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Ex Vivo Expansion of Hematopoietic Stem Cells from Human Umbilical Cord Blood-derived CD34+ Cells Using Valproic Acid

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Corresponding Author:	Luena Dr. Papa, Ph.D. Icahn School of Medicine at Mount Sinai Manhattan, New York UNITED STATES
Corresponding Author's Institution:	Icahn School of Medicine at Mount Sinai
Corresponding Author E-Mail:	luena.papa@mssm.edu
Order of Authors:	Luena Dr. Papa
	Luena Papa
	Mansour Djedaini
	Ronald Hoffman
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To the editors:

We were invited by Dr. Ronald Meyer to submit our manuscript entitled "***Ex Vivo* Expansion of Hematopoietic Stem Cells from Human Umbilical Cord Blood-derived CD34$^+$ Cells Using Valproic Acid**" for publication in the Journal of Visualized Experiments.

Umbilical cord blood (UCB) is an alternative source of hematopoietic stem cells (HSCs) for patients who require allogeneic stem cell transplantation. The use of UCB for this purpose is restricted due to the limited number of HSCs within a single UCB unit. Others and us have utilized different strategies and protocols to overcome this limitation. Such methods result in various degrees of expansion in HSC numbers. Importantly, *ex vivo* culture conditions in these protocols induce stress, leading to rapid cell proliferation, increased metabolic activity and loss of the primitive characteristics that define primary HSCs. Therefore, there is a great need to develop strategies and protocols that lead to expansion of fully functional HSC with characteristics that closely resemble primary primitive HSCs.

The method described in this manuscript utilizes a combination of a cytokine cocktail with a histone deacetylase inhibitor, valproic acid (VPA) that allows for the expansion of great numbers of HSCs from UCB-derived CD34$^+$ cells. *Ex vivo* expanded HSCs exhibit both phenotypic and primitive metabolic characteristics that resemble those of primary HSCs. Importantly, expanded HSC grafts generated with this protocol have the capacity to regenerate the hematopoietic system since they can differentiate into all hematopoietic cell lineages and establish long-term engraftment following

transplantation into myeloablated NSG mouse models. This strategy has been up-scaled into GMP grade and a NYSTEM-supported clinical trial is planned to start within 2018.

This protocol is rapid and relatively simple and cost effective. It is highly reproducible and generates two types of HSCs. The 7-day expansion protocol allows generation of HSCs that can be used for allogeneic HSC transplantation where more rapid as well as sustained multilineage engraftment is required. The 4-day expansion protocol can be used for genetic modification strategies that target long-term repopulation cells. This protocol might be beneficial for improving existing gene editing protocols and enable correction of genetic mutations in HSC transplantation-based therapies for diseases including β-thalassemia and sickle cell disease. Moreover, it can be applied to maintain great numbers of HSCs during manipulation of genes for studies focused on elucidation of gene functions in HSCs and in hematopoiesis.

Thus, the method described here can be used for *ex vivo* expansion of both human and murine HSCs and can be tailored to specific clinical applications as well as to a wide spectrum of investigations in basic research.

The authors have no conflicts of interest to declare related to this submission.

TITLE:

Ex vivo Expansion of Hematopoietic Stem Cells from Human Umbilical Cord Blood-derived CD34$^+$ Cells Using Valproic Acid.

AUTHORS & AFFILIATIONS:

Luena Papa[1], Mansour Djedaini[1], and Ronald Hoffman[1].

[1]Division of Hematology/Oncology, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

Corresponding Authors:

Dr. Luena Papa (Luena.Papa@mssm.edu)
Tel.: (212) 241-1331
Fax: (212) 876-5276

Dr. Ronald Hoffman (Ronald.Hoffman@mssm.edu)
Tel.: (212) 241-2296
Fax: (212) 876-5276

Email Address of Co-Author:

Mansour Djedaini (Mansour.Djedaini@mssm.edu

KEYWORDS:

umbilical cord blood, CD34$^+$ cells, ex vivo culture, VPA, HSCs, ex vivo expansion, transplantation, gene therapy.

SUMMARY:

Here, we describe the ex vivo expansion of hematopoietic stem cells from CD34$^+$ cells derived from umbilical cord blood treated with a combination of a cytokine cocktail and VPA. This method leads to a significant degree of ex vivo expansion of primitive HSCs for either clinical or laboratory applications.

ABSTRACT:

Umbilical cord blood (UCB) units provide an alternative source of human hematopoietic stem cells (HSCs) for patients who require allogeneic bone marrow transplantation. While UCB has several unique advantages, the limited numbers of HSCs within each UCB unit limits their use in regenerative medicine and HSC transplantation in adults. Efficient expansion of functional human HSCs can be achieved by ex vivo culturing of CD34$^+$ cells isolated from UCBs and treated with a deacetylase inhibitor, valproic acid (VPA). The protocol detailed here describes the culture conditions and methodology to rapidly isolate CD34$^+$ cells and expand to a high degree a pool of primitive HSCs. The expanded HSCs are capable of establishing both short-term and long-term engraftment and are able to give rise to all types of differentiated hematopoietic cells. This

method also holds potential for clinical application in autologous HSC gene therapy and provides an attractive approach to overcome the loss of functional HSCs associated with gene editing.

INTRODUCTION:

Ex vivo expansion of hematopoietic stem cells (HSCs) from umbilical cord blood (UCB) units holds great promise for HSC applications in regenerative medicine and transplantation therapy. Transplantation with UCB units has several unique advantages such as easy collection, high availability, minimal risk of infection, low risk of disease relapse, and low frequency of graft-versus host disease (GVHD). However, the major disadvantages of their use in clinical settings are the limited number of HSCs present within each UCB unit[1]. The insufficient number of HSCs results in delayed engraftment and hematopoietic recovery, risk of graft rejection, and aberrant immune reconstitution.

Currently, various methods and strategies have been developed to ex vivo expand the limited number of HSCs from UCBs. Combinations of different cytokine cocktails with small molecules or compounds in ex vivo cultures result in various degrees of expansion in HSC numbers[2-8]. Importantly, ex vivo culture conditions induce stress, leading to rapid cell proliferation, increased metabolic activity and loss of the primitive characteristics that define primary HSCs. Therefore, developing protocols that lead to expansion of a great number of functional HSCs with characteristics that closely resemble primary primitive HSCs are needed.

Serum-free cultures of $CD34^+$ cells isolated from UCBs and treated with valproic acid (VPA) result in the expansion of large numbers of primitive HSCs[4,9,10]. The HSC expansion is not solely due to the proliferation of the existing HSCs derived from UCBs. Instead, this expansion is due to the acquisition of a primitive phenotype combined with a limited number of cell divisions and proliferation[9]**.** Within the initial 24–48 h of incubation with a combination of cytokines and VPA, $CD34^+$ cells acquire a transcriptomic and phenotypic profile that characterize long-term HSCs. The significant increase in the percentage of HSCs is accompanied by a prompt increase in the number of HSCs (63 fold increase within 24 h of VPA treatment)[9]. Notably, the VPA-ex vivo expansion strategy expands HSCs with low metabolic activity, which further highlights their primitive characteristics.

The method described here provides conditions and treatments that lead to a significant degree of ex vivo expansion of primitive HSCs for either clinical or laboratory applications. This ex vivo expansion strategy uses a cytokine cocktail combined with VPA treatment. VPA is an FDA approved drug for treatment of bipolar disorders and other neurological diseases. The HSC expansion with VPA is prompt and occurs within 7 days, minimizing both the time of manipulation and the risk of contamination. Importantly, this protocol allows for the acquisition of long-term HSC phenotypic markers such as CD90 and CD49f within 24-48 h following treatment with VPA[9]. Expanded HSC grafts created with this protocol have the capacity to regenerate the hematopoietic system since they can differentiate into all hematopoietic cell lineages and establish long-term engraftment following transplantation into myeloablated NSG mouse models[4]. Moreover, this protocol is highly reproducible and allows efficient and rapid isolation of viable $CD34^+$ cells from UCBs, which is critical for industrialization of this procedure.

The VPA ex vivo expansion protocol also has the potential to overcome the significant loss of HSCs which occurs during gene editing[11]. Gene editing requires exposure to cytokines, which are necessary for cycling cells and activation of DNA-repair mechanisms. Due to the prompt effects of VPA treatment, this method might be beneficial for generation of a higher number of genetically modified cells within a period of time that is relevant for currently utilized gene modification protocols.

PROTOCOL:

The HSC ex vivo expansion protocol follows the guidelines of the Research Ethics Committee at Mount Sinai School of Medicine.

1. Buffer and media preparation

1.1. Prepare separation buffer 24 h prior to isolation of $CD34^+$ cells from UBCs by adding 2 mL of 0.5 M Ethylene Diamine Tetra-acetic Acid (EDTA) and 33 mL of 7.5% Bovine Serum Albumin (BSA) to 465 mL buffered saline (1x PBS). Mix gently and maintain the buffer overnight at 4 °C.

1.2. Warm media at room temperature on the day of purification.

2. Isolation of Mononuclear Cells (MNCs) from UCB unit

2.1. Dispense the whole UCB unit into a 75 cm^2 flask. Dilute and gently mix the UCB with an equal volume of PBS at room temperature.

2.2. Determine the number of tubes required for processing the whole UCB unit (one 50 mL conical tube can be used to process 35 mL of diluted UCB). Add 15 mL of density gradient media (see **Table of Materials**) to each tube and dispense the diluted UCB on the top of the density gradient media creating two layers.

NOTE: Dispense the diluted UCB very slowly while holding the tube at a 45-degree angle to prevent mixing of the density gradient media layer with the UCB.

2.3. Centrifuge at 400 x *g* for 30 min at a low acceleration and deceleration rate. After centrifugation, MNCs locate in the white layer (buffy coat) between the plasma and density gradient media layer.

2.4. Slowly aspirate 2/3 of the plasma without disturbing the buffy coat layer. Carefully transfer the buffy coat layer into a new 50 mL tube. Collect all MNCs from the same UCB unit into a 50 mL tube until reaching 25 mL. Use a new tube if the volume of collected MNCs exceeds 25 mL.

2.5. Add 25 mL of cold PBS into the tube containing 25 mL of MNCs and mix well. Centrifuge at 400 x *g* for 10 min at 4 °C.

NOTE: Cold PBS is important to prevent capping of antibodies on the cell surface and reduce non-specific labeling.

2.6. Carefully aspirate the supernatant and gently re-suspend the cell pellet in 50 mL of cold PBS.

2.7. Remove 20 µL of the cell suspension for counting. Count the cell number stained with acridine orange/propidium iodide staining by using an automated cell counter. Calculate the total number of MNCs.

NOTE: The cell count can be also performed by the trypan blue exclusion method using a hemocytometer.

2.8. Centrifuge at 400 x *g* for 15 min at 4 °C.

NOTE: If not required immediately, MNCs can be frozen at this stage.

3. Isolation of $CD34^+$ cells from UCBs

3.1. Prepare the $CD34^+$ antibody coupled with magnetic beads solution for isolation of $CD34^+$ cells from MNCs. Mix 300 µL of separation buffer with 100 µL of human FcR human IgG (blocking reagent) and 100 µL of CD34 magnetic beads for isolation of $CD34^+$ cells from each 10^8 MNCs. For isolation of a higher number of $CD34^+$ cells from (>10^8) MNCs, scale up reagents accordingly.

3.2. Carefully aspirate the supernatant from the tube centrifuged at step 2.8. Resuspend the pellet in the CD34 magnetic beads solution prepared in step 3.1 (use 500 µL of solution per 10^8 cells).

3.3. Mix gently and incubate at 4 °C for 30 min.

NOTE: Prepare the cell separator device (see **Table of Materials**) during the incubation period. Replace the storage solution with the working buffer as indicated by the manufacturer's instructions and run a washing program followed by a rinsing program.

3.4. Add cold cell separator running buffer to the tube containing the cell mixture until the tube is fully filled. Centrifuge at 400 x *g* for 15 min at 4 °C.

3.5. Aspirate the supernatant and resuspend the cell pellet in cold cell separator running buffer (2 mL/10^8 cells). Transfer the 2 mL of cell suspension mixture into 15 mL tubes.

3.6. Load 3 sets of 15 mL tubes into the cell separator rack prechilled at 4 °C. One set of tubes contains MNCs, the second set of tubes will be used to collect the negative fraction and the third set of tubes will be used to collect purified $CD34^+$ cells.

3.7. Load the rack into the cell separator device and run the **posseld2** preset program.

NOTE: An alternative method that utilizes manual magnetic separator can be used to replace the cell separator device[12] .

3.8. Centrifuge tubes with the positive fraction at 400 x *g* for 15 min at 4 °C. Aspirate the supernatant and resuspend purified $CD34^+$ cells in 1 mL of serum-free media.

3.9. Count the cells stained with acridine orange/propidium iodide using an automated cell counter.

NOTE: If not required immediately, purified $CD34^+$ cells can be frozen at this stage.

4. VPA ex vivo expansion of purified UCB-$CD34^+$ cells

4.1. Prepare sufficient volume of media to plate the purified $CD34^+$ cells at a density of 3.3 x 10^4 cells/mL. The culture media composition is serum Free culture medium (see **Table of Materials**) supplemented with 10 µL/mL pen/strep, 150 ng/mL stem cell factor (SCF), 100 ng/mL fms-like tyrosine kinase receptor 3 (FLT3 ligand), 100 ng/mL thrombopoietin (TPO), and 50 ng/mL interleukin 3 (IL-3).

4.2. Plate purified $CD34^+$ cells in a 12-well plate (5 x 10^4 $CD34^+$ cells/ 1.5 mL of media/ well) and culture at 37 °C in a 5% CO_2 humidified incubator.

4.3. Assess the purity of isolated $CD34^+$ cells and cell composition by FACS analysis as described below in step 6.3.

4.4. Add VPA at a final concentration of 1 mM into the cultures of cells treated for 16 h with cytokine cocktail.

4.5. Culture the cells for an additional 7 days at 37 °C in a 5% CO_2 humidified incubator.

NOTE: Media remains unchanged during the duration of the ex vivo expansion.

5. Antibody staining for flow cytometry analysis

5.1. Prepare antibody-staining solution to stain 2 x 10^4–6 x 10^4 cells and isotype solution to determine FACS gating strategy and determine the level of non-specific binding. Dilute antibodies (1/100 APC anti-CD34, 1/200 FITC anti-CD90) and the respective isotypes into 50 µL of separation buffer (composition of separation buffer is defined in step 1.1).

NOTE: Perform single staining of cells with each antibody for a FMO gating strategy. Total antibody compensation bead kit (see **Table of Materials**) can be used for compensation setup.

5.2. Homogenize the cell culture by pipetting multiple times. Count cells and pipet 5 x 10^4 cells into a 1.5 mL tube.

5.3. Add 1 mL of separation buffer and centrifuge at 400 x *g* for 15 min at room temperature.

5.4. Aspirate the supernatant and resuspend the pellet in 50 µL of the staining solution. Incubate cells for 30 min at room temperature.

5.5. Add 450 µL of separation buffer and centrifuge at 400 x *g* for 15 min at room temperature.

5.6. Aspirate the supernatant and resuspend the pellet in 100 µL of separation buffer. Keep cells on ice for at least 5 min until performing FACS analysis. Add 1 µL of 7-AAD to gate viable cells.

5.7. Load the stained cell sample on the FACS machine and acquire cells at the lowest flow rate.

5.8. For each fluorophore, analyze the isotype controls and single stained cells to set up gating for FITC (CD90), APC (CD34), and PerCP/Cy5.5 (7-AAD) staining.

5.9. Gate PerCP/Cy5.5 negative fraction and plot APC vs FITC to determine the percent of viable $CD34^+$, $CD90^+$, and $CD34^+CD90^+$ cells that defines the HSPC/HSC population in the culture.

6. Antibody staining for flow cytometry cell sorting

6.1. Resuspend the expanded cells by pipetting multiple times. Count the cell number and transfer the whole culture into a 15 mL or 50 mL tube.

6.2. Fill up the tube with cold separation buffer

6.3. Centrifuge at 400 x *g* for 15 min at 4 °C.

6.4. Prepare antibody-staining solution to stain 5 x 10^5–2 x 10^6 cells and isotype solution to stain 1 x 10^5 cells and determine FACS gating. Dilute 1/10 (APC anti-CD34), 1/20 (FITC-CD90) antibodies and the corresponding isotypes into 500 µL of separation buffer per each condition.

6.5. Prepare the single color compensation controls using a total antibody compensation bead kit according to manufacturer's instructions.

6.6. Transfer the supernatant from step 5.2 into a new tube and keep it at 37 °C. This culture media will be reused to culture the sorted cells.

6.7. Resuspend the cell pellet in cold separation buffer to obtain a concentration of 5 x 10^5 cells/ mL. Transfer 1 x 10^5 cells in 1.5 mL tubes for isotype staining and the remaining cells that will be sorted into other 1.5 mL tubes.

6.8. Centrifuge the tubes at 400 x *g* for 15 min at 4 °C.

6.9. Discard the supernatant and resuspend pellets of cells in isotype staining solution and the pellet of cells that will be sorted in the antibody staining solution.

6.10. Incubate for 30 min at 4 °C.

6.11. Add 450 µL of separation buffer and centrifuge at 400 x *g* for 15 min at 4 °C.

6.12. Resuspend the pellets with 2 mL of cold separation buffer.

6.13. To prevent clogs, filter the samples through a 40 µm cell strainer and place on ice until FACS.

6.14. Set-up cell sorter for sorting with the correct parameters.

6.15. Run isotype samples to set voltage and gain for forward and side scatter and identify negative fluorescence populations.

6.16. Run single color controls to set compensation coefficients and apply compensated parameter to collection protocol.

6.17. Run a small aliquot of the (Ab) sample (~50,000 events) to establish the gating strategy.

6.18. Set up sort decisions to collect the $CD34^+$ $CD90^-$ cell fraction.

6.19. Sort cells into collection tubes coated with 2 mL separation buffer.

6.20. After sorting, recount the cells and centrifuge them at 400 x *g* for 15 min at 4 °C.

6.21. Discard the supernatant and resuspend the pellet in the media recovered in step 5.5 to reach a final concentration of 3 x 10^4 cells/mL.

6.22. Plate $CD34^+$ $CD90^-$ purified cells in 12-well plates with 1.5 mL medium/well (5 x 10^4 $CD34^+$ $CD90^-$ cells/1.5 mL of media/well).

6.23. Assess the purity by FACS analysis using 50 µL of media containing cells.

6.24. Treat the cultures of $CD34^+CD90^-$ cells with VPA at 1mM final concentration.

6.25. Incubate at 37 °C in a 5% CO_2 humidified incubator.

REPRESENTATIVE RESULTS:

The ex vivo protocol described here increases the number of primitive HSCs generated from $CD34^+$ cells isolated from UCBs (**Figure 1**). Priming of $CD34^+$ cells for 16 h with a cytokine cocktail, followed by treatment with VPA for an additional 7 days, leads to a great degree of HSC expansion. Remarkably, the pool of expanded cells is highly enriched for HSCs, which are phenotypically defined by $CD34^+CD90^+$ markers. The total number of nucleated cells (TNCs) in cultures treated with the cytokine cocktail alone is significantly higher than the numbers of cells observed in cultures treated with cytokine cocktail and VPA (**Figure 2A**). Despite the higher number of TNCs, the number of HSCs in the cultures receiving the cytokine cocktail alone remains low during the entire expansion period compared to cultures treated with cytokine cocktail and VPA (**Figure 2B**). The greatest number of HSCs is generated in cultures treated with a combination of cytokine cocktail and VPA (**Figure 2B**). In particular, the greatest expansion in the numbers of HSCs is reached after 5–7 days following VPA treatment (**Figure 2B**). The increased number of HSCs correlates with a prompt increase in the percentage of HSCs, which is notable within 24 h of VPA treatment (**Figure 2C**). While the increase in the percentage of HSCs is high and maintained during the first 4 days of ex vivo culture, it declines progressively after 5–7 days of treatment with VPA (**Figure 2C**). However, this decrease is inversely correlated with an increase in the absolute number of HSCs.

Importantly, the rapid increase in the percentage of HSCs observed in VPA treated cultures is due to the acquisition of the CD90 phenotype. $CD34^+$ cells expressing CD90 phenotypic marker reaches almost 40%–45% of cells expanded in ex vivo cultures treated with cytokine cocktail and VPA for 4 days (**Figure 2C**,**D**). The acquisition of the CD90 phenotype is further confirmed by ex vivo expansion of the highly purified $CD34^+$ cells that lack expression of the CD90 marker. Within 24 h of VPA treatment, almost 75% of the ex vivo expanded cells in cultures initiated with sorted $CD34^+CD90^-$ cells express CD90 as opposed to 0% of the cells in cultures containing cytokine cocktail alone (**Figure 2E**). Importantly, the CD90 phenotype is highly retained during the first 4 days in ex vivo cultures treated with VPA (**Figure 2E**).

FIGURE LEGENDS:

Figure 1: Schematic presentation of ex vivo expansion culture. Purified $CD34^+$ cells from UCBs are primed for 16 h in ex vivo culture supplemented with a combination of the indicated cytokines. Cultures are treated with VPA (1mM) for an additional 7 days. The ex vivo expanded cells may then transplanted into myoablated NSG mice.

Figure 2: VPA treatment triggers acquisition of CD90 resulting in expansion of a great number of HSCs. (**A**) Absolute number of total viable nucleated cells expanded in ex vivo cultures[9]. Purified $CD34^+$ cells from UCBs described in section 3 treated with cytokine cocktail alone or a combination of cytokine cocktail and VPA as described in section 4 were counted by acridine orange/propidium iodide staining (n = 16). Numbers denote days of treatment with VPA, whereas

PC denotes the primary uncultured $CD34^+$ cells isolated from UCBs. (**B, C**) Absolute number and percentage of cells expanded throughout 7 days in ex vivo cultures treated with cytokine cocktail alone or a combination of cytokine cocktail and VPA (*n* = 21). Percentage of $CD34^+CD90^+$ cells is determined by flow cytometry analysis of cells stained as described in section 4. **(D**) Phenotypic analysis of sorted $CD34^+$ cells from UCBs (step 5.3) expanded in ex vivo cultures treated as indicated for 4 days. Left panel indicates the gating strategy using isotype staining whereas the other panels indicate stained cells expanded in cultures containing cytokine cocktail alone or a combination of cytokine cocktail with VPA. Numbers denote the percentage of $CD34^-CD90^+$ cells, $CD34^+CD90^-$ cells and $CD34^+CD90^+$ cells. (**E**) Percentage of $CD34^+CD90^+$ cells expanded in cultures initiated with sorted $CD34^+CD90^-$ cells from UBCs and treated with cytokine cocktail alone or a combination of cytokine cocktail and VPA for the indicated days. Percentage is determined by flow cytometry analysis (*n* = 4). N: number of biological replicates. Error bars with SEM; ****p ≤ 0.0001 as determined by negative-binomial models for A and B, Beta models for D and 2-way ANOVA for panel E. Panels A–C and E have been modified from Papa et al.[9]

DISCUSSION:

Herein, we present a protocol to rapidly expand to a significant degree the number of functional human HSCs from UCBs. The pilot and kinetic studies using this protocol indicate that the ex vivo expanded cells promptly acquire and retain the expression of several HSC phenotypic markers including CD90 as well as primitive HSC metabolic characteristics[9].

This ex vivo expansion protocol is relatively simple and reliable. Purification of $CD34^+$ cells with the cell separator device (see **Table of Materials**) is highly reproducible and rapid, allowing for fast recovery of isolated cells. This method results in a high yield of purified $CD34^+$ cells (>90%) as opposed to manual immunomagnetic separation. Moreover, this ex vivo expansion protocol does not require special and complex devices or fed-batch culture systems that need continuous supplies of large volumes of fresh media supplemented with cytokines[3,13]. Accordingly, this method limits costs. Importantly, this protocol allows expansion of the pool of HSCs within a short time, which is a key limiting factor for contamination frequency.

Several points should be considered for achieving primitive HSCs from $CD34^+$ cells using this protocol. $CD34^+$ cells expanded for 4 days in cultures treated with the cytokine cocktail and VPA lead to the generation of a pool of primitive long-term HSCs. These HSCs are characterized not only by the high expression levels of CD34, CD90 and CD49f, but also by a very primitive metabolic profile, which predominantly relies on glycolysis[9]. During more prolonged incubation with VPA for 7 days, expanded cultures however are more heterogeneous and contain a greater number of both long-term and short-term as well as differentiated cells as opposed to cells expanded for 4 days. This outcome is mainly due to the exhaustion of VPA in culture and increased number of cell divisions[9]. Thus, this protocol can be used to achieve expansion of two different pools of cells: 4-day and 7-day expanded cells. The 7-day expansion product can be used for allogeneic HSC transplantation where more rapid as well as sustained multilineage engraftment is required. The 4-day expansion protocol might be best suited for genetic modification strategies that target long-term repopulation cells. It is also likely that this combination of VPA and cytokine cocktail

can expand the pool of HSCs from $CD34^+$ cells at the same degree or higher when using media other than media (see **Table of Materials**) used in this protocol.

Current gene editing procedures are associated with a significant loss of HSCs, which limit their clinical application. The great number of primitive HSCs achieved within 2–4 days of VPA treatment in ex vivo cultures has the potential to overcome this loss in HSC numbers. Thus, this protocol might be beneficial for improving the existing gene editing protocols and enable correction of genetic mutations in HSC transplantation-based therapies for β-thalassemia and sickle cell disease. Moreover, it can be applied to maintain great numbers of HSCs during gene manipulations for studies focused on elucidation of gene functions in HSCs and in hematopoiesis. In conclusion, the method described here can be used for ex vivo expansion of both human and murine HSCs and can be tailored to specific clinical applications as well as to a wide spectrum of investigations in basic research.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure. 1

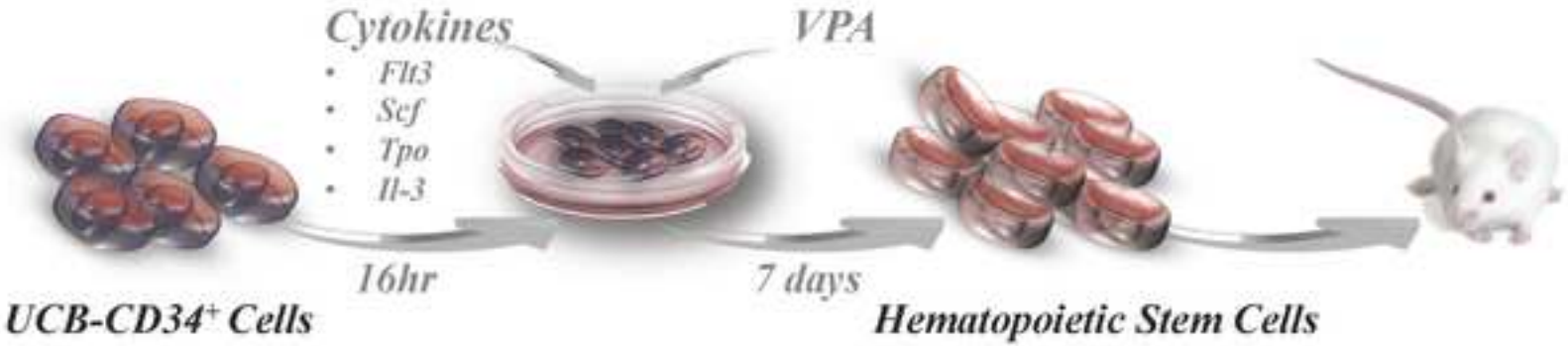

Figure.2

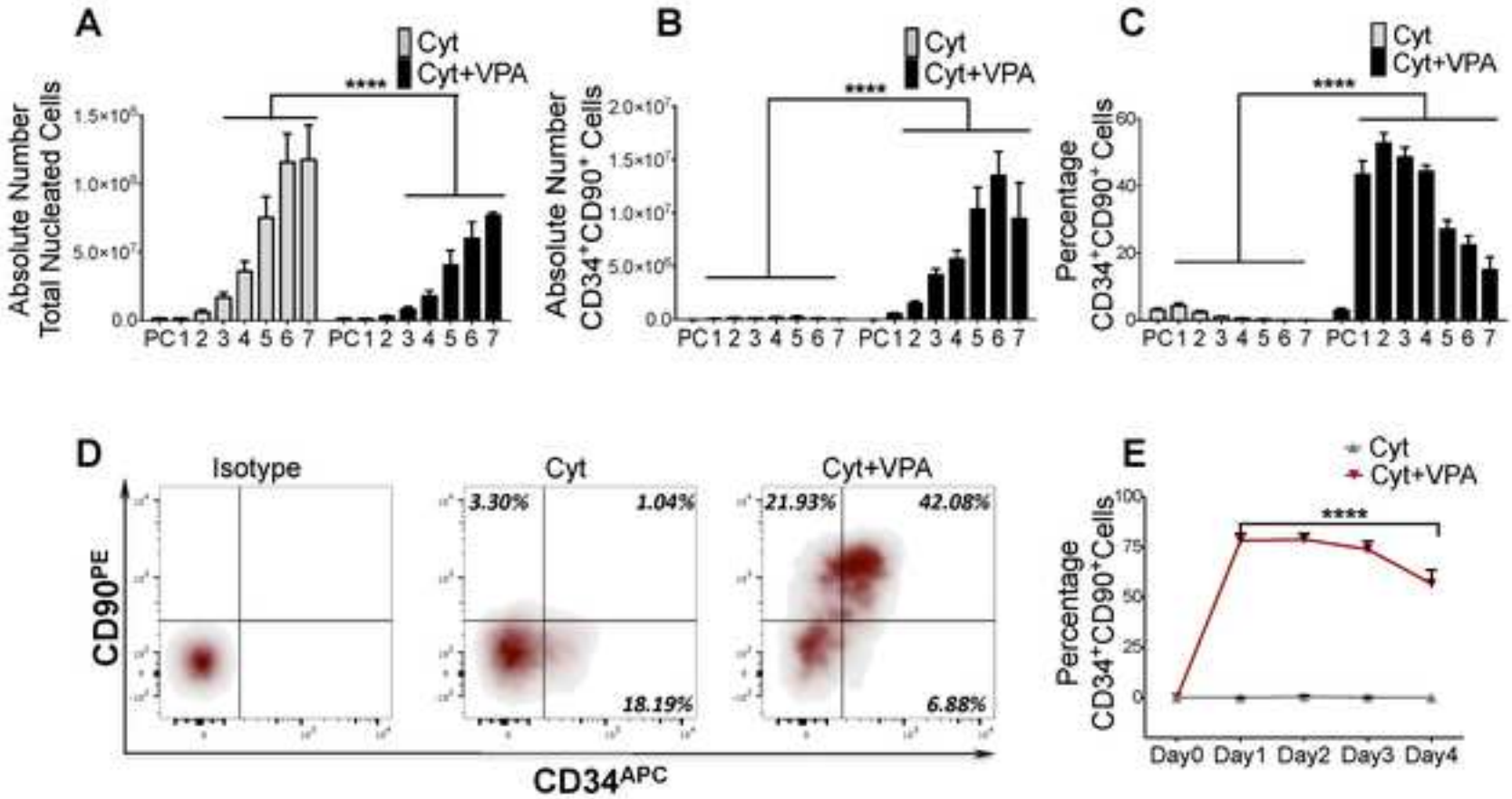

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Expansion Media	Sigma-Aldrich	Stemline II stem cell expansion media S0192-500ML
AO/PI (acridine orange / propidium iodide) staining solution for live/dead Mammalian nucleated cells.	Nexcelom	CS2-0106-25mL
APC Mouse Anti-Human CD34 Clone 581	BD BIOSCIENCE	555824
APC Mouse IgG1, κ Isotype Control Clone MOPC-21	BD BIOSCIENCE	555751
autoMACS Rinsing Solution	MACS Miltenyi Biotec	130-091-222
BD Falcon 15 ml Tube	BD Biosciences	352097
BD Falcon 5 ml Polystyrene Round-Bottom Tube	BD Biosciences	352063
BD Falcon 50 ml Tube	BD Biosciences	352098
Bovine serum albumine solution	Sigma-Aldrich	A8412
CD34 MicroBead Kit, contain cd34 beads and fcr blocking reagent, human	Miltenyi Biotec	130-046-703
Cell analyzer	BD Biosciences	FACS Canto II
Cell analyzer and sorter	BD Biosciences	FACS Aria II
Cell counter	Nexcelom	Cellometer Auto 2000 Cell Viability Counter
Cell separator device	autoMACS Pro Separator Miltenyi Biotec	
Counting Chambers	Nexcelom	CHT4-PD100-002
Density gradient media	GE Healthcare Bio-Sciences AB	17-1440-03

Ethylene diamine tetra-acetic acid (EDTA)	Sigma-Aldrich	E8008-100ML
FITC anti-human CD90 (Thy1) Clone 5E10	BIOLEGEND	328108
FITC Mouse IgG1, κ Isotype Ctrl (FC) Antibody	BIOLEGEND	400109
Inverted microscope		
PBS	Corning cellgro	21-040-CV
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
Recombinant Human Flt-3 Ligand Protein	R&D Systems	308FKN
Recombinant Human IL-3 Protein (IL-3)	R&D Systems	203-IL
Recombinant Human SCF Protein	R&D Systems	255-SC
Recombinant Human Thrombopoietin Protein (TPO)	R&D Systems	288-TP
Total Antibody Compensation Bead Kit	thermofisher	A10497
Umbilical Cord-blood	Placental Blood Program at the New York Blood Center	http://nybloodcenter.org/products-services/blood-products/national-cord-blood-program/cord-blood-101/
Valproic acid sodium salt	Sigma-Aldrich	P4543

Comments/Description

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Author(s): Iuena Papa, Mansour Djedaini and Ronald Hoffman

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CORRESPONDING AUTHOR:

Name:	Luena Papa
Department:	Hematology/Oncology
Institution:	Icahn School of Medicine at Mount Sinai
Article Title:	Ex vivo expansion of hematopoetic stem cells from human umbilical cord blood-deried CD34+ cells using valproic acid
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Dear Dr. Steindel,

We have received the reviewer's comments and we have addressed all their comments and concerns.

Editorial comments:
Changes to be made by the author(s):
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The manuscript has been thoroughly proofread.

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Permission from Journal of Blood Advances has been received in the form of an email and both a link to the editorial policy and the copyright link is uploaded in the Editorial Manager account.

3. Figure 1: Please change "16hr" to "16 h".
A change from hr to h is applied throughout the manuscript including Figure 1.

4. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.
Trademark and registered symbols have been removed and the materials are sorted in the alphabetic order.

5. Please revise lines 310-311 and 325-333 to avoid previously published text.
The text has been revised.

6. Please provide an email address for each author.
Email addresses for all the authors have been included.

7. Please expand the Summary to briefly describe the applications of this protocol.
Short abstract has been expanded (please refer to lines 68-72).

8. Please define all abbreviations (BSA, GMP, etc.) before use.
Abbreviations of all the reagents used in this protocol have been defined.

9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

The correct SI abbreviations have been used for all the units.

10. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.
Space has been included between all numerical values.

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All the commercial products have been referenced in the table of Materials and Reagents and all the commercial names have been removed from the main text.

12. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
The ethics statement has been included.

13. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
It has been corrected.

14. Line 205: Please provide the composition of GMP media. If it is purchased, please cite the Table of Materials.
It has been corrected.

15. Line 211: Please specify the source of UCB unit.
The source of UCB unit is stated in the table of Materials.

16. Line 273: Please describe how to assess the purity and cell composition by FACS analysis.
It has been specified "Assess the purity of isolated $CD34^+$ cells and cell composition by FACS analysis as described below in step 6.3".

17. Line 317: Is the supernatant discarded after centrifugation? Please specify.
The supernatant is discarded and this is specified in step 6.9.

18. A minimum of 10 references should be cited in the manuscript. For instance, please include applicable references to previous studies when describing advantages over alternative techniques.

Additional references have been included and the number of references is currently 13. Reference 2, 3,6 and 13 describes the advantages over the alternative techniques.

19. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
All references now appear as required.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a very well-written manuscript detailing a straightforward and reproducible method for rapidly expanding functional HSC from a single unit of cord blood. The protocol is preceded by a well thought-out and informative introduction, and the protocol is followed by a succinct, well-written Discussion making readers aware of some of the nuances of the expansion system. The protocol is sure to be of great interest to many readers in the HSC field.

Major Concerns:
None

Minor Concerns:
There are a couple of typos in lines 383 and 474.
The mistakes have been corrected.

Reviewer #2:

Manuscript Summary: This is a detailed protocol of the ex vivo expansion of human hematopoietic stem and progenitor cells using a cytokine cocktail and valproic acid.

Minor Concerns:

1. In the introduction the authors should explain in more details why the expansion of HSCs is due to the acquisition of a primitive phenotype and not simply proliferation.

An explanation has been currently added in the introduction part of this manuscript "**Within the initial 24-48 h of incubation with a combination of cytokines and VPA, $CD34^+$ cells acquire a transcriptomic and phenotypic profile that characterize long-term HSCs. The significant increase in the percentage of HSCs is accompanied by a prompt increase in the number of HSCs (63 fold increase within 24 h of VPA treatment)[9].** Moreover, regarding this point, we have added the appropriate reference in which the authors have provided detailed insights and explanations along with a great amount of data that includes phenotypic, transcriptomic and metabolic analysis as well as cell cycle analysis (Papa et al).

2. Can the authors define how many folds is the ex vivo expansion with VPA?
This has been defined in the introduction part of this manuscript "**The significant increase in the percentage of HSCs is accompanied by a prompt increase in the number of HSCs (63 fold increase within 24 h of VPA treatment)[9]".**

3. Define stemline II in line 205
Stemline II media has been defined in the table of Materials.

4. Can the authors include some alternative solutions for the readers that do not have the exact similar tools. For example for the autoMACS and the "posseld2" program.
The alternative method has been added in step 3.7 as a note followed by the appropriate reference.

5. Line 271: How many purified CD34+ cells do you put per well?
$5X10^4$ $CD34^+$ cells were plated in each well. This is clarified in steps 4.1 and 4.2.

6. Define how much antibody solution you need for a certain number of cells (line 279 etc)

The antibody solution and the concentration have been described in step 6.3: **"Prepare antibody-staining solution to stain $5x10^5$-$2x10^6$ cells and isotype solution to stain $1x10^5$ cells and determine FACS gating. Dilute 1/10 (APC anti-CD34), 1/20 (FITC-CD90) antibodies and the corresponding isotypes into 500 µL of separation buffer per each condition".**

7. The authors describe a 7-day protocol. Do they change the media? Renew the VPA or cytokines?

Media is not changed. This is currently clarified on line 344 or step 4.5 "**Media remains unchanged during the duration of the ex-vivo expansion".**

8. Why the total number of TNCs is important? Please explain

The total number of TNCs is important to show that the increase in the absolute number of HSCs in cultures treated with the combination of cytokine cocktail and VPA is not due to the increased number of cells but rather to acquisition of a primitive phenotype. We have included the following sentence in the "Representative Result" part of this manuscript "**The total number of nucleated cells (TNCs) in cultures treated with the cytokine cocktail alone is significantly higher than the numbers of cells observed in cultures treated with cytokine cocktail and VPA (Figure 2A). Despite the higher number of TNCs, the number of HSCs in the cultures receiving the cytokine cocktail alone remains low during the entire expansion period compared to cultures treated with cytokine cocktail and VPA (Figure 2B)".** Since this manuscript does not deliberate on the results but rather on the methods, a paper from our group that provides detailed insights on the ex vivo expansion is referenced.

Reviewer #3:

Manuscript Summary:
This is a very important method article for human HSC expansion fileld. It is well written and detail-oriented. To make it better, I have several concerns.

Major Concerns:

1. The authors claimed that they use 1mM VPA to culture CD34+ cells. This concentration sounds very high for expansion experiments. It would be better to give more information on how this concentration was determined.
VPA is an FDA approved drug used for treatment of bipolar disorders and epilepsy with very limited side effects. VPA treatment and other deacetylase inhibitors were previously tested and these studies have been described in several papers from our group including "Epigenetic reprogramming induces the expansion of cord blood stem cells" published in JCI, which is referenced in this manuscript. Among all the deacetylase inhibitors, VPA at a concentration of 1mM is the most efficient treatment to expand at a high degree the pool of very primitive HSCs without inducing either cell death or senescence. Interestingly, our evidence is consistent with an unbiased in vivo screening of 550 compounds in a transgenic zebrafish embryo model. Notably, this screen identified HDAC inhibitors and particularly VPA as a potent agonist of HSC expansion in vivo (Zebrafish In-Vivo Screening for Compounds Amplifying Hematopoietic Stem and Progenitor Cells: - Preclinical Validation in Human CD34+ Stem and Progenitor Cells. *Sci Rep*. 2017).

2. Why StemLineII medium was chosen? What about Stemspam SFEM medium? Maybe the authors could add some footnotes on similar questions as this.
Studies replacing StemlineII with different media including Stemspan SFEM are currently being performed and such possibility has been now stated in the

discussion part ". **It is also likely that this combination of VPA and cytokine cocktail can expand the pool of HSCs from CD34$^+$ cells at the same degree or higher when using media other than media (see Table of Materials) used in this protocol".**

Minor Concerns:
Page 6, Line 205, mediamedia might be media?

Mediamedia has been now corrected and replaced with media.

Reviewer #4:

Manuscript Summary:
The paper by Papa et al. describe a culture protocol to support the production of stem and progenitor cells in culture. The paper is well written, and the strength of this protocol is its simplicity, which I have to admit is admirable. So it could be very useful for labs with limited experience or for investigators looking for a straight forward protocol. A few minor recommendations are made.

Major Concerns:
none

Minor Concerns:

1. Introduction; if applicable, the author should direct readers to a good review on the matter of HSC expansion ex vivo.
Reviews focused on ex vivo expansions of HSCs are now added in the current manuscript as references numbered 1, 7 and 8.

2. Line 205; correct spelling error for "mediamedia"
Mediamedia has been now corrected and replaced with media.

3. For cell counts, not all labs are equipped with automated cell counters (on the contrary); author should provide alternative of manual cell counts with dilution of MNC samples in 3% acetic acid and use of hemocytometer.

The alternative method has now been mentioned in line: 281 or step 2.7. **"Note: The cell count can be also performed by using the trypan blue exclusion method with a hemocytometer".**

4. Controls for FACS; author should describe preparation of fluorescent minus one

(FMO) samples to appropriately set up regions/gates for cytometry. Isotype controls are better suited to determine the level of non-specific binding.
This has now been clarified in step 5.1.

5. Line 302; is it normal that the bullet is at 1, when it seems it should be at 7?
This has been corrected and number 6 replaces the bullet number 1.

6. The purpose of the sorting of the CD34+CD90- is to show that VPA culture promote the emergence of CD34+CD90+ cells. Am not sure that the whole section on this in the M&M is valuable. Most readers would probably want to produce CD34+CD90+ from CD34+ cells. Maybe this subsection can be removed, or else the context explained by a brief note. The results on that can be retained and presented as is. I leave it to the authors to make the final decision on this.'
The reason of including this data is to indicate and confirm that cultures supplemented with cytokine cocktail and VPA trigger transcriptomic alterations in $CD34^+$ cells that are associated with expression of primitive HSC markers. We have left unchanged this section as it appears in the previous submitted manuscript.

7. There is no mention in the text on feeding the culture…maybe the author need to state this as most would assume that the culture is fed with fresh media at least once.

This point is now clarified in the manuscript in step 4.5 "Media remains unchanged during the duration of the ex-vivo expansion".

Thank you for your consideration.

Luena Papa, Ph.D.
Assistant Professor
Division of Hematology and Medical Oncology
Mount Sinai School of Medicine
One Gustave L. Levy Place,
New York, NY 10029-6574
Tel: (212) 241-1331
Email: Luena.papa@mssm.edu