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Rapid Expansion of Human Hematopoietic Stem Cells by Automated Control of Inhibitory Feedback Signaling

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SUMMARY

Clinical hematopoietic transplantation outcomes are strongly correlated with the numbers of cells infused. Anticipated novel therapeutic implementations of hematopoietic stem cells (HSCs) and their derivatives further increase interest in strategies to expand HSCs ex vivo. A fundamental limitation in all HSC-driven culture systems is the rapid generation of differentiating cells and their secreted inhibitory feedback signals. Herein we describe an integrated computational and experimental strategy that enables a tunable reduction in the global levels and impact of paracrine signaling factors in an automated closed-system process by employing a controlled fed-batch media dilution approach. Application of this system to human cord blood cells yielded a rapid (12-day) 11-fold increase of HSCs with self-renewing, multilineage repopulating ability. These results highlight the marked improvements that control of feedback signaling can offer primary stem cell culture and demonstrate a clinically relevant rapid and relatively low culture volume strategy for ex vivo HSC expansion.

INTRODUCTION

Emerging data suggest that robustness and responsiveness in hematopoiesis is a property of the system, not an individual cell. The hematopoietic system is able to dynamically maintain appropriate proportions of cells of all hematopoietic lineages throughout the lifetime of the individual, both during homeostasis and in response to regenerative demand. This regulation depends on intercellular (between cell) communication networks, resulting from both local and systemic factor secretion (Kirouac et al., 2010). Hematopoietic stem cells (HSCs) must respond to and integrate cues from the microenvironment to ensure sustained production of all hematopoietic lineages (Rizo et al., 2006). As such, self-renewal versus differentiation decisions of HSCs critically depend on feedback-mediated paracrine factors and the associated signaling networks, both in vivo and in vitro.

Several decades of successful bone marrow transplantations have demonstrated the therapeutic importance of HSCs. The use of noninvasively accessible umbilical cord blood (UCB)-derived HSCs provides many advantages over bone marrow, including enhanced long-term immune recovery and decreased graft versus host disease (Gluckman, 2009; Wagner and Gluckman, 2010). However, because clinical studies have indicated that the most important factor for patient survival after UCB transplantation is infusing a cell dose above a minimum threshold of 3×10^7 cells/kg (Gluckman, 2009), low cell numbers in single UCB units have limited the suitability of UCB transplantation for adult patients. Methods to robustly increase the number of cells that give a rapid and sustained blood count recovery would enable the use of UCB in all patients (Hofmeister et al., 2007).

Strategies to expand HSC numbers in vitro have focused on identifying molecules that specifically target the stem cell population. Recent data demonstrating that there are multiple subpopulations of HSCs and that these cells are molecularly closely related (Benveniste et al., 2010; Dykstra et al., 2007) suggest that the opportunity of finding molecules that uniquely expand the long-term HSC pool (Antonchuk et al., 2002; Boitano et al., 2010; Delaney et al., 2010; Durand and Zon, 2010; Zhang et al., 2008) without impacting the distribution and growth of their more restricted progeny is challenging, especially after extended periods of time in culture. Herein we propose a fundamentally different and complementary approach, examining the impact of feedback signaling control on HSC output.

Feedback control is complicated by the fact that the hematopoietic system is regulated by a complex hierarchy of cellular and molecular networks. We have previously performed cell-cell

signaling network analysis to identify and experimentally validate the mode of action of endogenously produced HSC-inhibitory factors generated during in vitro UCB culture (Kirouac et al., 2010). Recognition that large numbers of these factors are produced in a time-dependent manner makes the addition of binding and signaling inhibitors challenging. We sought to identify a strategy to globally control feedback regulation. We performed computational simulations of in vitro hematopoiesis and iteratively predicted and evaluated candidate strategies for the reduction of endogenously produced negative regulators. In silico optimization resulted in the identification and development of a ''fed-batch'' culture strategy for HSC growth enhancement. By linking dynamic cell growth and endogenous factor secretion to a tunable media dilution algorithm, we experimentally confirmed predictions of significant enhancements in stem and progenitor growth, including an 80-fold increase in $CD34^+$ cells and an 11-fold increase in NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) repopulating cells (detected at limiting dilution after 16 weeks) within a 12-day culture period. Furthermore, this platform complements known HSC-enhancing factors and has provided insight into their mode of action. The integration of our strategy into an automated and closed-system bioreactor has produced a clinically relevant system for HSC expansion.

RESULTS

In Silico Design of a "Fed-Batch" Media Dilution Strategy

Hematopoietic culture systems generate a large number of endogenously produced soluble factors as a result of the rapid production of mature blood cells (Kirouac et al., 2010; Majka et al., 2001; Sautois et al., 1997). In order to investigate the impact of these secreted ligands, we first measured a sampling of the secreted factor profile under our previously optimized in vitro expansion conditions (Madlambayan et al., 2005) and found the rapid accumulation of many physiologically relevant ligands and large fluctuations resulting from periodic media exchanges (Figure 1A). Many of these factors have been reported to have inhibitory effects on human hematopoietic stem and progenitor cell expansion (Bonnet et al., 1995; Broxmeyer et al., 1995; Broxmeyer and Kim, 1999; Cashman et al., 1998; Fortunel et al., 2000; Zhang et al., 1995), and our previous studies have validated that several factors which are present at high levels in the culture system have a significant inhibitory effect on hematopoietic progenitor expansion, including TGF-β, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), and IP-10 (CXCL10) (Kirouac et al., 2010).

The large number and nonlinear nature of the secreted factor profiles limit the likely success of using molecularly targeted approaches to reduce the inhibitory impact of these factors and, instead, necessitates a global and unbiased strategy for feedback regulation. In order to identify and optimize an appropriate strategy, we performed computational simulations of cell population dynamics to investigate and predict the effect of candidate culture manipulations. Our computational approach (Kirouac et al., 2009) incorporated the effect of feedback signaling from differentiated cells on stem and progenitor cell expansion. This feedback is coded through different classes of paracrine signaling loops, as depicted in Figure 1B.

As a first step, we explored in silico strategies of regulating the effective concentration of the different classes (SF1–SF4) of secreted ligands. Under baseline conditions, with a full media exchange every 4 days, simulations depict a predominant accumulation of inhibitory factors (SF1 and SF2) (Figure 1C). As such, a net increase in stem and progenitor proliferation was predicted to result from minimizing the entire set of endogenously produced ligands. Rationalizing that the frequency of conditioned media removal would be an important parameter (Madlambayan et al., 2005), we first compared the effects of frequent full or partial media exchanges with our control culture process. The model predicted that a full media exchange every day (Figure 1D) or a half media exchange every 12 hr (Figure 1E) would outperform less frequent exchanges, emphasizing the importance of strong and frequent secreted factor regulation.

To limit periodic fluctuations and intermittent exposure to high concentrations of secreted ligands and to attain a continuous and tunable mode of media regulation, we simulated the effects of perfusion and fed-batch culture systems. A perfusion system (Figure 1F) is characterized by a continuous input of fresh media and output of spent media, while maintaining a constant volume, whereas a fed-batch system (Figure 1G) contains an input stream only, resulting in a continuous increase in culture volume. These cell culture systems are commonly used as feeding strategies for biopharmaceutical production (Farid, 2006), and previous studies have explored the use of perfusion systems for HSC culture (Koller et al., 1998). Simulations predicted that a fed-batch media dilution approach would achieve the most effective enhancement in stem and progenitor expansion and was predicted to outperform perfusion cultures and frequent media exchange strategies (Figures 1C–1G). Although all strategies act by reducing the concentration of accumulating secreted factors, the increasing culture volume of the fed-batch strategy additionally maintains lower cell densities, thereby slowing the rate and impact of endogenous factor accumulation.

The dilution rate (D) of a fed-batch culture system is defined as the input flow rate divided by the culture volume. This rate can be constant throughout the reaction period, proportional to the current volume of the culture or tuned based on predicted or measured parameters. As shown in Figure 1H, the volume of a culture initially containing 1 ml, will increase continuously with time depending on the defined dilution rate. Simulations predicted that the dilution rate would regulate the concentration of all secreted factors (Figure 1I), which in turn would regulate expansions of total nucleated cells (TNCs), colony-forming cells (CFCs), long-term culture-initiating cells (LTC-ICs), and SCID repopulating cells (SRCs) (Figure 1J). The slopes of the predicted increases in expansions are greatest from D = 0 to D = 1, suggesting that a significant impact on HSC expansion should be achievable at moderate dilution rates, while maintaining moderate media and cytokine needs.

Automated Closed System Fed-Batch Culture Enhances Progenitor Cell Expansion

We next undertook experiments to test the predicted superiority of the in silico optimized fed-batch strategy. These studies were

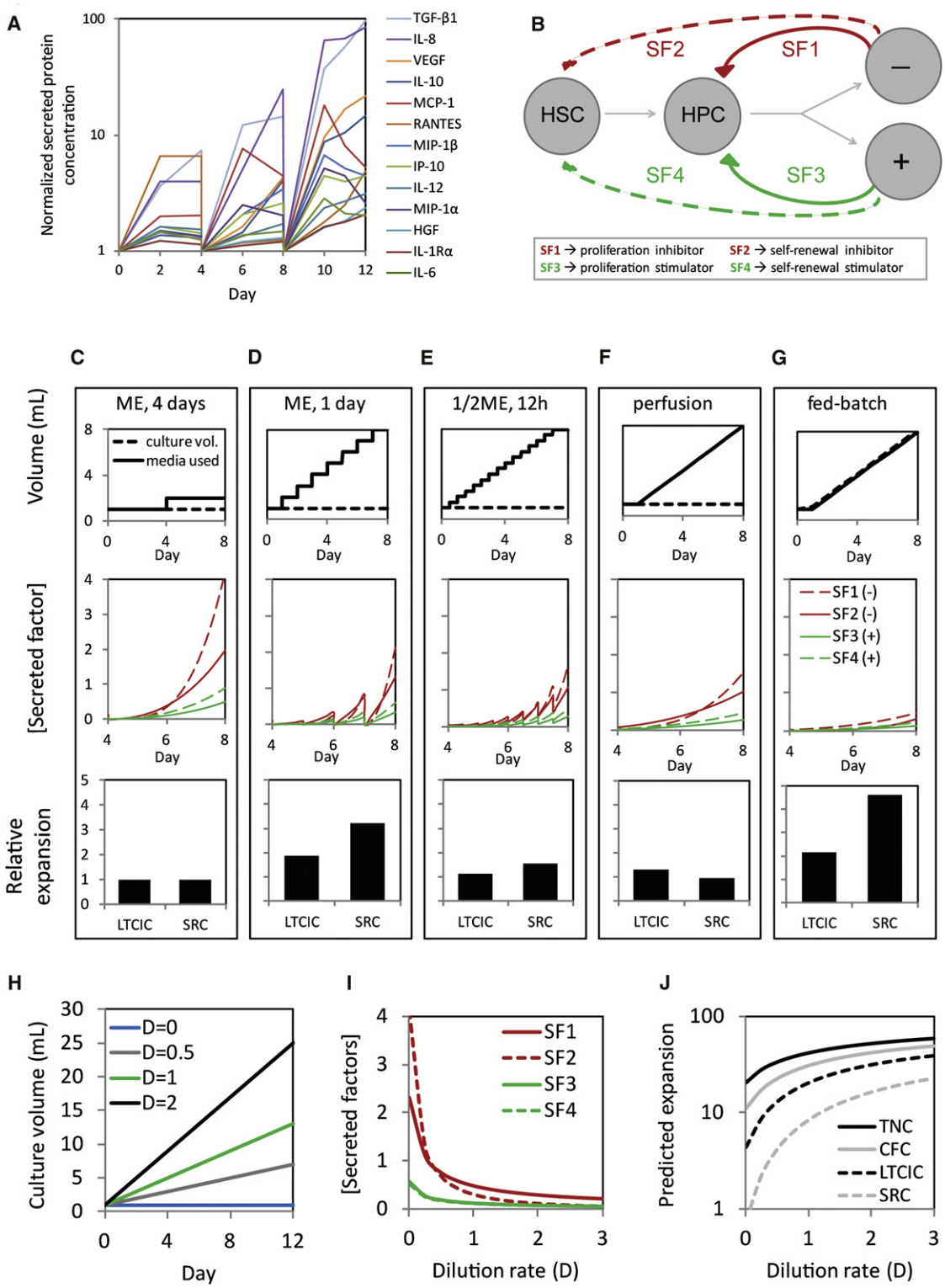

Figure 1. Computational Simulations Predict a Fed-Batch Strategy, at Moderate Dilution Rates, to Greatly Reduce Secreted Factor Concentrations and Give a Significant Enhancement of Expansion

(A) Time course of experimentally measured secreted factor concentrations from UCB culture, with complete media exchange every 4 days.

(B) Schematic of mathematical model, indicating the presence of paracrine feedback signaling. Groups of secreted factors are categorized depending on whether they act in a stimulatory or inhibitory manner and whether they impact stem cell self-renewal or proliferation.

(C–G) Simulated volume, secreted factor concentrations, and relative expansions under different media manipulation strategies: (C) "control" culture with complete media exchange (ME) every 4 days; (D) culture with complete media exchange every 24 hr; (E) culture with 50% media exchange every 12 hr; (F) perfusion

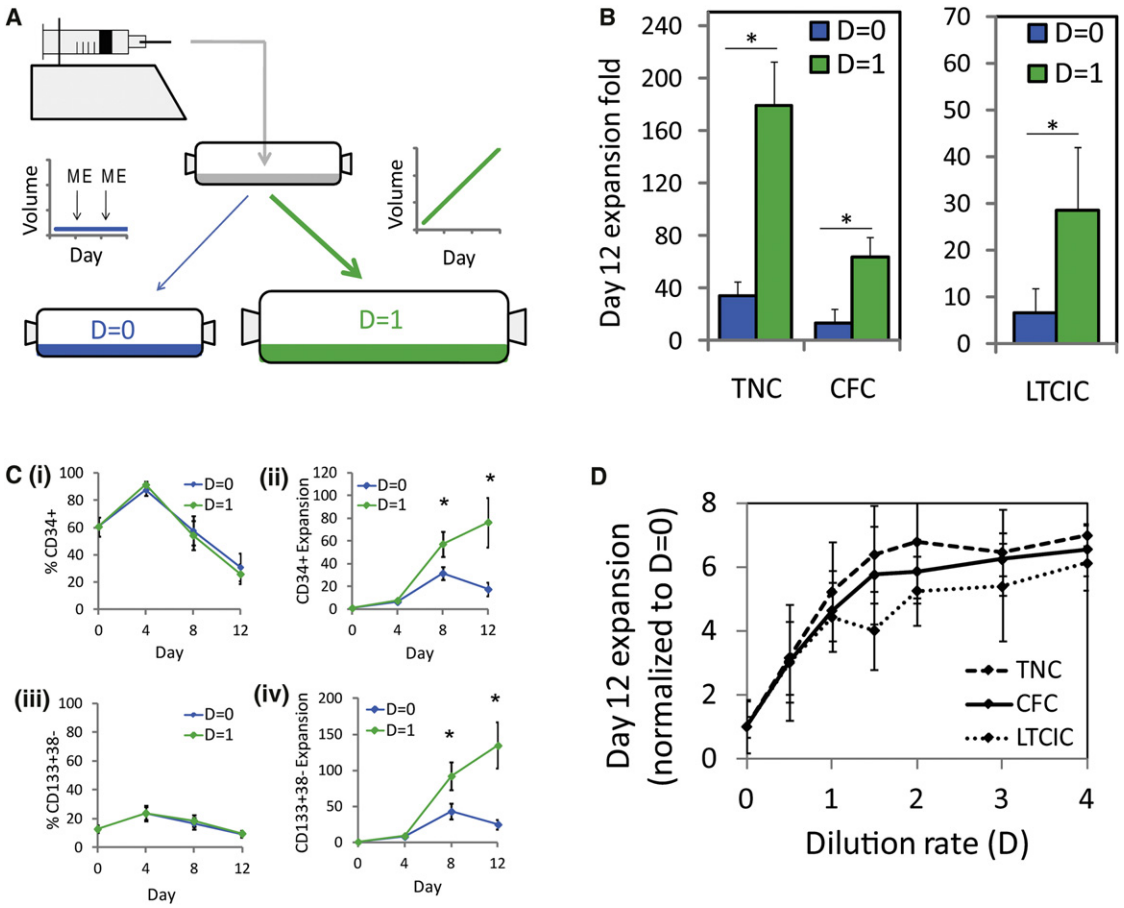

Figure 2. The Fed-Batch Strategy Is Experimentally Validated In Vitro to Give Significantly Improved Expansion of Progenitor Cells

(A) Schematic of experimental set-up comparing control (D = 0) strategy with 100% media exchange (ME) every 4 days to fed-batch (D = 1) strategy.

(B) Expansions of TNC, CFC, and LTC-IC, after 12 days of culture. n > 5.

(C) (i, iii) Surface marker analysis of $CD34^+$ and $CD133^+CD38^-$ frequencies throughout in vitro culture. (ii, iv) Expansion of $CD34^+$ cells and $CD133^+CD38^-$ cells, accounting for total cell expansion. n > 5.

(D) Normalized expansion of TNC, CFC, and LTC-IC after 12 days of culture, dependent on dilution rate. n = 3.

Data are expressed as mean ± SD. See also Figures S1 and S2.

facilitated with the use of an automated media delivery system (Csaszar et al., 2009). This system allows the user to control media input flow rates and enables the entire culture process to be performed in a closed system with minimal manual intervention. A constant flow rate, corresponding to a dilution rate of D = 1, was compared to our previously described optimal culture strategy (Madlambayan et al., 2005) (referred to here as D = 0) (Figure 2A).

We first confirmed that the D = 1 fed-batch strategy significantly outperformed the D = 0 control at our previously optimal day 8 endpoint (Figure S1). We next computationally predicted that the D = 1 strategy would achieve maximum expansions after 12 days of in vitro culture, because the regulation strategy inherent in the fed-batch system would allow for a positive rate of expansion to be maintained for a greater length of time (Figure S1). As shown in Figure 2B, the D = 1 strategy reached 12-day expansions of 179-fold (range 105- to 344-fold) of TNCs, 64-fold (22- to 166-fold) of CFCs, and 29-fold (14- to 53-fold) of LTC-ICs. Phenotypic analysis by flow cytometry (Figure 2C) indicated that the percentage of progenitor cells as measured by $CD34^+$ and $CD133^+CD38^-$ remained unchanged between the D = 0 and D = 1 cultures. However, the increased total cell expansion with the D = 1 strategy led to a significant increase in the absolute numbers of $CD34^+$ cells (80-fold) and $CD133^+CD38^-$ cells (135-fold). Similar results were found with $CD34^+CD90^+$ and $CD34^+CD49f^{hi}$ cells (Figure S1).

culture with one unit of media perfused every 24 hr; (G) fed-batch culture with one unit of media added every 24 hr. Conditions (D–G) are normalized to same media and cytokine requirements (one additional unit of media every 24 hr).

(H) Media volume requirements for a fed-batch culture at different constant dilution rates, assuming a 1 ml initial volume.

(I) Predicted effect of increasing constant dilution rate of fed-batch strategy on secreted factor concentrations.

(J) Predicted effect of increasing constant dilution rate of fed-batch strategy on population expansions.

To further validate our modeling predictions, we compared the fed-batch strategy to alternative strategies for the reduction of endogenously produced factors by investigating frequent media exchange approaches (Figure S2). Although small apparent improvements over the D = 0 cultures were achieved with frequent feeding strategies, these strategies produced significantly lower expansions than the D = 1 strategy, despite having the same overall media usage. We also assessed the effect of varying the dilution rate of the fed-batch culture. As predicted, the rate of increased expansion began to slow at dilution rates greater than D = 1, leading to diminishing returns of cell expansion at the expense of rapidly increasing media needs (Figure 2D). Taken together, these findings confirm our computational predictions of enhanced progenitor expansion achieved with a robust media dilution approach.

Global Maintenance of Subthreshold Levels of Inhibitory Factors Enables Fed-Batch-Mediated Expansion of Primitive UCB Cells

The observed enhancement in progenitor expansion was predicted to result from a global reduction in inhibitory paracrine factor levels in the culture media. To test this hypothesis, we investigated the effect of the fed-batch strategy on the output of specific mature blood cell types. Analysis of hematopoietic cell lineage markers did not reveal any change in the relative frequency of any of the differentiated cell types assessed ($CD14^+$, $CD7^+$, $CD41^+$, GyA^+, $CD33^+$, $CD19^+$, $CD56^+$, $CD8^+$, $CD4^+$) with the D = 1 strategy, as compared to the D = 0 control. Although the absolute number of cells in each of these populations was greater with the D = 1 strategy, resulting from the enhanced TNC expansion, the lower cell densities achieved with the D = 1 strategy resulted in lower concentrations (cells/ml) of each mature cell population (Figure S3).

We next asked how the reduction in mature cell concentrations would impact soluble factor concentrations. Figure 3A depicts the degree by which the concentration of each measured soluble factor was changed with the D = 1 strategy, as compared to D = 0. The nonlinear nature of factor accumulation means that the dynamics of individual factors and the degree by which the fed-batch strategy affects the concentration of specific factors varies; however, the net global reduction in factor concentrations provides an overall minimization of feedback inhibition in the culture system. Figure 3B highlights the concentration dynamics for several factors known to impact stem and progenitor growth, demonstrating the reduction of ligand concentrations achieved with the fed-batch strategy.

The functional effect of secreted ligand reduction on cell growth was further tested by culturing cells in media that had been previously conditioned for 8 days in a culture maintained with either the D = 0 or the D = 1 strategy. Exogenous cytokines (SCF, FL, TPO) were added to the conditioned media to ensure that the difference between the fresh and conditioned media was the presence of endogenously produced soluble factors. Both conditioned media led to a significant reduction in TNC expansions as compared to cultures utilizing fresh media, as expected. However, the expansion achieved with the D = 1 conditioned media were significantly greater than with the D = 0 conditioned media (Figure 3C). This shows the ability of reduced endogenous factor concentrations to better sustain primitive UCB cells in culture.

It remained unclear whether the fed-batch strategy was acting by simply regulating one or a few key inhibitory ligands or whether a more comprehensive (global) manipulation of the culture environment was important. To test this directly, we compared the D = 1 strategy to the targeted inhibition of TGF-β1, a known endogenous inhibitor of HSC growth (Fortunel et al., 2000). Targeted inhibition of TGF-β1 was achieved by adding the small molecule SB431542 to the culture every 4 days, a manipulation that yielded increases in progenitor cell expansions that approached those achieved by the fed-batch strategy on day 8. However, after 12 days of culture, TGF-β1 inhibition no longer had a significant effect on progenitor expansion (Figure 3D). This demonstrated that although some degree of culture regulation could be initially provided through the inhibition of one key endogenously produced factor, the impact of this approach is limited, particularly at the later time points of culture when the accumulation of many inhibitory factors is high, and targeting one factor alone is no longer effective.

In previous studies (Kirouac et al., 2010), we used expression analysis to identify 74 factors whose genes were upregulated during UCB culture. Many of these factors were hypothesized to be inhibitory to HSC growth and seven of these (TGF-β, TNFSF9, MIP-1α, MIP-1β, IP-10, NAP-2, SPARC) were experimentally validated to have an inhibitory effect on LTC-IC expansion. To investigate whether these factors contribute to HSC inhibition in an additive manner, we performed a combinatorial analysis of inhibitory soluble factors on $Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$ cells, which have been reported to be highly enriched for HSCs (Notta et al., 2011). The addition of TGF-β1 (10 ng/ml), MIP-1α (100 ng/ml), MIP-1β (100 ng/ml), and IP-10 (100 ng/ml) individually each caused a reduced expansion of this population after 7 days of culture, and the simultaneous addition of these four inhibitors produced a significant reduction of expansion, demonstrating that multiple inhibitory factors present in the culture system act in an additive manner (Figure 3E). Importantly, when lower concentrations (10 ng/ml each) of MIP-1α, MIP-1β, and IP-10 were used, the effect of the individual factors was negligible; however, the combination of the three led to reduced cell expansions (Figure 3F). This study illustrates how subthreshold levels of factors that individually do not provide significant inhibition can produce an inhibitory effect when acting in combination, highlighting the global nature of feedback inhibition.

In order to rule out the possibility that the fed-batch strategy was primarily acting by preventing critical metabolites from becoming limiting and inhibiting cell growth (Collins et al., 1997; Patel et al., 2000), we monitored the glucose and lactate concentrations of the media. Glucose levels did not become limiting under any of the culture conditions tested, including the D = 0 control, as shown by the fact that glucose concentrations below 7 mM were never observed (Figure S4). Furthermore, when glucose was added to the D = 0 culture in order to normalize the glucose levels to the corresponding D = 1 culture, no effect on expansion levels was observed (Figure S4). Lactate levels reached a maximum of 15 mM in the D = 0 cultures and the pH did not drop below 7.0, both of which were well within the range that support normal hematopoietic cell proliferation

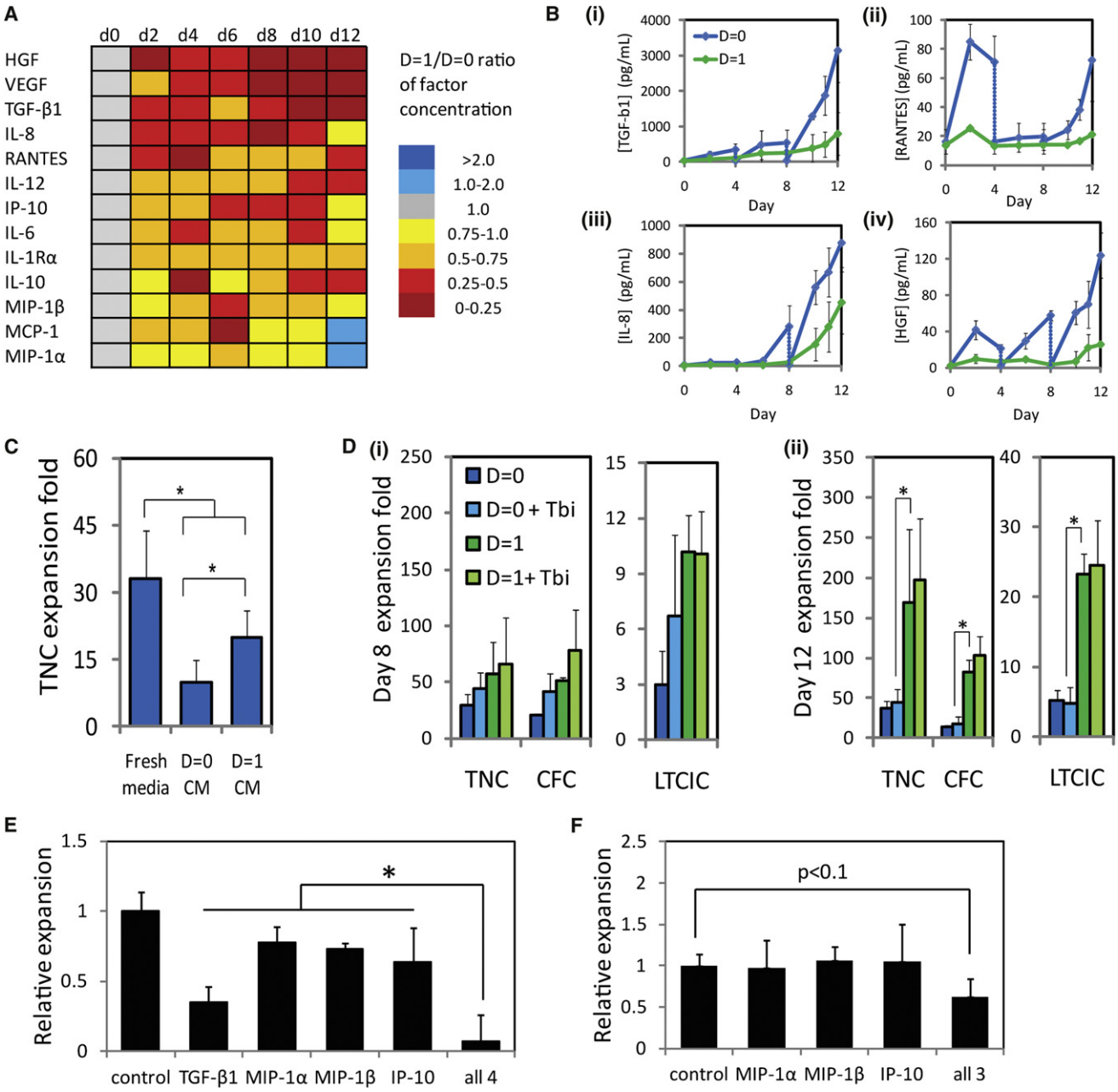

Figure 3. The Fed-Batch Strategy Enhances Expansion through the Global Reduction of Endogenously Produced Inhibitory Secreted Factors

(A) Heat map of secreted factor concentration ratios (D = 1/D = 0) showing the effect of the D = 1 strategy on secreted factor profiles as compared to the D = 0 strategy.

(B) Representative samples of reduction in secreted factor concentrations for: (i) TGF-β1; (ii) RANTES; (iii) IL-8; (iv) HGF with the D = 1 strategy as compared to the D = 0 strategy. n = 5.

(C) TNC expansion after 8 days of D = 0 culture with either fresh media or conditioned media (CM) from day 8 of previous D = 0 or D = 1 cultures. n = 3.

(D) Comparison of the fed-batch D = 1 strategy to the addition of a TGF-β1 small molecule inhibitor, SB431542, based on expansions of TNC, CFC, and LTC-IC at (i) day 8 and (ii) day 12. n = 3.

(E) Inhibitory factors, TGF-β1 (10 ng/ml), MIP-1α (100 ng/ml), MIP-1β (100 ng/ml), and IP-10 (100 ng/ml) were added to a sorted HSC population ($Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$), and total cell expansion was assessed after 7 days of culture. n = 3.

(F) Low concentrations of inhibitory factors, MIP-1α (10 ng/ml), MIP-1β (10 ng/ml), and IP-10 (10 ng/ml) were added to a sorted HSC population ($Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$), and total cell expansion was assessed after 7 days of culture. n = 3.

Data are expressed as mean ± SD. See also Figures S3 and S4.

(Figure S4; Patel et al., 2000). Collectively, these studies support the interpretation that the global reduction of inhibitory ligands was providing the supportive conditions for improved progenitor growth.

Transplantation Studies Show an 11-fold SRC Expansion under Fed-Batch Conditions

To determine whether the enhanced expansions observed with the progenitor populations would also apply to long-term repopulating HSCs (LTR-HSCs), we performed transplantation studies with the NSG mouse model (McDermott et al., 2010). Repopulation was quantified by bone marrow analysis for human hematopoietic cell contribution 16 weeks after transplantation, and freshly isolated (day 0) Lin^- UCB cells were compared to cells cultured for 8 or 12 days. Figure 4A shows representative flow cytometry plots of repopulated and nonrepopulated mice. For each condition tested, a dose response of average human contribution was seen, as determined by the quantification of human CD45 and HLA-ABC double-positive populations (Table S1).

All positively repopulated mice were found to have multilineage repopulation, as indicated by human cells that were positive for a myeloid lineage marker (CD33), a B cell lymphoid lineage marker (CD19), a T cell lineage marker (CD3), and an erythroid lineage marker (GyA). Representative plots are shown in Figures 4B and 4C and full details are presented in Table S2. Human progenitor cells were detected from 16-week transplanted mice by both surface marker analysis ($CD34^+$ and $CD133^+$) and CFC assays (Figures 4D and 4E). Cells from 8 mice repopulated for 16 weeks with day 12 D = 1 cells (19%–62% human cells in the marrow of primary recipients) were retransplanted into secondary mice to determine whether the culture-expanded cells had retained LTR-HSC activity. Five out of eight secondary mice were positive for human cells (human contribution ranging from 0.5% to 2.1%), all of which showed multilineage repopulation (Table S3), indicating that the expanded cells are able to maintain their long-term repopulation potential in vivo.

In order to quantify LTR-HSC expansion, limiting dilution analyses were performed (Figure 4F). The frequency of LTR-HSCs in the fresh Lin^- cells was 1 in 14,700 (95% CI: 1/8,659 to 1/24,979, n = 26). After 8 days of culture, the D = 1 culture produced a corrected LTR-HSC frequency of 1 in 1,940 (1 in 110,000 corrected for the concomitant 57-fold expansion of TNCs, 95% CI: 1/1,195 to 1/3,149, n = 33), giving a 7.6-fold LTR-HSC expansion, relative to the fresh cells. The D = 0 strategy gave a 3.6-fold LTR-HSC expansion, as reported previously (Ito et al., 2010; Madlambayan et al., 2005) (1 in 121,000 corrected for a 28-fold TNC expansion to give a 1 in 4,330 corrected frequency, 95% CI: 1/2,223 to 1/8,428, n = 17). We predicted that the 12 day culture would give the greatest LTR-HSC expansion, based on both computational simulations and progenitor assays. Indeed, the corrected LTR-HSC frequency after 12 days of culture was 1 in 1,334 (after a 1 in 233,000 frequency was corrected for 178× total cell expansion, 95% CI: 1/759 to 1/2,345, n = 24), producing an 11-fold LTR-HSC expansion. LTR-HSC frequencies were used to determine SRC numbers per 10^6 cells (Figure 4G).

Collectively, these results demonstrate that the fed-batch D = 1 culture strategy is effective at expanding clinically relevant numbers of mature cells, progenitor cells, and LTR-HSCs in a short (12 day) culture time with an automated closed system bioprocess.

The Fed-Batch Strategy Complements the Effects of Other of HSC-Enhancing Factors and Provides Insight into Their Modes of Action

The fed-batch strategy provides a means to assess the effect of feedback signaling under different conditions and thus serves as a platform to interrogate the mode of action of factors known to enhance blood stem and progenitor cell growth (Figure 5A). We hypothesized that HSC culture additives could be classified into two major categories depending on whether they act directly on HSC self-renewal or act indirectly on a mature cell population, which feeds back positively on HSCs. If the mode of action is HSC self-renewal specific, the fed-batch strategy should minimize inhibitory feedback signals, providing an enhancing environment for HSCs growth. Alternatively, if the mode of action is non-stem cell autonomous, the fed-batch strategy should dilute the mature cell population and dilute the soluble signaling molecules that have been produced. In this case, the fed-batch strategy should reduce the impact of the factor added.

To test this hypothesis, we investigated the interaction between the fed-batch culture and two known HSC growth-supportive factors, the aryl hydrocarbon receptor antagonist (SR1), and the transcription factor HOXB4. SR1 has been shown to enhance $CD34^+$ and HSC outputs by inhibiting HSC differentiation (Boitano et al., 2010). We thus predicted that the fed-batch strategy would complement the effect of SR1 by reducing the impact of endogenous inhibitory feedback signaling. Figure 5B demonstrates that under our culture conditions, SR1 produced an increase in LTC-IC expansion with both the D = 0 and D = 1 strategy, and the absolute levels of LTC-IC expansion were significantly enhanced with the D = 1 strategy. Primitive cell phenotypes were also enhanced with the addition of SR1 (Figure S5). To validate that this factor was acting directly on a population with a very primitive phenotype and not through a feedback-mediated response, we added the molecule to the $Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$ population. Figure 5C shows that treatment with SR1 leads to a significant increase in primitive ($CD34^+CD133^+CD90^+$) cells in this assay, illustrating that it is directly targeting a population that is highly enriched in HSCs. Given the apparent additive impact of SR1 and the fed-batch strategy on the output of primitive cell phenotypes, it is possible that combining these technologies under the conditions described herein will enhance the number of LTR-HSCs above that which has been obtained with the D = 1 strategy alone (Figure S5). However, because the $CD34^+CD133^+CD90^+$ phenotype has not been validated under these culture conditions, limiting dilution long-term transplantation studies are required to confirm the potential additive effects between these two technologies.

We next investigated the transcription factor HOXB4, which has been shown to increase stem and progenitor expansion by either viral overexpression or delivered as a TAT-HOXB4 soluble protein (Antonchuk et al., 2002; Csaszar et al., 2009; Krosl et al., 2003). HOXB4 has been shown to cause increases in the production of $CD41^+$ megakaryocytes (Zhong et al., 2010), a finding that we reproduced in our culture system with the TAT-HOXB4 protein (Figure S5). Notably, we have previously reported that $CD41^+$ cells have a stimulatory feedback effect on LTC-IC

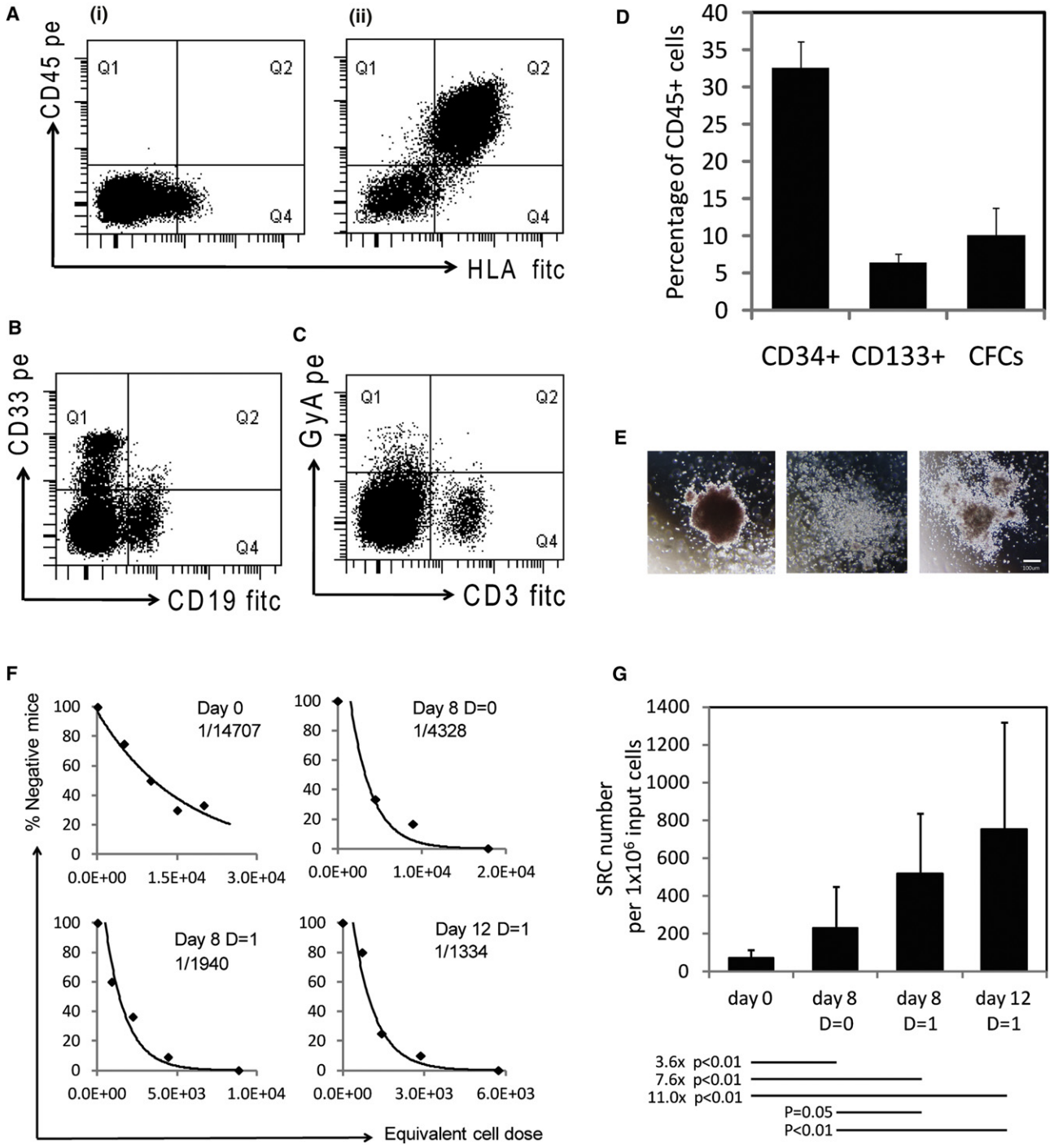

Figure 4. In Vivo SRC Assay with D = 1 Fed-Batch Strategy Shows an 11-fold Multilineage Expansion of LTR-HSCs

(A) Representative flow cytometry images of bone marrow analysis of (i) nonrepopulated and (ii) repopulated recipient mice. The quantification of the double-positive quadrant was used to determine the percentage donor contribution for each recipient.

(B and C) Representative images showing multilineage repopulation of recipients, as measured by $CD33^+$, $CD19^+$, GyA^+, and $CD3^+$.

(D) Quantification of progenitor cells in repopulated recipient mice.

(E) Representative images of CFCs formed with human cells from repopulated mice.

(F) Limiting dilution curves quantifying LTR-HSC contribution of uncultured (day 0) Lin^- cells; day 8 cells cultured with the D = 0 strategy; day 8 cells cultured with the D = 1 strategy; and day 12 cells cultured with the D = 1 strategy. LTR-HSC frequencies corrected to day 0 equivalent cell numbers are indicated for each. Data are fit with exponential curves.

(G) LTR-HSC expansions were used to calculate SRC numbers, based on 1×10^6 Lin^- cell input.

Results show the pooled data from two independent experiments. Data are expressed as mean ± 95% CI. See also Tables S1, S2, and S3.

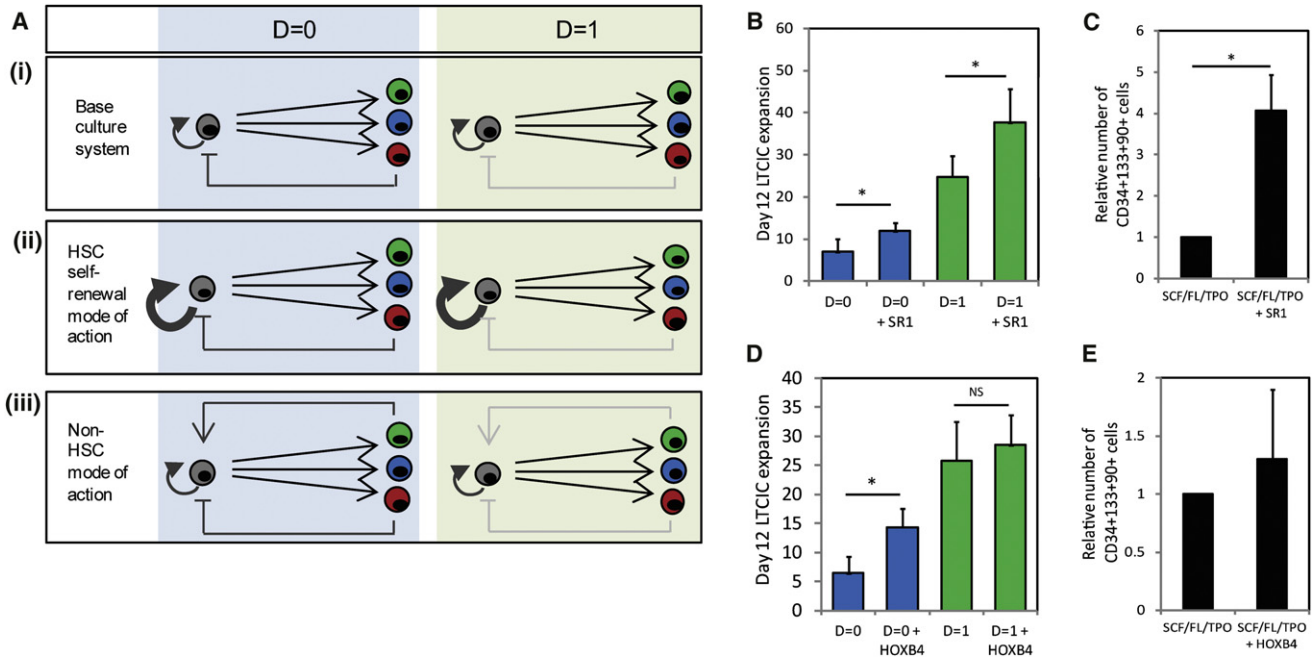

Figure 5. Fed-Batch System Complements the Effects HSC-Enhancing Molecules and Provides Insight into Their Mode of Action

(A) Schematic of in vitro expansion mode of action. (i) Under base conditions, static (D = 0) culture systems balance HSC self-renewal and feedback inhibition. The fed-batch (D = 1) strategy minimizes feedback, while allowing for self-renewal. (ii) The addition of a factor that targets HSC self-renewal gives increased self-renewal under both D = 0 and D = 1 conditions but the impact will be in enhanced with D = 1 conditions resulting from the reduced feedback. (iii) The addition of a factor with a positively acting indirect mode of action will increase stimulatory feedback signaling while maintaining self-renewal. Under D = 1 conditions, this stimulatory feedback will be reduced that will reduce the impact of the added factor.

(B) The addition of the aryl hydrocarbon receptor antagonist (SR1) gives a significant increase in LTC-IC expansion under both D = 0 and D = 1 conditions. n = 5.

(C) When added to a sorted HSC population ($Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$), SR1 shows an increase in $CD34^+CD133^+CD90^+$ numbers. n = 3.

(D) The addition of TAT-HOXB4 yields a significant increase in LTC-IC expansion under D = 0 conditions but not D = 1 conditions, suggesting a non-HSC mode of action. n = 5.

(E) TAT-HOXB4 shows minimal effect when added to the sorted $Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$ population. n = 3.

Data are expressed as mean ± SD. See also Figure S5.

expansion (Kirouac et al., 2010). Thus, we hypothesized that the mode of action of HOXB4 on UCB HSCs was, at least in part, resulting from an indirect feedback-mediated effect, as has also been suggested in ESC-derived hematopoiesis (Jackson et al., 2011). As Figure 5D shows, TAT-HOXB4 produced a significant increase in LTC-ICs when added to D = 0 conditions but the impact of the molecule was reduced with D = 1 conditions. Furthermore, TAT-HOXB4 did not produce a significant increase in expansion when added to $Lin^-Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$ cells (Figure 5E). Collectively, these data provide further insight into the feedback signaling control mechanism that underpins the HSC-supportive effects of the fed-batch system.

DISCUSSION

A robust strategy to generate ex-vivo-expanded HSCs will enable the use of UCB for transplantation in patients for whom a single cord blood unit would not contain the desired progenitor content and ensure that a much greater proportion of current and future banked UCB units are applicable for use by any patient meeting the HLA matching criteria. In this study, we have computationally interrogated and experimentally validated a highly tunable hematopoietic progenitor cell expansion strategy that can produce 11 times more blood stem cells than originally present over a 12 day culture period. The short culture time and continuous tight regulation of cell densities also allows this system to have reduced media volume needs as compared to other expansion strategies, which is an important feature for cost-effective clinical implementation. This system has been designed to be adaptable for direct scale-up to accommodate cell numbers needed for human transplantations and we are planning to integrate this technology into clinical trials in the near future.

The fed-batch strategy relies on simplifying the complexity of dynamic and heterogeneous hematopoietic culture systems. In doing so, the need for targeted inhibition of individual endogenously produced factors is eliminated. We have previously shown evidence that mature hematopoietic cells and their associated secreted factors have a net inhibitory effect on stem cell self-renewal during in vitro culture (Madlambayan et al., 2005). Strategies to overcome this typically involve a significant amount of undesired manipulation and handling and provide only a temporary solution, because undesired factors will quickly reaccumulate after each manipulation. Perfusion cultures have been shown to enhance UCB progenitor cell expansion (Koller et al., 1998), but these cultures are subject to the challenge of high

cell densities and rapid factor reaccumulation. This challenge can be overcome with the use of higher perfusion flow rates, but this then fails to maintain desired usage limits of media and cytokines. The fed-batch strategy has the benefit of slowing the rate of factor accumulation through the continuous dilution of both endogenous factors and the cells that secrete these factors.

All current expansion strategies that rely on the direct regulation of the hematopoietic stem and progenitor cell populations are subject to the unregulated accumulation of inhibitory endogenous factors which, if unaccounted for, limit achievable expansion levels. Recent reports of human HSC expansion with apparently stem-cell-autonomous factors include SR1, producing a 17-fold LTR-HSC expansion (analyzed 16 weeks posttransplantation) after 21 days of in vitro culture, the immobilized Notch Delta-1 ligand described by Delaney et al. (2010) producing a 15.6-fold and 6.2-fold in vivo repopulation cell expansion (analyzed at 3 and 9 weeks posttransplantation, respectively) after 17–21 days of culture, and the growth factors Angiopoietin-like 5 and IGFBP2 (Zhang et al., 2008), producing a 14-fold repopulating cell expansion (analyzed at 8 weeks posttransplantation in NOD/SCID recipients) after 10 days of culture. The fed-batch strategy reported here gives a LTR-HSC expansion of comparable magnitude with a 12 day culture time and a conservative 16 week posttransplantation analysis. Because this strategy affects nonautonomous feedback regulation and acts on the microenvironment of the culture system, it suggests strong potential to synergize with primarily autonomously acting expansion strategies, the combinations predicted to lead to greater and more sustained cell expansions.

It is clear that in vitro (and in vivo) hematopoiesis is dynamic and regulated, at least in part, through nonlinear feedback control. The trajectories of factor secretion vary widely among individual factors and do not always correlate with the exponential trajectory of total cell expansion. Factor concentration dynamics may follow the dynamics of specific lineage subpopulations or may result from multiple interacting feedback networks. Moving to nonlinear media dilution strategies is one example of how more sophisticated dilution dynamics can be predicted to further tune regulatory interaction and HSC growth in the system. In order to maximize this control, a feedback-regulated system in which a set of critical factors is measured, either ''offline'' by ELISA assay or ''online'' with the adaption of current technologies to quantify multiple soluble factors in real-time (Klostranec et al., 2007), could be linked to a threshold level process control mechanism. This should allow for the dilution strategy to be tuned in real-time and would provide a means to account for sample-to-sample biological variability, ensuring that optimal expansion can be achieved for each specific cord blood unit.

The fed-batch strategy is a globally acting expansion strategy amenable for clinical use, alone or in combination with other expansion protocols. It also provides a platform with which to more closely study the dynamic nature of the in vitro hematopoietic cell culture system. We have illustrated how the fed-batch strategy has different effects when acting in combination with factors that directly or indirectly enhance HSC growth. These studies provide insight into the mode of action of the aryl hydrocarbon receptor antagonist SR1 and the TAT-HOXB4 protein, through the differing effect that the D = 1 strategy has on their efficacy. More broadly, they demonstrate that regulating feedback signaling can act to reduce inhibitory feedback, thereby allowing factors that target HSC self-renewal to act with maximal impact.

The ability to modulate secreted factor concentrations and measure corresponding functional outputs of cell expansions will allow for a more precise study of links between specific endogenous protein secretion and lineage subpopulations and their associated cell-cell interactions. This strategy serves as a robust clinically relevant system for rapid and automated in vitro cell expansion as well as a platform for further study of the regulation of cell-cell interactions in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Mathematical Simulations

The model that we previously developed and described (Kirouac et al., 2009) was used to run all culture simulations with MATLAB 2009 software (Mathworks, Natick, MA). The model simulates in vitro hematopoietic culture by incorporating self-renewal and differentiation of cell populations and soluble factors secreted by mature cells. For this study, the model was adapted to simulate fed-batch and perfusion strategies by adjusting the rate of change of secreted factor concentrations. The fed-batch strategy incorporated a continuous input stream, which resulted in an increase in culture volume and a dilution of all cells and all endogenously produced secreted factors. The perfusion strategy included a continuous input and output stream, resulting in a constant cell culture volume and a continuous reduction of endogenously produced soluble factors. See Supplemental Experimental Procedures for details.

Umbilical Cord Blood Cell Collection and Processing

UCB samples were collected from consenting donors according to ethically approved procedures at Mt. Sinai Hospital (Toronto, ON, Canada). Mononuclear cells were obtained as previously described (Kirouac et al., 2009). Lineage-negative (Lin^-) progenitor cells were isolated from the mononuclear cell fraction with the StemSep system or EasySep system with the human progenitor cell enrichment kit (StemCell Technologies, Inc., Vancouver, BC, Canada), according to the manufacturer's protocol.

Cell Seeding and In Vitro Culture

Freshly isolated Lin^- cells were seeded at an initial density of 1×10^5 cells/ml in serum-free IMDM media (GIBCO, Rockville, MD) with 20% BIT serum substitute (StemCell Technologies) and 1% Glutamax (GIBCO). The media was supplemented with 100 ng/ml Stem Cell Factor (SCF, R&D Systems, Minneapolis, MN), 100 ng/ml FMS-like Trysine Kinase 3 Ligand (FL, R&D Systems), 50 ng/ml Thrombopoietin (TPO, R&D Systems), and 1 μg/ml low-density lipoproteins (LDL, Calbiochem, La Jolla, CA). The syringe loaded pumping system was assembled and connected to the cell culture bag, as previously described (Csaszar et al., 2009). The initial cell suspension was injected into a 2-port 12 ml culture bag (VueLife, American Fluoroseal Corporation, Gaithersburg, MD) and maintained on an orbital shaker at 37°C and 5% CO_2. The pump was set to deliver the desired volume of media (based on the user-defined dilution rate) to the cell culture. Media delivery was automated to occur at 0.5 hr intervals at a flow rate of 30 μl/min, for a semicontinuous delivery. For SR1 studies, SR1 was added to fresh media at 0.75 μM as previously described (Boitano et al., 2010). For TAT-HOXB4 studies, TAT-HOXB4 was produced and delivered semicontinuously as previously described (Csaszar et al., 2009; Krosl et al., 2003).

Cell Assays

Colony-forming cell (CFC) assays and long-term culture-initiating cell (LTC-IC) assays were performed as previously described (Kirouac et al., 2009). Surface marker staining was performed with conjugated human antibodies: CD4, CD7, CD8, CD14, CD19, CD33, CD34, CD38, CD41, CD49f, CD56, CD90, CD133, and GyA (BD Biosciences, San Jose, CA).

7-AAD dye was added to assess cell viability and isolate live cells for quantification. All samples were analyzed on a FACSCanto flow cytometer (BD Biosciences).

Sorted Cell Assay

Freshly isolated Lin^- cells were sorted for $Rho^{lo}CD34^+CD38^-CD45RA^-CD49f^+$ with a FACSAria flow cytometer (BD Biosciences), according to the gating strategy previously described (Notta et al., 2011). 40 sorted cells were dispensed per well in a 96-well plate in the above-described media and cultured for 7 days. After culture, cells were assessed for total cell number and number of $CD34^+CD133^+CD90^+$ cells by flow cytometry.

Limiting Dilution Transplantation Studies

All animal studies were performed according to procedures approved by appropriate animal ethics boards. Female NSG mice were sublethally irradiated (250 rad) <24 hr before transplantation. Uncultured Lin^- cells (n = 26) or cells cultured for 8 days (n = 50) or 12 days (n = 25) were transplanted at limiting dilution via tail vein injection. Mice were sacrificed 16 weeks after transplantation and bone marrow was collected from femurs and tibias. Cells were assessed by flow cytometry. Mice were scored positive for human repopulation if at least 0.5% of bone marrow cells were positive for both human CD45 and human HLA-ABC. For secondary transplantations, 33% of the harvested bone marrow cells of positively engrafted mice were transplanted into secondary NSG recipients. Bone marrow harvest and analysis was performed after 16 weeks. All limiting dilution analysis was performed with the L-calc software (StemCell Technologies, Inc.). Limiting dilution analysis was based on the combined data of two independent transplantation studies.

Secreted Factor Analysis

Secreted factor concentrations were sampled in duplicate from conditioned media samples using the Human Cytokine 30-Plex panel (Invitrogen, Burlington, ON, Canada), designed for the Luminex microsphere detection platform (Luminex Co. Austin, TX), to screen for EGF, Eotaxin, FGF-β, G-CSF, GM-CSF, HGF, IFN-α, IFN-γ, IL-1β, IL-IRA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10 (CXCL10), MCP-1 (CCL2), MIG (CLCL9), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), TNF-α, and VEGF. Samples were prepared and assessed with a BD FACSCanto flow cytometer, as previously described (Kirouac et al., 2009). TGF-β1 was analyzed separately in parallel, with a TGF-β1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's directions.

Glucose and Lactate Assay

Conditioned media samples were analyzed for glucose levels, with the Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen), according to the manufacturer's protocol. In the subsequent glucose normalization study, D-glucose was added to the D = 0 cell culture media every 2 days. Lactate concentrations were analyzed with a L-lactate assay kit (Eton Bioscience, San Diego, CA), according to the manufacturer's protocol.

Statistical Analysis

Statistical significance was computed via a Student's t test. All error bars represent the standard deviation of three or more biological replicates. Asterisks indicate statistical significance between conditions of $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at doi:10.1016/j.stem.2012.01.003.

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