

# 3-D Perfusion Bioreactor Process Optimization for CD34+ Hematopoietic Stem Cell Culture and Differentiation towards Red Blood Cell Lineage

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## Abstract

Process optimization for *in vitro* cellular engineering of CD34+ hematopoietic stem cell (HSC) culture expansion and differentiation towards red blood cell (RBC) lineages continues to remain a multifaceted challenging operation. This work focuses on three process aspect experiments with the goal of providing improved conditions for the culture of HSCs towards RBC lineages in four-compartment hollow fiber based bioreactors. In a first set of experiments, ideal conditions for the expansion and differentiation of CD34+ HSCs into RBCs were determined by testing the impact of initial cell plating density (3,000 cells/mL versus 20,000 cells/mL), the frequency of replenishing medium, and the transfer to new wells for expansion using 2D transwell plate cultures over 28 days. Results show that a lower density of 3,000 cells/mL and more frequent media changes promote higher levels of cell expansion. In a second independent set of experiments, hollow fiber bioreactor cultures were used to assess if cell inoculation and harvest from such a bioreactor technology platform are potentially damaging HSCs, yielding unfavorable outcomes. Four 8-mL cell chamber volume laboratory scale bioreactors were inoculated with an initial HSC seeding density of 20,000 cells/mL each, perfused for 4 hours, and then harvested to determine the percent recovery. Cells were effectively recovered from the bioreactors, and in follow-up 2D conventional plate cultures the recovered cells expanded as well as the control cultures, indicating that inoculation and harvest procedures are not a source of mechanical injury or cell loss during bioreactor culture. Finally, a third independent set of experiments used multiple 8-mL laboratory scale bioreactors with an initial HSC seeding density of 20,000 cells/mL. Cells were cultured at three time intervals for 8 to 11 days (n=10), 12 to 14 days (n=15), or 15 to 22 days (n=3) with fold-expansion results of 106.0 ± 94.0, 999.5 ± 589.6, and 456.3 ± 33.6, respectively. Although additional studies are necessary for complete large scale-up RBC optimization, the results of these studies have led to a methodical understanding of improved conditions for HSC culture in hollow fiber perfusion bioreactor systems.

**Keywords:** Inoculation; Hydroxyapatite; Perfusion; Stem cell niche; Transwell cultures; 3D bioreactors; CD34+ hematopoietic stem cells

## Introduction

Although significant advances have been made towards the *in vitro* culture of human CD34+ hematopoietic stem cell (HSC) populations, bioreactor culture processes and differentiation into red blood cells (RBCs) *in vitro* have yet to be fully optimized for a therapeutic production of RBCs [1]. The *in vivo* expansion and differentiation of hematopoietic progenitor populations is known to be highly dependent on their stem cell niche environment. Stem cell niches are characterized through cell-cell interactions of the various cell types, including stroma cells, cell-substrate / matrix interactions [2], interactions and the combination and concentration of soluble factors [3]. In addition, substrate interactions with the bone marrow niche hydroxyapatite provide a mechanical stabilization and protection of these components. Recreating such a niche *in vitro*, in order to enable cell production behaviors similar to the human body, is particularly challenging (Table 1). Furthermore, the *in vitro* cellular engineering process of creating RBCs starting from progenitor cells involves a very systematic detailed approach (Figure 1). Compared to conventional two-dimensional (2D)

plate cultures with static medium conditions, bioreactor systems that can provide a dynamic three-dimensional (3D) perfusion more closely mimic the bone marrow stem cell niche and allow for spatial cell-to-cell interaction. Table 2 summarizes some information of relevance for the development of RBC bioreactor culture technology [4-13].

We previously demonstrated a proof of principle expansion and differentiation of human CD34+ HSCs towards RBC lineages using a four-compartment hollow fiber based 3D perfusion bioreactor that provides integral oxygenation and high performance mass exchange with low gradients, thus making it suitable for larger scale cell production that approaches the cell densities under which RBC production is performed *in vivo*. High initial cell seeding density (800,000 cells/mL) studies in a 2-mL scale bioreactor system showed a total fold expansion of 105 ± 33 (n=3). Successive studies conducted in an 8-mL cell compartment scale bioreactor demonstrated cell harvesting capabilities, sequential cell removal, and passaging of cells [14]. Although our previous results were promising, a number of

Cell Type	Cell Concentration (cells/mL)	Lifespan (Days)	Production rate (billions per day)
RBCs	5×10 <sup>9</sup>	120	208
Platelets	2×10 <sup>9</sup>	8	125
WBCs	5×10 <sup>6</sup>	0.5	50
Total Production			383

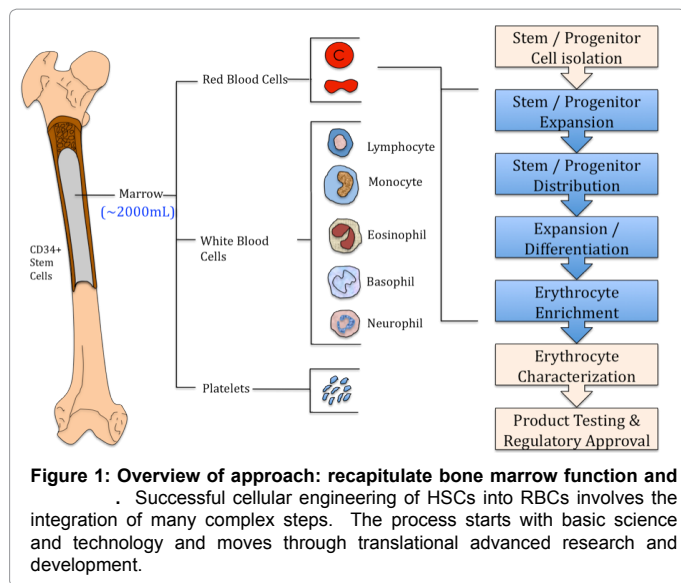
**Table 1: *In vivo* human blood cell production rates (steady state, normal conditions).** The optimal goal for cellular engineering is to mimic bone marrow function and efficiency according to the body's natural blood cell production rates; red blood cells (RBCs), white blood cells (WBCs) [9].

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processes associated with variable efficiency of *in vitro* cell expansion and erythrogenic differentiation of HSCs must be optimized for continuous RBC production in these bioreactor systems.

Dynamic perfusion bioreactor systems differ as compared to conventional static 2D cultures in terms of culture medium feed mode and rate, 3D medium perfusion, oxygen supply / CO<sub>2</sub> removal, and waste medium removal [15]. Because of the complexity, the level of variability in expansion and differentiation characteristics can be caused by a number of controlled bioreactor inputs [16]. The cell inoculation density and mechanical cell harvest procedures that could potentially injure cells are two critical steps and can affect follow-up cell culture or lead to cell loss, thus yielding misleading expansion totals.

This work focused on three experimental studies with the ultimate goal of providing improved bioreactor conditions for the culture of HSCs towards RBC lineages in the described technology. We worked on three objectives in order to optimize bioreactor culture conditions: (1) We sought to determine how cultures are affected by higher and lower seeding densities and more frequent medium changes. This was performed simulating general bioreactor conditions in 2D transwell cultures that are independent of the platform we promote. (2) We proceeded to determine if the cell inoculation or harvest procedures in the specific bioreactors are mechanically stressing HSCs, and if cells can be effectively recovered from the bioreactor after short periods of culture using standard perfusion protocols when employing an 8-mL culture volume bioreactor scale system. (3) Increasing the cell expansion within one bioreactor while extending the culture time was investigated to enhance differentiation towards RBC lineage, using an initial cell seeding density of 20,000 cells/mL in 8-mL volume bioreactor systems.

## Materials and Methods

### Simulation of varying bioreactor feed rates and densities using 2D transwell cultures

**HSC isolation and pre-cultivation in standard 2D culture:** Postpartum placentas were procured under informed consent at Celgene Cellular Therapeutics (CCT), with donor eligibility documentation and undergoing a series of quality control tests, including serology, bacteriology and HLA typing. Total nucleated cells as the source of HSCs were generated by treating the donor matched umbilical cord blood and

Aspect	Literature
RBCs	Lifespan – 120 days [4,5,9,10,11] Turnover rate in human body – 4-5×10 <sup>12</sup> RBCs every 3 weeks [5] Mean half-life of a heterogeneous normal donor RBC – 28 days [5,6,10] Concentration <i>in vivo</i> – 5×10 <sup>9</sup> cells/mL [9] Length of time that RBCs can be conserved <i>in vitro</i> – 30 days [6]
Cord Blood	Number of CD34+ cells in a cord blood donation – 2-5×10 <sup>6</sup> cells [8,9] Theoretical yield from one cord blood donation – 10-50 packed RBC units (assuming 5 million CD34+ cells in CB donation and generation of 4-22 million RBC from each CD34+ cell [6])
Adult bone marrow	Daily production of 0.1 units of RBCs, 2.5 units of platelets and 10 <sup>11</sup> neutrophils. [8,10] Under stress these production rates can be increased. [9] In vivo perfusion rate is approximately 0.1mL per cc of marrow/minute [9] Oxygen tension values reported from 10-50 mmHg [9] Frequency (ratio) of HSCs in the bone marrow – 1 cell in 10,000 [6] A volume approximating 2L of adult human bone marrow can generate 9×10 <sup>9</sup> cells/hour (not all RBCs) [11] Cell growth densities approaching 1×10 <sup>9</sup> are comparable to bone marrow. [11] Production flux = [(blood volume) × (cell concentration)] / lifespan [9] Typical human blood volume = 5 L [9] Total blood cell production in humans – ~400 billion cells/day [9] Size of human hematopoietic system – approx. 500-1000 billion cells [1 to 2 kg at approximately 5×10 <sup>8</sup> cells/mL] [9] Bone marrow produces as many cells as it is made of over a 1-3 day period. [9]
Blood Collection / Supply	Number of donor units of whole blood collected per year – 80 million [8] Developing nations give 39% of blood that is collected but account for 82% of the population → shortages in supply [8] Number of units of whole blood collected in the USA in 2006 – 15.7 million [8,12] Shelf life of transfusable RBC product – 42 days (increasing time = possible toxicity) [7,10,11,12] Only a few days supply of type O blood available at any given time in the USA and other blood groups have even lower supply [11]
RBC units	Number of cells in concentrate – 2×10 <sup>12</sup> (2.5×10 <sup>12</sup> cells/mL) [4,5,7,8] Minimum mean age – 5.0 days [10] Maximum mean age – 42.0 days [10] Median – 15.0 days [10] Shelf life – 42 days in U.S. FDA approved additive solution according to American Association of Blood Banks guidelines. [7,10] Average cost per RBC unit (O+, leukofiltered, not irradiated, not cytomegalovirus (CMV)-negative – \$153.68 (range \$143-\$164) [10] Number of RBC units transfused in 2011 – 13,785,000 [13]

**Table 2: Relevant RBC supporting facts and literature.** RBC production aspects that are of interest for the development of RBC production systems with corresponding literature references.

placenta derived stem cells with ammonium chloride (catalog# 07850, Stem Cell Technologies) to remove the erythrocytes. The subsequent purification of HSCs was performed using RoboSep<sup>®</sup> automated cell isolation system and EasySep<sup>®</sup> Human Progenitor Cell Enrichment Kit (catalog# 19056, Stem Cell Technologies). The purity of isolated cell populations was measured using CD34+ cell surface marker expression as determined using fluorescence activated cell sorting (FACS) analysis. After isolation, cells were shipped overnight from Warren, New Jersey to Pittsburgh, Pennsylvania. HSCs were received from CCT and an initial cell count/viability assessment was performed. Cells were then placed in 25-cm<sup>2</sup> static t-flask culture at a density of 100,000 cells/mL

using expansion and differentiation culture medium and placed in an incubator with a 5% CO<sub>2</sub> atmosphere, 95% humidity, and 37°C for two days prior to the start of the experimental culture period. Following the pre-culture period, a final cell count/viability assessment was performed using a Countess™ Automated Cell Counter (Invitrogen) prior to plating the cells.

**Progenitor cell expansion and erythroid differentiation:** Expansion and differentiation of HSC into RBC was performed as previously described [14].

**2D transwell plate cultures:** In order to compare the effect of cell density and medium exchange intervals a series of 2D plate cultures were created for experimental testing. A description of the plates that were created can be seen in Table 3, while Table 4 shows the details of the experimental strategy. Cells were placed in 12-well plates with transwell inserts (Corning, USA) to allow daily medium exchange in the bioreactor simulation protocol without loss of cells during aspiration. Four replicate wells were plated for each experimental condition using 2 mL of CCT expansion and differentiation medium per well. The two densities chosen were 20,000 and 3,000 cells/mL. The 20,000 cells/mL reflects the density commonly used when culturing HSCs according to an established protocol (the CCT expansion protocol). The 3,000 cells/mL reflects the actual density in the bioreactor if 20,000 cells/mL are inoculated and the entire circuit volume is considered. The two volumetric medium exchange rates compared were an established CCT expansion protocol and a bioreactor simulation (BR Simulation). Cells were maintained according to CCT expansion protocol by diluting one volume of cell culture supernatant with 3 or 4 volumes of fresh medium on days 4 and 7, respectively. Further maintenance of medium exchange was performed on days 11 and 18 (as of day 4) and days 14 and 21 (as of day 7). Cell number and viability were determined by trypan blue exclusion method on culture expansion days using a Countess™ Automated Cell Counter (Invitrogen). The medium exchange rate in the bioreactor simulation for an actual bioreactor feed rate of 1ml/hr was approximated in 2D by replacing 0.45 mL of culture medium every 12 hours during the entire culture period. The combination of variables in Table 3 also examines whether or not expansion of cells into new wells according to the CCT protocol is beneficial as compared to not expanding the cells, which is equivalent to the conditions inside of the bioreactor. The experiment was repeated two times for a total of three experimental trials.

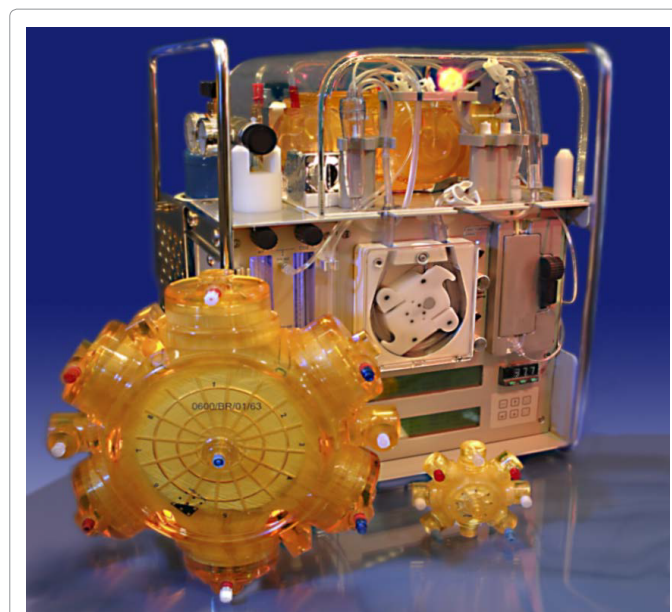
#### Four-hour harvest of CD34+ HSC bioreactor recovery testing

**Bioreactor technology:** The 8-mL scale laboratory bioreactor consists of three independent interwoven capillary systems that facilitate counter-current medium perfusion and decentralized oxygenation with low gradients (Figure 2). The structure of the capillary network is described in detail elsewhere [17-19].

**Bioreactor assembly and priming:** Bioreactors and tubing sets

Cell Density (cells/mL)	Medium Exchange Protocol	Expansion (Yes/No)
3,000	CCT	Yes
20,000	CCT	Yes
3,000	BR Simulation	No
20,000	BR Simulation	No
3,000	BR Simulation	Yes
20,000	BR Simulation	Yes

**Table 3: Summary of 2D transwell plate culture methodology using CD34+ HSCs.** The two cell densities compared were 3,000 and 20,000 cells/mL. Celgene Cellular Therapeutics (CCT) medium exchange and bioreactor simulation (BR simulation) protocols were utilized. The expansion (Yes/No) column refers to expanding into new wells.



**Figure 2: Four-compartment hollow based bioreactor systems.** Bioreactor perfusion, temperature regulation, and oxygenation device (background) that can accommodate up to four bioreactors of various scales. Two bioreactors for two different culture volume scales are shown in the front (left: 800-mL cell compartment volume bioreactor, right 8-mL volume bioreactor). The 8-mL bioreactor platform was used for experimental studies 2 and 3.

were assembled under sterile conditions in a laminar airflow biosafety cabinet according to standard protocol and were then set up on an appropriate perfusion device (StemCell Systems, Germany). The bioreactors were primed with 200 mL of 70% ethanol solution (to remove any particulate material or plasticizers from the medium fibers) and then 500 mL of Dulbecco's phosphate buffered saline (without Ca or Mg) (Invitrogen Gibco, USA) to flush out the ethanol solution. Next, 500 mL of Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen Gibco, USA) was used followed by 500 mL of IMDM supplemented with 10% fetal bovine serum (FBS; Invitrogen, Gibco) and 1% penicillin-streptomycin to prime the medium fibers. Finally, 200 mL of expansion and differentiation medium supplemented with 1% penicillin-streptomycin was used to prime the system to equilibrium.

**2D pre-culture and inoculation into 8-mL scale bioreactors:** HSC isolation and 2D 2-day preculture was performed according to a procedure previously described [14]. Following the pre-culture period a final cell count/viability assessment was performed prior to bioreactor inoculation preparation.

Syringes containing 160,000 viable cells in 10 mL of CCT expansion and differentiation medium were prepared for each of the four 8-mL laboratory scale bioreactors to be used in the study giving a bioreactor density of 20,000 cells/mL. For consistency, when referring to the cell density in the bioreactor, a volume of 8 mL will be used since our previous publication density was calculated with respect to the cell chamber volume (a volume of 2-mL for the 2 mL cell compartment bioreactor). A 16-gauge needle was attached to each syringe to aid in piercing the septum on the cell inoculation port. Additionally, an extra syringe containing 10 mL of CCT expansion and differentiation medium was prepared for each bioreactor. Prior to cell inoculation the media flow into and out of the cell compartment was clamped to make sure that cells were trapped inside of the bioreactor. The arterial medium flow into the cell inoculation port was also clamped.

Inoculation of the cells into the 8-mL laboratory scale bioreactor was performed under sterile conditions. Cell inoculation was accomplished by injecting the contents of the syringe at a slow rate to avoid cell injury. Following inoculation the cells were then ‘chased’ into the cell compartment with 10 mL of fresh CCT expansion and differentiation medium and perfusion of the cultures was initiated.

**4-hour culture and cell harvest using 8-mL scale bioreactors:**  
Following inoculation the cells were perfused with CCT expansion and differentiation medium for 4 hours using a bioreactor perfusion system feed rate of 2 mL/hr and a recirculation rate of 20 mL/min.

At the end of this period each reactor was transferred under the laminar airflow biosafety cabinet and 400 mL of IMDM was flushed

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
<b>Cell Plating Condition:</b> 3,000 cells per mL CG protocol feed regime Expansion	-	-	-	3,000/CG/Exp/1a	-	-	3,000/CG/Exp/1aI			
							3,000/CG/Exp/1aII			
							3,000/CG/Exp/1aIII			
							3,000/CG/Exp/1aIV			
				3,000/CG/Exp/1b	-	-	3,000/CG/Exp/1bI	-	-	3,000/CG/Exp/1bII
										3,000/CG/Exp/1bIII
										3,000/CG/Exp/1bIV
										3,000/CG/Exp/1bV
				3,000/CG/Exp/1c	-	-	3,000/CG/Exp/1cI	-	-	3,000/CG/Exp/1cII
										3,000/CG/Exp/1cIII
										3,000/CG/Exp/1cIV
										3,000/CG/Exp/1cV
				3,000/CG/Exp/1d	-	-	3,000/CG/Exp/1dI	-	-	3,000/CG/Exp/1dII
										3,000/CG/Exp/1dIII
										3,000/CG/Exp/1dIV
										3,000/CG/Exp/1dV

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<b>Cell Plating Condition:</b> 3,000 cells per mL BR simulation feed regime No Expansion	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
<b>Cell Plating Condition:</b> 20,000 cells per mL CG protocol feed regime Expansion	-	-	-	20,000/CG/Exp/1a	-	-	20,000/CG/Exp/1aI			
							20,000/CG/Exp/1aII			
							20,000/CG/Exp/1aIII			
							20,000/CG/Exp/1aIV			
				20,000/CG/Exp/1b	-	-	20,000/CG/Exp/1bI	-	-	20,000/CG/Exp/1bII
										20,000/CG/Exp/1bIII
										20,000/CG/Exp/1bIV
										20,000/CG/Exp/1bV
				20,000/CG/Exp/1c	-	-	20,000/CG/Exp/1cI	-	-	20,000/CG/Exp/1cII
										20,000/CG/Exp/1cIII
										20,000/CG/Exp/1cIV
										20,000/CG/Exp/1cV
				20,000/CG/Exp/1d	-	-	20,000/CG/Exp/1dI	-	-	20,000/CG/Exp/1dII
										20,000/CG/Exp/1dIII
										20,000/CG/Exp/1dIV
										20,000/CG/Exp/1dV

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<b>Cell Plating Condition:</b> 20,000 cells per mL BR simulation feed regime No Expansion	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
<b>Cell Plating Condition:</b> 3,000 cells per mL BR simulation feed regime Expansion	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	3,000/BR/Exp/1a	0.9 mL media exchange	0.9 mL media exchange	3,000/BR/Exp/1aI			
							3,000/BR/Exp/1aII			
							3,000/BR/Exp/1aIII			
							3,000/BR/Exp/1aIV			
				3,000/BR/Exp/1b	0.9 mL media exchange	0.9 mL media exchange	3,000/BR/Exp/1bI	0.9 mL media exchange	0.9 mL media exchange	3,000/BR/Exp/1bII
										3,000/BR/Exp/1bIII
										3,000/BR/Exp/1bIV
										3,000/BR/Exp/1bV
				3,000/BR/Exp/1c	0.9 mL exchange	0.9 mL exchange	3,000/BR/Exp/1cI	0.9 mL exchange	0.9 mL exchange	3,000/BR/Exp/1cII
										3,000/BR/Exp/1cIII
										3,000/BR/Exp/1cIV
										3,000/BR/Exp/1cV
				3,000/BR/Exp/1d	0.9 mL exchange	0.9 mL exchange	3,000/BR/Exp/1dI	0.9 mL exchange	0.9 mL exchange	3,000/BR/Exp/1dII
										3,000/BR/Exp/1dIII
										3,000/BR/Exp/1dIV
										3,000/BR/Exp/1dV
Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7			
<b>Cell Plating Condition:</b> 20,000 cells per mL BR simulation feed regime Expansion	0.9 mL media exchange	0.9 mL media exchange	0.9 mL media exchange	20,000/BR/Exp1a	0.9 mL media exchange	0.9 mL media exchange	20,000/BR/Exp1aI			
							20,000/BR/Exp1aII			
							20,000/BR/Exp1aIII			
							20,000/BR/Exp1aIV			
				20,000/BR/Exp1b	0.9 mL media exchange	0.9 mL media exchange	20,000/BR/Exp1bI	0.9 mL media exchange	0.9 mL media exchange	20,000/BR/Exp1bII
										20,000/BR/Exp1bIII
										20,000/BR/Exp1bIV
										20,000/BR/Exp1bV
				20,000/BR/Exp1c	0.9 mL exchange	0.9 mL exchange	20,000/BR/Exp1cI	0.9 mL exchange	0.9 mL exchange	20,000/BR/Exp1cII
										20,000/BR/Exp1cIII
										20,000/BR/Exp1cIV
										20,000/BR/Exp1cV
				20,000/BR/Exp1d	0.9 mL exchange	0.9 mL exchange	20,000/BR/Exp1dI	0.9 mL exchange	0.9 mL exchange	20,000/BR/Exp1dII
										20,000/BR/Exp1dIII
										20,000/BR/Exp1dIV
										20,000/BR/Exp1dV

**Table 4: 2D transwell Petri culture well set-ups, feed regime, and expansion schematic.** Each individual cell in the tables above represents a separate well. Two mL of media was used per well. Blue labeled cultures represent actual practical well follow up whereas the Red labeled cultures represent theoretical, or no follow-up. Although not all wells were followed up on, the theoretical number of wells (Blue and Red) was taken into account when calculating the total expansion rates. There were a total of 2 repeats for each cell plating condition. The experiment was repeated three times for statistical significance. Although the schematic above shows maintenance for 7 days, we maintained the cultures over 28 days.

in a sterile manner through the cell compartment into a series of 50 mL conical polypropylene tubes (Falcon, BD Biosciences). The cell suspensions were then centrifuged at 400 g for 8 minutes, supernatants were aspirated and the cell pellets were combined and counted using the Countess Automated Cell Counter (Invitrogen) to determine total cell recovery.

**2D follow-up culture of cells recovered from 8-mL scale bioreactors:** The cells recovered from the 8-mL bioreactor were then plated in 2D at a density of 20,000 cells/mL with 2 mL of expansion and differentiation media per culture well. 6-well tissue culture polystyrene

plates with transwell inserts (Corning, USA) were used to allow sampling of the cultures without cell loss. Additionally, a 2D control culture of cells from the same population that were never inoculated into the bioreactor was plated at a density of 20,000 cells/mL with 2 mL of expansion and differentiation media per well.

Cells were maintained according to CCT protocol by diluting one volume of cell culture with 3 or 4 volumes of fresh medium on days 4 and 7, respectively. Further maintenance was performed on days 11 and 18 (as of day 4) and days 14 and 21 (as of day 7). Cell number and viability were determined on culture expansion days using a Countess Automated Cell Counter (Invitrogen).

## 8-mL scale laboratory scale bioreactor culture

**Bioreactor assembly and priming:** Bioreactors and tubing sets were assembled and primed as described above.

**2D pre-culture and inoculation into 8-mL scale bioreactors:** HSC isolation, 2D 2-day preculture, and inoculation were performed according to the procedure described above.

**2D control cultures:** HSCs were cultured using six-well plates (Falcon, BD Biosciences) at a density of 20,000 cells/mL in a medium volume of 2 mL in parallel with bioreactor cultures to check for proper cell growth and to easily identify potential bacterial or fungal contamination. On day 4, one volume of cell culture was diluted in three volumes of fresh medium. On day 7, one volume of cell culture was diluted with four volumes of fresh medium. Afterwards, media were replenished at day 11 (as of day 4), day 14 (as of day 7), day 18 (as of day 4) and day 21 (as of day 7) following the procedure as of day 4 and day 7, respectively.

**Cultivation of cells in bioreactors for a long-term 8-mL scale bioreactor study:** Cells at an initial seeding density of 20,000 cells/mL were cultured in 8-mL scale bioreactors for three time intervals; (n=10) for 8 to 11 days, (n=15) for 12 to 14 days, and (n=3) for 15 to 22 days. For each bioreactor culture, cells were harvested when lactate production reached a plateau by completely rinsing with 400mL of Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Gibco) supplemented with penicillin-streptomycin (1%v/v) through the capillary cell compartment network at a flow rate of 20mL/min. During rinsing each bioreactor was vigorously shaken to enhance cell liberation from the capillary network. The harvested cell suspension was centrifuged (475 g, 8 min) and analyzed with respect to the cell yield, viability (using an Improved Neubauer hemacytometer with Trypan blue staining), and surface marker expression. Cell surface marker expression was determined by FACS using a variety of fluorescently labeled antibodies; CD34 fluorescein isothiocyanate- (present on HSCs), CD71 allophycocyanin- (proliferating marker during RBC development), CD45 and CD235a phycoerythrin- (present on RBCs) conjugated antibodies. Cells were also stained with a monomeric cyanine nucleic acid stain, TOPRO-3 iodine, to determine enucleation (all flow cytometry reagents were obtained from BD Biosciences). Quantification of surface marker expression was conducted using a FACSVantage SE DiVa (Becton Dickinson, Franklin Lakes, NJ) flow cytometer and running DiVa v4.1.2 softwareC.

**Measurement of metabolic parameters in bioreactors:** The partial pressures of O<sub>2</sub> and CO<sub>2</sub> and the pH values in the recirculation medium were regularly measured using a blood gas analyzer (ABL 5 Blood Gas Analyzer, Copenhagen Radiometer, Copenhagen, Denmark), and the culture pH was maintained between 7.35 and 7.45 by adjusting the air: CO<sub>2</sub> gas ratio on the perfusion system while maintaining the same overall gas flow rate. In parallel, lactate dehydrogenase (LDH) levels were monitored daily using a bioassay (QuantiChrom LDH Kit, BioAssay Systems) as an indirect measurement of cell viability. A culture showing decreasing metabolic activity, defined as a decrease in lactate production or an increase in LDH concentration, was used as a criterion to determine the harvest day for bioreactor cultures unless specific time points for harvest or passaging were required in the experimental plan.

Concentrations of glucose and lactate in samples from the culture perfusate were analyzed (YSI 2300 STAT Plus Glucose & Lactate Analyzer, USA) and the medium flow rate was adjusted, if necessary, by

increasing from initially 1 mL/hr to a maximum feed rate of 4 mL/hr. The daily parameter assessment was calculated as mg/dL or U/d per 10<sup>7</sup> cells, if not otherwise indicated.

## Statistical Analysis

Values are given as means ± standard error of the mean (SEM) in the text and in figures, if not otherwise indicated.

## Results

### Simulation of varying bioreactor feed rates and densities using 2D transwell cultures results

The following conditions were compared as shown in Figure 3: 1) 3,000 cells per mL density (3000)/CCT Protocol (CG)/Expansion (Exp), 2) 20,000 cells per mL density (20000)/CG/Exp, 3) 3000/Bioreactor Simulation (BR)/No Expansion (noExp), 4) 20000/BR/NoExp, 5) 3000/BR/Exp, and 6) 20000/BR/Exp. According to the Figure 3, results indicate that low density and high medium exchange results in higher expansion. Although low-density and high medium exchange results were favorable, we chose to use to use a density of 20,000 cells/mL (or 3,000 cells/mL if you are to consider the entire circuit volume of approximately 55mL) for cell harvesting and long-term culture studies for scale-up volumetric practicality reasons. Furthermore, the Countess Automated Cell Counter has a lower limit of 1.0x10<sup>5</sup> cells/mL making cell counting at 3,000 cells/mL unfeasible.

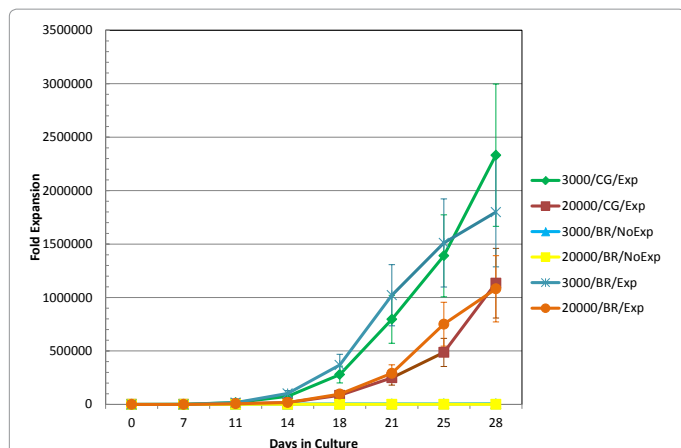
### Four-hour harvest of CD34+ HSC bioreactor recovery testing results

A total of 1.6x10<sup>5</sup> cells (20,000 cells/mL) were inoculated into each of the four 8-mL bioreactors listed in Table 5. The bioreactors were perfused for four hours according to the parameters listed in the Materials and Methods section and then harvested to determine total cell recovery, which is summarized below in Table 5.

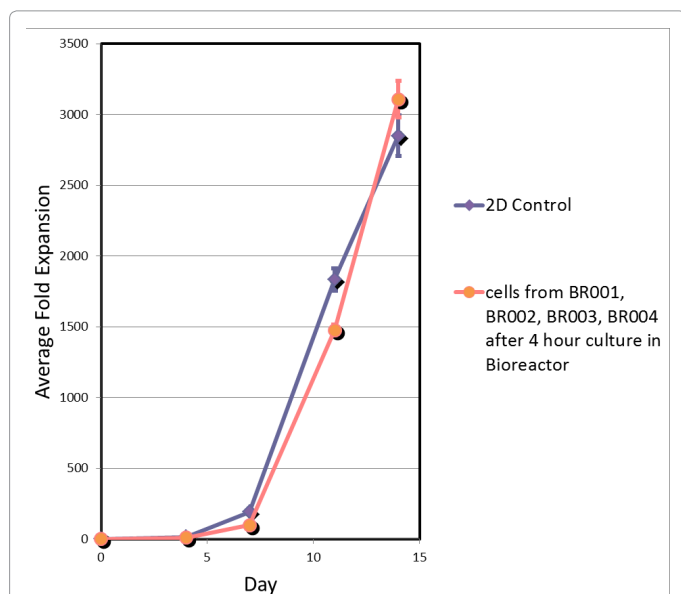
It can be readily seen that cell recovery from the bioreactors was somewhat variable in nature, ranging from 50-100% of the total amount of cells inoculated. Additionally, the viability differed somewhat between reactors and was generally lower than the measured viability of 82% prior to cell inoculation. The low viabilities are likely not able to be fully attributed to cell death since the cells were only in the reactors for a total of 4 hours. The limitations of the cell counting device could be responsible for some of the observed variability in these results. The lower limit of accuracy for the counting device is 1.0 × 10<sup>5</sup> cells/mL and a significant fraction of the counts that were performed were listed as 'below accurate level'. When a sample is below this level the counts and viabilities can vary tremendously, even when measuring the same sample. It should be noted that harvest tests performed at CCT (results not shown) using an assay that can accurately count cells at very low densities (CyQuant, Invitrogen Molecular Probes, USA) demonstrated high cell recovery from bioreactors at low densities.

Previously published studies demonstrated high (> 90%) recovery of cells after culture. Given the variation that was observed in this study, the use of such an assay could have proven to be more reliable given the low cell inoculation density. That aside, it was shown that a significant fraction of the cells could be recovered from the bioreactors after short periods of culture time.

The recovered cells were then plated in 2D at a density of 20,000 cells/mL and the culture behavior over time was observed by cell counting. The rationale behind this methodology was to verify if the cells were damaged by either the inoculation or harvest procedures,



**Figure 3: 2D transwell petri dish culture results.** The results shown are for three independent cell population repeats and indicate low density and high medium exchange result in higher expansion. In two out of the three repeats, cell cultures reached logarithmic growth. The large SEM error bars shown in the graph resulted because one cell population repeat did not reach logarithmic growth. Bioreactor volumetric media simulation (BR), Celgene Cellular Therapeutics volumetric media protocol (CG), expansion (or passaging of cells) (Exp), no expansion (NoExp).



**Figure 4: Four-hour harvest of CD34+ HSC bioreactor recovery testing expansion results.** The above graph shows the average fold expansion of CD34+ HSCs initially cultured for four hours in the 8-mL laboratory scale bioreactor and transferred to 2D plate cultures. The results are for a single cell population (pooled cells after harvest from four 8-mL scale bioreactors) with SEM error bars representing technical replicates.

which would result in decreased expansion when compared to a control culture containing cells that were not cultured in a bioreactor. The fold expansion results for these cultures can be seen in Table 4.

The orange curve in Figure 4 represents average of fold expansion values from the four bioreactors that were harvested after four hours of perfusion culture. It can clearly be seen that the cultures expanded very much to the same extent as the control culture that was plated with cells that were never in a bioreactor. Such an observation indicates that the cells were likely not damaged to a significant degree during either the inoculation or harvest procedures. Since a significant portion of the

cells were recovered from the bioreactors and expanded accordingly in 2D, the reproducibility issues in the bioreactors likely do not stem from cell loss or cell damage issues.

### 8-mL scale laboratory scale bioreactor culture results

Based on fold expansions (Figure 5) the optimal time to harvest a bioreactor is approximately 14 days after inoculation with an initial HSC seeding density of 20,000 cells/mL. Bioreactors cultured longer than 14 days for the time period 15 to 22 days produced lower fold expansions. Such a result could be due to the differentiation medium and overexposure to the growth factors for an extended period of time or important factors secreted by the cells being washed away in the waste. FACS results (Figure 6) indicated that the CD34+ receptor no longer existed in any of the cell suspensions harvested from the bioreactors. Harvested cells at days 8-11, day 12-14, and 15-22 expressed the CD71 receptor at  $86.7 \pm 5.8\%$ ,  $84.2 \pm 7.0\%$ , and  $88.2 \pm 2.4\%$  respectively, which indicates many cells are still in a proliferative stage of erythropoiesis. Interestingly, the presence of the CD45 receptor, a common leukocyte antigen expressed on all nucleated HSCs, decreased consistently with longer culture time in the bioreactor (days 8-11:  $95.0 \pm 1.2\%$ , days 12-14:  $43.6 \pm 17.7\%$ , days 15-22:  $8.3 \pm 4.2\%$ ). Furthermore, harvested cells from the bioreactors expressed the CD235a receptor (present on mature RBCs) at  $44.6 \pm 18.5\%$  for days 8-11,  $57.4 \pm 6.1\%$  for days 12-14, and  $28.3 \pm 4.1\%$  for days 15-22. Finally, several bioreactor cell cultures expressed the CD235a receptor and did not have a nucleus (days 8-11:  $51.3 \pm 15.9\%$ , days 12-14:  $19.9 \pm 5.6\%$ , days 15-22:  $25.9 \pm 1.1\%$ ), which indicates the cells possess RBC-like characteristics. Overall, flow cytometry results indicate that the bioreactor culture environment promoted differentiation of HSCs toward RBC lineages.

### Discussion

Advances have been made towards the *in vitro* culture of HSCs, but culture processes have yet to be optimized for the production of RBCs. A number of processes associated with variable efficiency of *in vitro* expansion and erythrogenic differentiation of HSCs must be enhanced. The expansion of cells in both 2D plate cultures and 3D perfusion culture techniques are affected by any variables, including initial cell inoculation density, cell density of expanding cells during culture, medium / nutrition exchange per day and medium supply / removal mode over the day, perfusion versus diffusion conditions in the cell environment, metabolite gradients, oxygen supply and gradients, and culture pH regulation and gradients (that is controlled by the oxygenator CO<sub>2</sub> removal rate in case of bicarbonate buffered medium and integral oxygenation).

In a previous publication, feeding in 2D conventional cultures was performed with a medium change every 3-4 days, whereas when the 3D perfusion bioreactor was used, culture media feed continuously ranged from 1-4 mL/h (adjusted based on concentrations of glucose and lactate

Bioreactor	BR001	BR002	BR003	BR004
Total cell count	$2.25 \times 10^5$	$1.2 \times 10^6$	$1.1 \times 10^5$	$8.0 \times 10^4$
Live cell count	$1.25 \times 10^5$	$9.5 \times 10^4$	$7.0 \times 10^4$	$6.0 \times 10^4$
Dead cell count	$1.0 \times 10^5$	$3.0 \times 10^4$	$3.0 \times 10^4$	$2.5 \times 10^4$
Viability (%)	56.0	75.0	68.5	72.5
Number of 2D cells plated	$8.0 \times 10^4$ (2 wells)	$8.0 \times 10^4$ (2 wells)	$4.0 \times 10^4$ (2 wells)	$4.0 \times 10^4$ (2 wells)

**Table 5: Four-hour harvest of CD34+ HSC bioreactor recovery results.** Cell recovery from 8-mL laboratory scale bioreactors after 4 hours of perfusion culture with expansion and differentiation media. The recovered cells were then plated in 2D cultures and expanded for 14 days.

from the culture perfusate samples) [14]. The previous results using a four-compartment 3D perfusion bioreactor technology with integral oxygenation for the culture of HSCs towards RBC lineages encouraged us to formulate and proceed with the following questions: First, was a more frequent medium exchange, as possible in dynamic bioreactor feed regime, affecting the cell expansion? Second, was the initial and/or subsequent cell density affecting cell expansion? And, third, was frequent medium exchange affecting cell growth when cells are grown at different densities?

In a first set of experiments, we used a transwell plate culture model with simulated bioreactor conditions, as the associated high numbers of experiments could not realistically be performed in independent bioreactor runs. 2D transwell cultures can provide a simple tool for an initial understanding of density, feed regime, and expansion aspects between conventional 2D cultures and 3D bioreactor cultures. Overall, the results indicate that low density and high medium exchange results in more significant fold expansion rates. Interestingly, expansion factors differ strongly between cell donors/isolations but are consistent within a single cell population experiment. In one of the repeats, the cell did not enter logarithmic growth, but that was the case in the controls as well (data not shown). This indicates a strong impact of variables beyond the experimental conditions, such as donor, procurement, isolation and

initial culture—suggesting that primary cultures derived from umbilical cord blood represent a suboptimal cell source for a larger scale RBC production. As the expansion factors did not differ as much within a single donor cell population used for each of the experimental groups, we assume that the overall outcome is acceptable, and therefore a cell density of 20,000 cells/mL was selected for the second experiment, a cell harvesting and long term 8-mL scale bioreactor study.

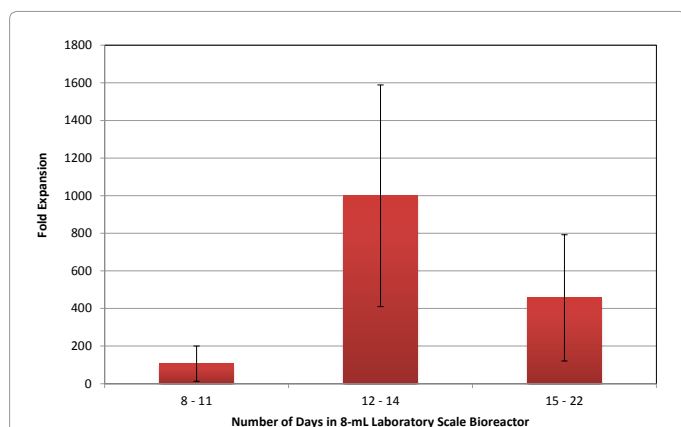
For the previously published experiments [14] and bioreactor studies described here, an 8 mL volume was used to calculate the initial seeding density because the bioreactor cell chamber entraps that volume. For example, if  $1.6 \times 10^5$  cells are inoculated, the initial seeding density is 20,000 cells/mL [ $(1.6 \times 10^5 \text{ cells}) / (8 \text{ mL}) = 20,000 \text{ cells/mL}$ ]. However, if the total circuit volume of approximately 55 mL medium (that circulates through the cell chamber) is considered for the calculation, the density reduces by approximately 7-fold. For example, if  $1.6 \times 10^5$  cells are inoculated, the initial seeding density is instead 3,000 cells/mL [ $(1.6 \times 10^5 \text{ cells}) / (55 \text{ mL}) = 3,000 \text{ cells/mL}$ ].

When translating findings from static 2D culture experiments into dynamic 3D perfusion bioreactor cultures, both density calculations should be considered because the bioreactor cultures may perhaps depend on soluble products of the cells that are likely diluted in the entire medium volume. Furthermore, the cell concentration within the cell chamber should be considered as cell-to-cell interactions and concentration of mediators around the cells are important aspects which can ultimately impact expansion and differentiation.

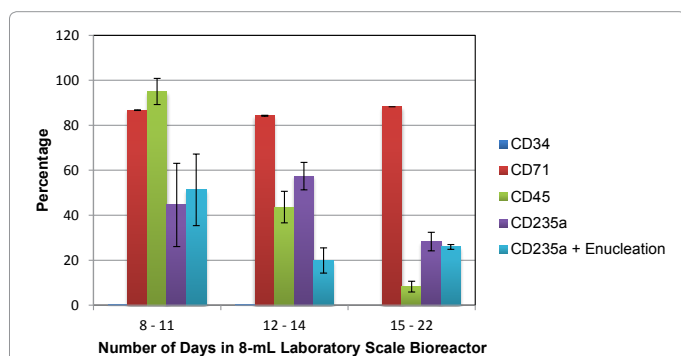
In the second independent experiment on excluding potential cell injuries during cell inoculation and harvesting into and from a bioreactor, HSCs could be effectively recovered from the 8-mL laboratory scale bioreactor systems after four hours of perfusion culture, and could subsequently be expanded in follow-up 2D plate cultures to the same degree as a control culture that was plated with cells that were never exposed to bioreactor conditions. Consequently, the mechanical impact during cell inoculation or harvest is unlikely to contribute to the discussed variability in culture results. However, there are many more variables in the in-bioreactor growth conditions, which could possibly contribute to a large variability in bioreactor cultures that we did not test.

In order to further improve cell production, in a third independent experiment, we used an 8-mL scale bioreactor with an initial cell seeding density of 20,000 cells/mL cell chamber volume. Increasing the expansion within one bioreactor while trying to extend the culture was possible, which is important for studies on further enhancing CD34+ to enhance differentiation towards RBC lineage. The approximate 1000-fold expansion result indicates that a suitable time to harvest from this bioreactor technology with its employed initial inoculation density and feed rate combination is approximately 2 weeks after inoculation. The initial seeding density appears to be a critical factor, as by decreasing the initial cell seeding density in the 8-mL bioreactor, we were able to improve the expansion by 10 fold as compared to our previous results [14].

Given the limitations in the number of CD34+ HSC yield per donor blood unit, we are able to isolate from one given cord blood unit on average 200,000 cells, a cell amount that allows for a manageable initial bioreactor seeding density. Based on the FACS analysis results, the 3D perfusion bioreactor conditions enhanced differentiation of HSCs towards RBC lineages. Again, the fold-expansion inconsistency between experiments in our opinion is mostly related to cord blood



**Figure 5: Average fold expansion in 8-mL laboratory scale bioreactor cultures.** Several bioreactors were seeded with an initial density of 20,000 cells/mL and cultured for various periods of time to assess an optimal time point for cell harvest.



**Figure 6: FACS data for 8-mL laboratory scale bioreactor cultures.** Shown is FACS data for 8-mL laboratory scale bioreactors with initial 20,000 cells/mL inoculation density. Cell suspensions were collected after each bioreactor harvest and analyzed for various FACS makers including CD34, CD71, CD45, CD235a, and CD235a + enucleation.



donor-to-donor variability. However, other technological aspects have to be considered, such as perfusion gradients due to cell sedimentation inside the bioreactor cultures. Such aspects could be further studied in modified bioreactors that would allow a semi-continuous sampling of small amounts of cells from different points in the culture space during the experiments. Overall, the three experiments led to the here described improved HSC Culture methods for high cell density hollow fiber membrane based 3D perfusion bioreactors.

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#### Competing Interest Statement

The bioreactor prototypes were purchased from Stem Cell Systems GmbH, Berlin, Germany, a University spin-off of the Charité, Berlin. J.G. owns shares of the company. No other author has a financial interest in the company.

#### Author Contributions

G.H. designed and planned the study, conducted experiments, supervised, interpreted data, participated in data collection and data interpretation, and wrote the manuscript; T.M., E.S., and C.P. supervised, provided technical assistance during experiments, participated in data collection and data interpretation, and provided useful discussions; K.Z. contributed to bioreactor development and parameter characterization; J.G. designed and performed the study, supervised and performed system application, supervised data analysis, interpreted data, and wrote the manuscript.

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