Combination of SB431542, Chir9901, and Bpv as a Novel Supplement in the Culture of Umbilical Cord Blood Hematopoietic Stem Cells

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Research

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Abstract

**Background:** Small molecule compounds have been well recognized for their promising power in generation, expansion and maintenance of embryonic or adult stem cells. The aim of this study was to identify a novel combination of small molecules in order to optimize the *ex vivo* expansion of umbilical cord blood derived-CD34\(^+\) cells.

**Methods:** Considering the most important signaling pathways involved in the self-renewal of hematopoietic stem cells, CB-CD34\(^+\) cells were expanded with cytokines in the presence of seven small molecules including SB, PD, Chir, Bpv, Pur, P\(_\mu\) and NAM. Eliminativism approach was used to find the best combination of selected small molecules for effective *ex vivo* expansion of CD34\(^+\) cell. In each step, proliferation, self-renewal, and clonogenic potential of the expanded cells as well as expression of some hematopoietic stem cell related genes were studied. Finally, the engraftment potential of expanded cells was also examined by the mouse intra-uterine transplantation model.

**Results:** Our data shows that simultaneous use of SB431542 (TGF-\(\beta\) inhibitor), Chir9901 (GSK3 inhibitor) and Bpv (PTEN inhibitor), resulted in a 50-fold increase in the number of CD34\(^+\)CD38\(^-\) cells. This was further reflected in approximately 3 times increase in clonogenic potential of the small molecule cocktail-expanded cells. These cells, also, showed a 1.5-fold higher engraftment potential in the peripheral blood of NMRI model of in utero transplantation. These results are in total conformity with up-regulation of HOXB4, GATA2 and CD34 marker gene as well as CXCR4 homing gene.

**Conclusion:** Taken together, our findings introduce a novel combination of small molecules to improve the yield of existing protocols used in the expansion of hematopoietic stem cells.

Introduction

Umbilical cord blood (UCB) as one of the most valuable and convenient source of hematopoietic stem cells (HSCs) has a great potential for treatment of various haematological, non-haematological disorders and cancers (1–4). However, limited number of HSCs in a UCB unit has limited its use to the young patients. In this regard, *ex vivo* expansion is one of the main solutions for acquiring sufficient numbers of HSCs (5, 6). Therefore, in recent years, many efforts have been made to identify the factors affecting the self-renewal of the umbilical cord blood hematopoietic stem cells.

The use of small molecules in the field of hematopoietic stem cell research has grown rapidly in recent years, as they are good tools for controlling the variety of cellular processes (7). There are different approaches to select small molecules in HSCs expansion; induction of self-renewal, inhibition of lineage commitment differentiation, inhibition of HSC apoptosis. In the present study, we hypothesized that the best expansion is achieved when the proliferation, survival and self-renewal pathways are induced while the apoptosis and differentiation pathways are inhibited. Therefore, through data mining, a limited set of seven small molecules were selected which are as following:
• SB431542 (SB) and Purmorphamin (Pur) that regulate TGFβ and SHh pathways and are associated with the proliferation of HSCs.
• PD0325901 (PD) and Chir9901 (Chir) that regulate Wnt/β-catenin and ERK pathways and play important role in HSCs differentiation.
• Bisperoxovanadium (Bpv) and Pifithrin-µ (Pµ) are associated with the pathways related to HSC survival like Akt and P53.
• Nicotinamide (NAM) that facilitate the transcriptional epigenetic changes of chromatin.

The main question was whether a cocktail of these small molecules along with SCF, TPO and Flt3L could improve the self-renewal and transplantation potential of ex vivo expanded cells. To find the best combination, the eliminative approach was used, in which the components of a system are removed one by one; then the interaction between the other components is investigated and the system is re-constructed. Here, we report that a cocktail consisting of SB, Chir and Bpv is effective in promoting the cord blood hematopoietic stem cell proliferation while their stemness and in vivo engraftment potential maintained.

Methods

Ethical approval

All the experiments in this study were reviewed and approved by the Research Ethics Committee of Royan institute and was conducted in accordance with the ethical principles and the national norms and standards for conducting Medical Research in Iran (IR.ACECR.ROYAN.REC.1398.189).

HSC isolation

Schematic illustration of procedure was shown in Supplementary Fig. 1. Umbilical cord blood (UCB) samples were obtained from Royan Cord Blood Bank. Collection of UCB was performed with the informed consent of the mother. To isolate CD34⁺ cells, Hydroxyethyl starch (Grifols, Spain), lymphoprep™ (Stem Cell Technology Inc.) density-gradient centrifugation and immuno-magnetic selection kit (Miltenyi Biotec, Germany) was used. Highly purified (>90%) CD34⁺ cells were confirmed by flowcytometry (Partec PAS system, USA) and then prepared to expand in different culture condition.

Cell viability assay

To find the proper dose of small molecules, the cell viability test was used. Briefly, cells were seeded into 96-well plates at a density of 1.0 × 10⁴ cells/well in different concentration of small molecules for 24h. Control cells received an equal amount of 10% FBS-IMDM medium with no small molecule. For MTS assay, 100 μL of MTS (Promega) was subsequently added to each well and then incubated in the dark at 37 °C for at least 1 h. The absorbance was measured at 490 nm. All groups were normalized to the same
control group and significant data was calculated using one-way ANOVA. All data were collected from five independent experiments.

**Cell culture**

Umbilical cord blood CD34+ cells were cultured in the serum-free StemSpan™ medium (Stem cell technology Inc.) supplemented with three basic cytokines: 100 ng/mL stem cell factor (SCF), 100 ng/mL Fms-related tyrosine kinase 3 ligand (Flt3-L), and 50 ng/mL thrombopoietin (TPO), all from R&D. Three small molecules named SB431542 (SB), Chir9901 (Chir), Bpv at various concentrations were added at the same time. Cytokines only served as a positive control. The cells were maintained at 37°C in a humidified atmosphere containing 5% of CO2 for 10 days. Cell numbers and CD34/45 expression were determined and analyzed on days 0 and 10.

**Immunophenotyping of expanded cells**

Viable cells were enumerated by the trypan blue exclusion method and cellular expansion fold was calculated based on the initial inputs. Cells were collected and stained with an anti-human CD34 monoclonal antibody conjugated to phycoerythrin (PE; BD Pharmingen™) and an anti-human CD38 monoclonal antibody conjugated to allophycocyanin (PerCP-Cy™5.5, BD Pharmingen™), together or separately. The appropriate isotype control antibodies were used for setting the Partec PAS system. At least $10^4$ events were acquired and data was analyzed using FlowMax software.

**Colony-forming assay**

Colony-forming units (CFUs) were generated by seeding 300 CD34+ cells into 1.1 ml methylcellulose media (H4434, Stem Cell Technologies, Canada) diluted with IMDM + 2% FBS at a ratio of 1/10. The colonies including burst-forming units-erythroid (BFUs-E), CFUs granulocyte-macrophage (CFUs-GM), CFUs granulocyte-erythrocyte-macrophage-megakaryocyte (CFUs-GEMM), were scored on day 14-16 at 4X magnification under an inverted microscope.

**RNA extraction and qPCR**

Total RNA was isolated using QIAzol lysis reagent. Integrity and quality of RNA samples were checked using a Nanodrop (ND-1000) spectrophotometer. 1 µg of the total RNA was subjected to reverse transcription using oligo-dT and PrimeScript™ 1st strand cDNA kit (Takara, Japan). Transcript levels were determined using the SYBR Green master mix and Corbett Rotor-Gene 6000. The GAPDH-normalized transcript data are shown as relative expression levels in the small molecules cocktail compared to the corresponding level in positive control group. The primer sequences for qRT–PCR are listed in Supplementary Table 2.

**Animals and xeno-transplantation study**
Transplantation procedure was done as explained previously (8). Briefly, on embryonic days E11.5–E13.5, each NMRI embryo injected intraperitoneally with 2-3×10^4 fresh CD34^+ cells or their entire progeny following 10 days expansion. Evaluation of chimerism was performed monthly up until age 4 months after growth factor treatment beginning at 3 weeks of age as explained previously. After staining the peripheral blood with anti-Human CD45, at least 10^5 cells were analyzed on a Partec system. Engraftment defined as detection of 0.2% or more human CD45 cells.

**Statistical analysis**

All the data were presented as mean ± SD of at least three different biological replicates. Statistical comparisons between the groups were examined by two-tailed Student’s t-test assuming unequal variances. P<0.05 was considered statistically significant difference.

**Results**

**Optimization of small molecules doses for HSC expansion**

The aim of dose finding was to set appropriate concentrations of selected small molecules which was not cytotoxic for HSCs. We selected initial concentrations of small molecules based on previous studies (Supplementary Table 1). The UCB-HSCs cultivated in SB (10 μM), Bpv (5 μM), NAM (2.5 μM) and Pur (4 μM) were viable. The result was in consistent with the other studies (Fig. 2). However, predetermined concentrations of PD (1 μM), Chir (3 μM) and Pμ (10 μM) were toxic for UCB-HSCs. Therefore, lower concentrations of PD (0.25 μM), Chir (0.37 μM) and Pμ (2.5 μM) were added to the culture medium (Fig. 1).

**SB, Chir and Bpv are sufficient for ex vivo expansion of UCB-CD34^+ cells**

We next did some serial experiments (Supplementary Fig. 1). In first round of experiments, isolated UCB-CD34^+ cells were cultured in presence of cytokines (SCF, TPO and Flt3L) and selected small molecules. In other groups (small molecules) SMs were deleted one by one from the pool of 7 SMs. Although, individual removal of SB, Chir, Bpv, Pur, NAM and Pμ did not make significant differences in total nuclear cells (TNCs) number compared to the 7SMs group, removal of PD yielded increased total number of mononuclear cells (Fig. 2A). The precise effect of PD on ex vivo expansion of CD34^+ cells has been discussed before (9). Additional round of small molecules removal showed that, deletion of NAM and Pur from the cocktail increased the fold expansion of TNCs and CD34^+ cells. Furthermore, the groups lacking NAM and Pur had a higher colony forming potential, especially CFU-GM, compared to other groups containing small molecules (Fig. 2B). In next round, by removing Pμ the number of CD34^+ cells, CFU-GM and CFU-GEMM colonies was increased significantly compared to the PC group (Fig. 2C). In the final round removal of SB, Chir, or Bpv reduced the expansion of CD34^+CD38^- cells and abolished formation of CFU-GM and CFU-GEMM colonies, showing that these are essential for CD34^+ cell expansion (Fig. 2D). Although, there was no significant difference between the 3SMCs and the positive control in terms of TNC
expansion, removal of Bpv slightly increased the TNC fold expansion compared to 3SMs group (118 to 140). Moreover, exclusion of each of the remaining three SMs (SB, Chir, or Bpv) had a dramatic negative impact on the expansion CD34+CD38− cells. Expansion with these three SMs (SB, Chir and Bpv) produced a 2.7-fold increase in the number of CD34+CD38− cells relative to positive control (17 vs. 47). Finally, a CFU assay was performed to determine if the optimal SM cocktail actually promotes the expansion of hUCB-HPCs. As shown in Fig. 2D, the number of total CFUs increased more than 3-fold when CD34+ cells were expanded in the presence of SB, Chir and Bpv for 10 days compared to the positive control. The expanded cells generated significantly more BFU and CFU-GM than the positive control (p<0.01). However, the number of GEMMs in SM group was slightly greater than that of the PC, but the difference was not statistically significant (p>0.05).

The ability of 3SMs cocktail to enhance the short-term engraftment potential of ex vivo expanded CD34+ cells in the in utero transplanted NMRI mice

In order to evaluate the in vivo functional capability of the expanded CD34+ cells, we used in utero transplantation model (8). We transplanted 30-50×10³ freshly isolated hUCB-CD34+ cells or the cells harvested from the cultures with the same number of input hUCB-CD34+ cells in the presence or absence of SMs cocktail into NMRI mouse embryos, E11.5-E13.5. 2 weeks after birth, born mice were treated with human hematopoietic growth factors SCF (4ng/g), IL-3 (4ng/g) and G-CSF (50ng/g) for one week. As shown in Fig.3, by treatment with human hematopoietic factor, the hCD45+ chimerism was distinctly increased compared with initial values, 4 and 8 weeks post transplantation. 16 weeks after transplantation, the average human cell engraftment in the peripheral blood of the mice transplanted with freshly isolated hUCB CD34+ cells was about 1%. While, the percentage of CD45+ cells in 3SMs and positive control transplanted mice was 9 times and 3.4 times (3.6±1 and 3.2±0.3) respectively, compared to the unexpanded cell recipients (Fig. 3). In the other words, ex vivo expansion of hUCB CD34+ cells with SM cocktail resulted in 1.5 fold increase in human cell engraftment compared to the positive control.

Ability of the optimal SMs cocktail to modulate the cell signaling pathways

Subsequently, RT-qPCR was performed in order to determine the expression of typical genes involved in HSC stemness. The result shows that the relative expression of the two major genes involved in the proliferation and self-renewal of HSCs, including HOXB4 and GATA2 as well as the HSC-specific marker, CD34, have significantly increased in the presence of 3SMs cocktail after normalization to the level of the PC group. Furthermore, the expression of the CXCR4 gene involved in the migration and transplantation of HSCs has increased dramatically in the presence of 3SMs cocktail. The expression of other genes associated with self-renewal, such as ABCG2, Notch and Bmi1, does not show a significant difference between the groups (Fig. 4).

Discussion
In recent years, small molecules have been widely used in the field of stem cell research. So far, there have been numerous indications for the successful use of small molecules to inhibit apoptotic and differentiation processes during hematopoietic stem cell reproduction (7). It seems that combination of two or more small molecules may produce a better result. For example, the effect of chir and insulin (10), chir and rapamycin (11) along with SCF, TPO and Flt3L have been reported to enhance the proliferation of mouse hematopoietic stem cells. The proliferation of human hematopoietic stem cells has also been studied in the presence of various combinations of 5Aza, TSA, VPA and NAM (12-14). Notably, based on the cell status, synergistic/antagonistic interactions may have been created between the small molecules. As a result, the simultaneous use of small molecule compounds can produce unpredictable results compared to their individual use. To our knowledge this is the first study in which expansion of CD34+ cells is targeted through the simultaneous modulation of proliferation, differentiation and apoptosis signaling pathway (Fig. 5).

In this study, a cocktail of seven small molecules were selected to target the TGFβ, ERK, Wnt, Akt, Hedgehog and P53 signaling pathways as well as the cell epigenome. Then, their best combination to induce efficient HSC expansion was screened through eliminative approach. To successful expansion of UCB-CD34+ cells, SCF, TPO and Flt3L which greatly affect the HSC signaling pathways were also added to the culture medium. Our experiments conducted us to this notion that addition of SB, Chir and Bpv to the HSC conventional HSC culture medium increases the efficiency of ex vivo expansion of CD34+ cells with 50-fold enhancement in the number of CD34+38- cells. The small molecule cocktail can also augment colony formation ability of expanded cells (Fig. 2). All these changes were associated with up-regulation of HOXB4, GATA2 as well as CD34 gene. Moreover, here, higher engraftment potential and higher percentage of human CD45 cells in infused mice confirm the in vivo potential of the expanded cells in the presence of small molecule cocktail.

In overall, according to our findings, the best result is obtained by simultaneous controlling of PTEN/Akt, Wnt/β-catenin and TGFβ signaling pathways. In such a way that Bpv leads to exiting the cells from the quiescence and proliferation through inhibiting PTEN and enhancing the Akt pathway. On the other hand, Chir indirectly inhibits the differentiation process through GSK3 inhibition and β-Catenin activation. All of these events occur while TGFβ, the most important apoptotic pathway is inhibited by SB (Fig. 5).

PI3K-AKT pathway is one of the most important pathways affecting a wide range of stem cells cellular signaling molecules (15). In particular, many apoptotic proteins such as Bim and Bcl-2 can be inactivated by the pathway. AKT, also, inhibits certain cell cycle inhibitors such as P21 and P27 and activates Cyclin D, which in turn leads to exit from G0 and entry into the cell cycle (16). Furthermore, Akt facilitates the migration of HSCs and their binding to the bone marrow stromal cells through induction of integrin expression (17, 18). PTEN is a tumor suppressor protein that inhibits the PI3K-AKT pathway. Actually, inhibition of PTEN leads to increased survival, proliferation, self-renewal as well as incomplete differentiation potential of embryonic stem cells (19) and also in vitro proliferation of HSCs (10).
Wnt pathway not only plays a critical role in development of embryonic stem cells (20), but also in proliferation and differentiation of adult stem cells including HSCs (21, 22). The major effects of Wnt are applied through β-catenin which can increase the self-renewal and proliferation of HSCs, even independently of Wnt pathway (23, 24). According to previous studies, accumulation of the β-catenin, following GSK3 inactivation, facilitates maintenance the pluripotency state of embryonic and adult stem cells (25, 26).

TGFβ is one of the major negative regulators of HSC proliferation (27). The pathway, specifically, inhibits cell cycle progression through induction of P57 expression; which, in turn leads to CyclinD-Cdk4/6 and CyclinE-Cdk2 inactivation. P38MAPK is also a downstream molecule of TGFβ pathway which its inhibition results in decreased in vitro apoptosis and aging of HSCs (27). JNK is another downstream target of TGFβ which activates some apoptotic factors such as Bcl2, Bad. Therefore, inhibition of TGFβ pathway not only leads to P57, P38MAPK, and JNK inhibition which is associated with cell cycle promotion, but also inhibits the apoptotic pathways (28-30).

In overall, a cocktail of SB431542, Chir99021 and Bpv, which respectively inhibits the TGFβ differentiation pathway and activates the Wnt and Akt pathways, can be used to improve the conventional protocol of HSC expansion.

**Abbreviations**

UCB: Umbilical cord blood, HSCs: Hematopoietic stem cells, SMs: Small molecules, SB: SB431542, Pur: Purmorphamin, PD: PD0325901, Chit: Chir9901, Bpv: Bisperoxovanadium, Pμ: Pithrin-μ, NAM: Nicotinamide, CFUs: Colony-forming units, BFUs-E: Burst-forming units-erythroid, CFUs-GM: CFUs Granulocyte-macrophage, CFUs-GEMM: CFUs Granulocyte-erythrocyte-macrophage-megakaryocyte

**Declarations**

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and in supplementary figures.
Ethics approval

All the experiments in this study were reviewed and approved by the Research Ethics Committee of Royan institute and was conducted in accordance with the ethical principles and the national norms and standards for conducting Medical Research in Iran (IR.ACECR.ROYAN.REC.1398.189).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors’ Contributions

M.Z., E.A.; Performed all *in vitro* experiments, analyzed the data and wrote the manuscript. M.H.A.; Performed *in vivo* experiment and analyzed the *in vivo* data. M.E.; Contributed to concept and design, financial support, and final approval of the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

The cytotoxic effect of selected small molecules (SB, Bpv, NAM, Pur, PD, Chir and Pμ) on UCB-HSCs. In each graph, the middle column corresponds to the reference concentration of the small molecules based on literatures. Two-point lower and Two-higher concentrations were selected for cytotoxic assay. Cell viability was measured by MTS assay post 48 hours incubation with small molecules. The negative control in each group was used for normalization of data. Bars indicated as mean ± SD at least five independent replicates. * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001.
Figure 2

Characterization of expanded UCB-CD34+ cells in the presence or absence of different combination of small molecules. TNC fold expansion, CD34+ cells percentage, fold expansion of CD34+ cells and colony forming potential of UCB-CD34+ cells was evaluated in each experiment. (A) 7 SMs cocktail (SB, PD, Chir, Bpv, NAM, Pur, Pμ) and its derivative groups (B) 6 SMs cocktail (SB, Chir, Bpv, NAM, Pur, Pμ) and its derivative groups (C) 4 SMs cocktail (SB, Chir, Bpv, Pμ) and its derivative groups (D) 3 SMs cocktail (SB,
CD34+ cells cultivated in presence of SCF, FLT3L and TPO was used as positive control. Fold expansion was determined by dividing the total number of viable cells expressing the phenotype at the end of the culture by the input number of viable cells expressing the same phenotype (n=3). Statistically significant difference compared with positive control group, *P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001.

Figure 3

Mean human engraftment levels in the peripheral blood of NMRI mice fetal transplanted with expanded hUCB-CD34+ cells. (A) The percentage of human CD45 cells in the peripheral blood of newborn mice. Each bar indicated mean±SD for at least 6 independent samples. ****P ≤ 0.0001. (B) Each shape indicates the percentage of human CD45 expression in the peripheral blood of one newborn mouse. Mice with ≥0.2% human cells were considered chimeric.
**Figure 4**

Treatment by SB, Chir and Bpv modifies the gene expression of UCB-CD34+ cells. Bars represent the mean fold-changes of gene expression in the 3 SMs-expanded cells relative to the positive control group detected by quantitative real-time PCR (n=3), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 vs. positive control.

**Figure 5**

The molecular mechanisms which through them SB, Chir and Bpv modulate proliferation, differentiation and survival of hematopoietic stem cells.

**Supplementary Files**
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