regulated by

GSK-3 β in cardiomyoblasts (20). Myc expression was shown to promote haematopoietic progenitor cell expansion (21), thus, we propose that BIO may act at least in part through up-regulation of c-myc. Ectopic expression of v-Myc, the viral variant of c-Myc, increased clonogenic potential of HPC resembling the effect of BIO on *ex vivo*-cultured HPC. We hypothesized that β -catenin-mediated up-regulation of c-myc in BIO-treated cells acted to improve regenerative potential of *ex vivo*-expanded HPC. Other mechanisms triggered by GSK-3 β inhibition in human HPC cannot be excluded; GSK-3 β is known as a multifunctional kinase that modulates phosphorylation of a number of substrates (23). Our data suggest that transient pharmacological inhibition of GSK-3 β may provide a novel approach to improve early engraftment of *ex vivo*-expanded stem cells.

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