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Production of erythrocytes from directly isolated or Delta1 Notch ligand expanded CD34+ hematopoietic progenitor cells: process characterization, monitoring and implications for manufacture

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Abstract

Background aims. Economic ex vivo manufacture of erythrocytes at 10¹² cell doses requires an efficiently controlled bio-process capable of extensive proliferation and high terminal density. High-resolution characterization of the process would identify production strategies for increased efficiency, monitoring and control. Methods. CD34⁺ cord blood cells or equivalent cells that had been pre-expanded for 7 days with Delta1 Notch ligand were placed in erythroid expansion and differentiation conditions in a micro-scale ambr suspension bioreactor. Multiple culture parameters were varied, and phenotype markers and metabolites measured to identify conserved trends and robust monitoring markers. Results. The cells exhibited a bi-modal erythroid differentiation pattern with an erythroid marker peak after 2 weeks and 3 weeks of culture; differentiation was comparatively weighted toward the second peak in Delta1 pre-expanded cells. Both differentiation events were strengthened by omission of stem cell factor and dexamethasone. The cumulative cell proliferation and death, or directly measured CD45 expression, enabled monitoring of proliferative rate of the cells. The metabolic activities of the cultures (glucose, glutamine and ammonia consumption or production) were highly variable but exhibited systematic change synchronized with the change in differentiation state. Conclusions. Erythroid differentiation chronology is partly determined by the heterogeneous CD34⁺ progenitor compartment with implications for input control; Delta1 ligand-mediated progenitor culture can alter differentiation profile with control benefits for engineering production strategy. Differentiation correlated changes in cytokine response, markers and metabolic state will enable scientifically designed monitoring and timing of manufacturing process steps.

Key Words: bio-process, erythrocytes, ex vivo, hematopoiesis, manufacture, metabolism

Introduction

Red blood cell transfusions are one of the most common clinical interventions worldwide. This process is currently supported by approximately 90 x 10⁶ annual voluntary blood donations each year. However, the donation system is increasingly stretched, leading to demand for a manufactured red blood cell product to reduce the logistical issues with donor cells. These issues include a projected donor/recipient imbalance in aging populations, low donor recruitment and transmissible disease risk in developing countries, sustainability of immune matched supply to high-demand patient groups (e.g., patients with sickle cell anemia), and the (much debated) detrimental effects of storage on erythrocytes (1). Consequently, there has been considerable recent interest and scientific progress in the production of clinically transfusable red blood cells from a renewable stem cell source.

Successful protocols to produce late-stage maturity erythroid cells have been developed based on both umbilical cord-derived hematopoietic stem cell (HSC) populations and human pluripotent stem cell populations. An HSC source has been used more recently to generate an erythrocyte population that was successfully transfused in a human proof-of-principle study; the transfused cells underwent final maturation in vivo and persisted for approximately 1 month after transfusion (2). Human pluripotent stem cell-based approaches have not yet reached the clinic but are advanced in terms of quality of final red
cell product (3). The latter approach benefits from an unlimited quantity of input cell line material compared with a requirement for repeated primary sourcing with HSCs. However, the human pluripotent stem cell starting cells have a longer and more complex path of differentiation and require more process control to reach a final product.

A possible method to overcome the limited mature lineage yield from primary HSCs would be to increase proliferation of the early-stage progenitors. Clinical demand for transplantable HSCs as part of leukemia treatment regimens has motivated numerous developments to support early progenitor proliferation, including novel biologic mediators, such as Notch ligand Delta1, or chemical mediator approaches, such as inhibition of the aryl hydrocarbon receptor (4).

Multiple other approaches are at the non-clinical stage, such as regulation of inhibitory feedback networks through control of common culture parameters (5).

As the biologic protocols for progenitor proliferation and myeloid/erythroid differentiation become better defined, greater attention needs to be paid to the manufacturing challenges of ex vivo erythrocyte production. The challenge, regardless of source stem cell population, is dominated by a cost of goods issue. A single unit of blood contains approximately $2 \times 10^{12}$ cells, far greater than the quantity required by most candidate cell therapies. To reach a 10-L final production volume for a unit of blood, densities on the order of $1 \times 10^9$ cells mL$^{-1}$ would need to be achieved; generating 100 units of blood from a $1 \times 10^6$ cell starting population would require approximately 28 population doublings (PDs). The purity and concentration of cultured product determine the scale and cost of downstream processing requirements. Appropriate manufacturing systems and processes are critical to delivering sufficiently high production densities and efficient generation of end product per starting cell material, with economically viable inputs of culture consumables, facility and machine time and labor.

Early approaches to these scale-up challenges focused on suspension culture and differentiation of HSCs; this step would need to be addressed regardless of the progenitor source. Agitated bag systems have been successfully applied (6) and, in our laboratories, scaled down stirred tank processing (7). The latter process sustained cell proliferation beyond densities of $1 \times 10^7$ cells mL$^{-1}$. Proliferative capacity issues can potentially be addressed through prolonging culture to increase the number of blood cells per input cell, although this protracted proliferation appears to reduce the quality of the terminal differentiation process (6).

Engineering a process to achieve such a high level of efficiency necessarily requires a greater degree of control, which is contingent on tighter process tolerances; this requires a sufficiently high-frequency and precise characterization of the physical state of the process (both cell population and environment) to enable data-driven process modifications and implementation of appropriate monitoring and responses. Such an approach would also facilitate identification of markers that can accurately report the functional state of the bio-process. High-frequency monitoring is particularly important for stem cell differentiation because of rapid changes in the cell population and consequently its response to the process environment. It is suggested that a fundamental issue with many stem cell-based processes is a lack of adequate monitoring data throughout the process.

To address these issues, we used two different HSC populations: directly isolated cord blood CD34+$^+$ cells and CD34+$^+$ cells after 7 days of pre-expansion with Delta1 Notch ligand. The latter population potentially has production benefits associated with enhanced progenitor expansion (8). Cells were profiled for differentiation state and metabolite consumption throughout the previously reported erythroid protocol in a suspension bioreactor (7). We have identified unreported patterns in the differentiation profile, tools with which to manipulate these patterns and an effective process monitoring marker conserved throughout all process conditions and input cells. In light of these, we discuss implications for further manufacturing development of a blood product.

Methods

Cells

Two different cryopreserved input cell populations were obtained: (i) mixed donor CD34+$^+$ umbilical cord-derived cells enriched via negative selection using RoboSep automated cell separator (AllCells, Alameda, CA, USA) and (ii) umbilical cord-derived CD34+$^+$ cells after 7 days of pre-expansion with Notch Delta1 ligand in conditions previously described (9) (provided courtesy of Professor Irwin Bernstein, Fred Hutchinson Cancer Research Center, Seattle, WA, USA).

Bioreactor inoculation and maintenance

The ambr bioreactor (TAP Biosystems, Royston, UK) is a scaled-down version of a classic stirred tank bioreactor operating at micro-scale (10–15 mL), using multiple disposable micro-bioreactors, with individual, automated, online monitoring and control of pH, oxygen gassing, temperature and stir rate. The system has been validated for 2-L scale-up equivalence (10). Before cell defrost, ambr vessels were loaded with 14 mL of medium and stabilized for temperature (37°C), oxygen delivery and pH. Automated
Figure 1. Representative growth characteristics and differentiation profiles (assessed by Hb expression and size change) of Delta1 ligand pre-expanded CD34+ cells and directly isolated CD34+ cells across the culture period. (A) Both cell populations showed similar growth profiles in the bioreactor (accounting for the pre-expansion of the former group). (B) Bi-modal differentiation pattern, conserved under every test condition, was observed with a Hb peak at day 13 and again at day 22 of culture. The timing was equivalent for both cell types. The magnitude of the early peak and subsequent trough in Hb expression were greater in the unexpanded CD34+ cells. (C) Cell size changes (flow cytometry forward scatter) support the bi-modal differentiation events. A significant small population is detected at day 13 coinciding with the first Hb expression peak. A split between large and small cell populations exists up to the end of the process. The balance of this split depends on the originating cell types. The CD34+ cells show a relatively synchronous shift in cell size. Delta1 pre-expanded cells maintain a more mixed population size throughout the period of day 13–20 and beyond. (D) At day 22 of the process, the cell population can be categorized into quadrants as large/small size and high/low CD71-expressing populations. The most mature erythroid population, small cells with low CD71 expression, would be expected to synchronize with the high Hb expression at day 13 and day 22 of the process. However, this population is clearly distinctive only at day 22 suggesting a later state of maturity is achieved in the second peak of differentiation in both input cell populations. The percentage of the population in this state is annotated. A representative histogram showing the proportion of cells expressing the erythrocyte marker, CD235a, that are positive (nucleated) or negative (enucleated) for the nuclear stain Syto16 and a benzidine Wright-Giemsa-stained micrograph are also shown to illustrate terminal enucleation occurring at this point in the culture. Arrows point to enucleated cells in the micrograph, and these cells appear in the left peak of the plot (i.e., CD235a expressing and no nuclear stain). An erythrocyte positive control at equivalent scale is inserted at the top left of the micrograph for comparison. (E) To determine the response of the two differentiation peaks to a process intervention designed to enhance erythroid differentiation, supplementation with SCF and dexamethasone was stopped after 11 days of culture. This caused sustained CD235a expression across the trough between the two differentiation events and a shortened trough in Hb expression. The bi-modal pattern of differentiation was conserved in both input cell populations.
anti-foam additions (20 μL of 1% solution; Sigma-Aldrich, Dorset, UK) were made every 48 h. Culture pH was maintained by automated sodium bicarbonate (20 μL of 1 mol/L solution; Sigma-Aldrich) additions at 2-h intervals (if required) and regulated carbon dioxide gassing. Impeller speed was 450 rpm.

Input cells were thawed and inoculated to the preconditioned bioreactor. Cells were cultured with a control protocol comprising 1E5 cells/mL in Iscove’s modified Dulbecco’s medium with GlutaMAX-I (Invitrogen, Paisley, UK) supplemented with cytokines and reagents for erythroid cell proliferation and differentiation (R&D Systems, Abingdon, UK, unless otherwise stated): bovine serum albumin 1% (Sigma-Aldrich), insulin 10 μg/mL (Sigma-Aldrich), transferrin 330 μg/mL, stem cell factor (SCF) 100 ng/mL, insulinlike growth factor-I 40 ng/mL (Sigma-Aldrich), dexamethasone (Dex) 1 μmol/L (Sigma-Aldrich), penicillin-streptomycin 1% (Invitrogen), Pluronic F-68 0.10% (Sigma-Aldrich). Replacement of culture medium was conducted through centrifugation and re-suspension of cells at 1E5/mL in fresh medium every 2–3 days.

In addition to these control culture conditions, a range of cell culture variables reported to affect proliferation and differentiation were explored, including culture densities (4E4 to 1E6 cells mL⁻¹), 5% oxygen (11), low pH (7.2) (12) and addition or concentration changes of supplementary mediators: vascular endothelial growth factor (10 ng/mL)/insulinlike growth factor-II (250 ng/mL) for initial 6 days (13), 5% Plasmanate supplement (13), 1 IU/mL erythropoietin (low), no lipids (14), no supplementation of SCF/Dex after 11 days (13).

Culture analysis

Cells and culture medium were sampled for offline analysis at each exchange of culture medium. Metabolite analysis was conducted using a Nova BioProfile FLEX (Nova Biomedical, Runcorn, UK) in accordance with manufacturer’s instructions and specific cell consumption rates calculated as follows:

\[
\text{Specific consumption} = \frac{CN_i \times \left\{ \frac{\ln(CN/CN_i)}{(T - T_i)} \right\} - 1}{r} 
\]

where \( r = \frac{\ln(CN/CN_i)}{(T - T_i)} \) and \( T = \text{time at start of culture period} \), \( T_i = \text{time at end of culture period} \), \( CN_i = \text{cell number at } T_i \) and \( CN = \text{cell number at } T \).

Online cell counting and viability (trypan blue membrane integrity) was conducted (Vi-Cell; Beckman Coulter, Inc, Brea, CA, USA) to calculate cell seeding densities. Cell apoptosis or necrosis was also measured using Guava Nexin Reagent (annexin V binding and 7-amino-actinomycin D penetration) and Guava flow cytometer (Millipore, Watford, UK) per manufacturer’s protocol. PDs were calculated as follows:

\[
\text{PD} = \frac{\left\{ \frac{\ln(CN/CN_i)}{(T - T_i)} \right\} \times 3.33}{(T - T_i)} 
\]

Cell samples were taken for flow cytometry analysis of erythroid lineage markers. Antibodies were

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**Figure 1. (continued).**

C

7 day Notch ligand expanded cells

Day 10  Day 13  Day 15  Day 17  Day 24

Unexpanded CD34+ cells

Day 8  Day 13  Day 15  Day 19  Day 22

Side Scatter
pre-conjugated CD235a-PE, CD71-FTTC, CD34-PE, CD45-PE, CD117-PECy5, fetal hemoglobin (Hb)-PE (BD Biosciences, Oxford, UK) and appropriate isotype controls. Cell samples were washed in flow cytometry staining buffer (R&D Systems) and incubated with antibody as per manufacturer’s instructions for 30 min at 4°C. Cells were washed twice in flow cytometry staining buffer and fixed with 2% paraformaldehyde (Sigma-Aldrich). The fetal Hb staining was pre-fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences) per manufacturer’s protocol. A Guava Flow Cytometer was used for analysis, and geometric mean (Gmean) values were used to determine cell expression. The Gmean was calculated as the average between the start and end of any given culture period.

Statistics and modeling
Minitab (Minitab Inc, State College, PA, USA) was used to conduct best subset regression, statistical comparisons (analysis of variance) and correlation analysis (Pearson correlation coefficient). Where Pearson values are compared, a Fisher r-to-z transformation was calculated and applied to assess the significance of the difference between correlations.

Results
Growth and differentiation trends
Initially, we sought to characterize the time profile of erythroid expansion and differentiation in scalable suspension culture format from two different umbilical cord hematopoietic progenitor populations: directly isolated CD34⁺ cells and CD34⁺ cells that had been pre-expanded for 7 days with Delta1 Notch ligand.

Frequent growth and phenotype measurements showed that both input cell populations differentiate toward a late-stage hemoglobinized erythroid phenotype at similar rates (Figure 1A,B). Both cell populations also showed a bi-modal erythroid differentiation pattern; Hb expression peaked in synchrony with the detection of a small cell size sub-population after approximately 2 weeks and 3 weeks of culture (Figure 1B,C). The small cells in the second differentiation peak contained a CD71low expressing cell population indicating late-stage mature erythrocytes and demonstrated enucleation observed by microscopy and CD235a/nuclear co-staining (Figure 1D). Although the gross trends in maturation were comparable between input cell populations, the two differentiation events were significantly more distinct in the directly isolated CD34⁺ population relative to the cells that had been pre-expanded with Delta1 Notch ligand. This is evident in both the reduced magnitude of the dip in Hb expression between weeks 2 and 3 and the lower synchronicity of cell size distribution changes throughout the process in the Delta1 pre-expanded cells.

A total proliferative advantage of Delta1 pre-expanded cells was observed relative to the primary CD34⁺ sourced cells indicating the potential for increased yields through early progenitor manipulation. However, there is clearly significant sample-to-sample variability [previously reported progenitor makeup of cord blood (15)] (Figure 1, Table I).

To determine the response of the bi-modal differentiation peaks to a previously reported method for enhancing late stage differentiation [SCF and glucocorticoid withdrawal (13)], supplementation with these cytokines was stopped after 11 days of culture, before the initial differentiation. In this instance, differentiation was enhanced, assessed by Hb and CD235a expression, in both input cell types. The trough in expression of the markers over the third week of culture also was reduced in magnitude and shortened, most notably in the CD34⁺ input cell population (Figure 1E).

Relationship between culture history and growth rate (directly isolated CD34⁺ cells)
Identifying process markers that are effective at predicting the functional state of a cell population in a bio-manufacture process is important to enable meaningful process monitoring for precisely timed process interventions. These process markers are of critical importance in stem cell differentiation processes where cell populations and their bio-manufacture demands change rapidly. As described in the methods section, different progenitor cell populations were exposed to a range of different physical and cytokine conditions to cause differing rates and levels of erythroid differentiation; this generated a data set that we could use to identify conserved relationships over a wide range of operating conditions.

Cell growth rate in the bioreactor was the initial target for modeling because it is a primary bioprocess functional output. Given that the cells were in an erythroid progenitor tailored expansion and differentiation medium, the maturity of the cell population on the erythroid lineage commitment pathway was proposed as the key determinant of the cell responsiveness to the growth factor cocktail and the cell growth rate. The corollary to this is that the number of PDs the cells have passed through (i.e., the more responsive they have been) is likely to be a primary determinant of population maturity.

In support of this hypothesis, a strong relationship was identified between the PD rate of the cells
and cumulative population doublings (CPDs). The relationship between CPDs and PD rate, particularly in the later phase of culture, could be strengthened if we calculated an adjusted cumulative population doubling (ACPD) figure that inflated CPDs to account for cumulative cell death within cultures (assessed by percentage of trypan blue positive). A least-square fit was used to identify a cell death coefficient to generate the best relationship with PD rate: ACPD was defined as CPDs + 0.7 CD. Nonlinear regression was used to fit a function to the relationship between ACPD and PD per day (Figure 2A).

Regression equation (1):

\[ R = -0.2811 + 0.3737 \times M - 0.03159 \times M^2 + 0.00680 \times M^3 \]

\[ S = 0.13, R - Sq = 87\% \]

(coefficient P values < 0.001)

where R is the growth rate estimate (PDs per day) and M is the maturity estimate (ACPD).

The relationship between PD rate and ACPD was stronger than the relationship with culture duration, suggesting that the cell population response was determined to a greater degree by its proliferation history rather than culture time and that ACPD could be a useful metric for predicting proliferative state of the culture under variable operating conditions.

**Relationship between phenotype and growth rate**

Calculation of CPD or ACPD depends on the availability of process history data. Such data might not be available during production. Error accumulates throughout the process, and a single measurement with a large error (or one that could not be made at all) has an effect on the accuracy of all subsequent calculations. Although ACPD is both an intuitively meaningful parameter representing

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**Table I. Notch cells expansion and primary CD34⁺ expansion.**

<table>
<thead>
<tr>
<th></th>
<th>Notch cells expansion 1</th>
<th>Notch cells expansion 2</th>
<th>Notch cells expansion 3</th>
<th>Primary CD34⁺ cells expansion 1</th>
<th>Primary CD34⁺ cells expansion 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch expansion phase (PD rates)</td>
<td>6.83</td>
<td>6.65</td>
<td>7.16</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Suspension expansion (PD rates)</td>
<td>7.73</td>
<td>9.84</td>
<td>8.54</td>
<td>13.9</td>
<td>11.4</td>
</tr>
<tr>
<td>Total (PD rates)</td>
<td>14.56</td>
<td>16.67</td>
<td>15.7</td>
<td>13.9</td>
<td>11.4</td>
</tr>
<tr>
<td>Total (fold expansion)</td>
<td>2.4E4</td>
<td>1.04E5</td>
<td>5.3E4</td>
<td>1.6E4</td>
<td>2.7E3</td>
</tr>
</tbody>
</table>

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**Figure 2.** The relationships between cell PDs/day and both historical cumulative proliferation and CD45 marker expression over a range of operating conditions (dot per bioreactor). (A) Cumulative population doublings adjusted for cell death (ACPD) had a strong relationship with the average PD rate (Rsq = 88%). It was considered to be a good measure of population maturity. (B) CD45 expression had an excellent correlation with the adjusted cumulative PDs relative to a range of other relevant markers (R – Sq = 94%) making this marker an effective alternative indicator of functional state for the cultures. (C) A CD45-derived growth rate prediction determined from these relationships has a good correlation with actual growth rate for cells undergoing erythroid proliferation and differentiation from freshly isolated CD34⁺ cells (Rsq = 76%). (D) This predictive ability is substantially retained when applied to an alternative cell input population, CD34⁺ cells previously expanded with Delta1 ligand (Rsq = 62%).
population maturity and a good predictor of a key physiologic parameter (growth rate), measures of population maturity that do not require historical data have greater practical robustness from a bio-manufacturing perspective. We investigated if any directly measured marker or marker combinations had a robust relationship with ACPD that could be combined with the known non-linear functional effect of ACPD to predict culture behavior.

We conducted multiple linear regression to determine any linear relationships between the ACPD and the marker profile of the cells measured by mean population expression of CD34, CD71, CD235a, CD45, CD117 (SCF-R) and Hb. CD45 was the best independent predictor of ACPDs with a remarkably strong correlation (R - Sq = 94%, P ≤ 0.001) (Figure 2B). Combination with additional markers did not increase the strength of the relationship.

Regression equation (2):

$$M_{CD45} = 26.31 - 10.23 \log_{10} F_{CD45}$$

$$S = 1.23, \quad \text{Rsq} = 94\%$$

(coefficient P values ≤ 0.001)

where $M_{CD45}$ is an alternative directly measured estimate of the functional state of the population, previously measured by ACPD, and $F_{CD45}$ denotes mean fluorescence for the marker CD45.

The relationship between CD45 and ACPD can be combined with the non-linear relationship defined between ACPD and growth rate to provide CD45 monitoring of the growth rate of the cell population during the erythroid differentiation process. This is demonstrated by a linear correlation of the predicted population growth rate (derived from substituting $M_{CD45}$ into regression equation 1) and the actual population growth rate (R - Sq = 76%, P ≤ 0.001) (Figure 2C).

**Relationship of phenotype to growth rate: robustness for cells pre-expanded with Delta1 Notch ligand**

To test the robustness of the relationship determined with directly isolated CD34+ cell data, we used the Delta1 expanded HSC population. These cells had been exposed to an alternative expansion regimen, Delta1 Notch ligand, and are shown to follow a different differentiation profile. ACPD and $M_{CD45}$ maturity values were both used to calculate the predicted population growth rate using regression equation 1. $M_{CD45}$ (R - Sq = 62%, P ≤ 0.001) provided a better correlation of real growth rate than the direct ACPD derived value (R - Sq = 51%, P ≤ 0.001) (significance of difference between correlations $P = 0.1$) (Figure 2D). This improved prediction with CD45 is hypothesized to be due to the cumulative error associated with ACPD. The strong relationship between CD45 and ACPD and the ability of the marker to predict proliferative rate of culture over diverse operating range and input cells suggests robustness that could be useful for monitoring process state in erythrocyte production.

**Establishing relationships between phenotype and metabolic activity**

Given that the growth rate of the cells could be predicted by measures of cell population maturity, we proposed that other bio-manufacture critical attributes, such as metabolic behavior, could be predicted using the same tools. We investigated the relationship of ammonia production and glutamine and glucose consumption per cell with ACPD for both input cell populations.

Metabolic activity per cell varied substantially across the differentiation and expansion process (Table II). However, there were distinctive and conserved patterns in both processes relative to the proliferative history of the cells. The cultures had a peak of metabolic activity (glucose use and ammonia production) after approximately 11 ACPDs followed by a substantial decline to 13 of 14 ACPDs that approximate the bi-modal differentiation pattern (Figure 3). Although the pattern was conserved for either input cell source, the drop from a high to low metabolic state was more pronounced in the directly isolated CD34+ cells, compared to those pre-expanded with Delta1 ligand; this mirrors the greater separation of the bi-modal differentiation process in the former cells (Figure 3).

To understand the relationship between the differentiation cycle and specific metabolic activity further, we withdrew SCF and Dex at day 11 of culture (previously established as a means of enhancing the pace of late-stage erythroid differentiation). Early withdrawal of these growth factors led to earlier and higher levels of differentiation (CD235a, Hb) and briefly enhanced proliferation (Figure 4A,B). After approximately 11 ACPDs, the more rapidly proliferating and maturing cells showed a suppression of glutamine consumption and

| Table II. Range of specific metabolite consumption and production rates observed over the proliferation and differentiation process. |
|---|---|---|---|---|
| | Mean | SD | Minimum | Maximum |
| Glutamine/cell/day (Delta1) | 4.19 | 2.22 | 0.37 | 10.64 |
| Glucose/cell/day (Delta1) | 0.91 | 0.47 | 0.00 | 1.92 |
| Ammonia/cell/day (Delta1) | 1.90 | 1.20 | 0.70 | 7.27 |
| Glucose/cell/day (CD34+) | 2.27 | 4.22 | 0.00 | 36.47 |
| Glucose/cell/day (CD34+) | 1.56 | 1.86 | 0.00 | 13.15 |
| Ammonia/cell/day (CD34+) | 0.96 | 1.22 | 0.08 | 8.93 |

Ranges and mean values are shown (glutamine E^-6 mmol/cell/day, glucose E^-6 g/cell/day, ammonia E^-6 mmol/cell/day).
ammonia production per cell (Figure 4C,D). A clear effect on glucose consumption was not observed. The effect of higher growth and lower nutrient consumption appears counter-intuitive. We compared the rate of cell proliferation with glutamine consumption using data from across the growth period. A linear relationship between glutamine consumption and growth rate was present when SCF and Dex supplementation was maintained and differentiation was relatively suppressed. However, withdrawal of the growth factors led to a high proliferative and low metabolite consuming state and loss of linear relationship (Figure 4E). This finding strongly indicates that both rate of proliferation and differentiation status have separate and potentially confounding relationships with metabolic behavior, and culture metabolic demand would be a function of both properties.

**Discussion**

The objective of these studies was to provide a high-resolution characterization of the erythroid proliferation and differentiation process to improve design and monitoring of suspension scale-up and manufacture. The data indicate cyclic patterns in differentiation and metabolic behavior that would need to be addressed in a manufacturing scenario. The data also identify rapid process change and robust predictive process measurements that inform the extent of process monitoring that would be required to optimize and control an erythrocyte production process.

**Expansion and proliferation characteristics: implications for manufacture**

The two temporally distinct differentiation events from the CD34+ cord cell population suggest at least two levels of erythroid progenitor maturity within the initial cell population. The median frequencies of three distinct progenitor sub-populations with erythroid potential in the CD34+ cord blood compartment have been reported previously: HSCs 6.6%, common myeloid precursors 3.2%, megakaryocyte erythroid precursors 0.12% (15). The early differentiation peak observed in the bioreactor is likely to reflect the latter...
two progenitor populations rapidly responding to the interleukin-3/erythropoietin-rich culture environment, corresponding to a 2-week single-wave expansion/differentiation life cycle for these committed progenitors. The later maturing population is likely to be derived from the HSC compartment, which would take longer to become responsive to the erythroid progenitor targeted cytokines. The wide variability in progenitor distribution among donors provides an explanation for the variable sample-to-sample proliferative capacities we have reported (15). The implication that a relatively small proportion of the CD34$^+$ compartment contains cells with erythroid potential would be a significant cause of the lag phase at the start of culture.

The Notch ligand, Delta1, has complex developmental effects. It is primarily reported as a regulator of hematopoietic precursors, inhibiting myeloid differentiation, enhancing precursor expansion including rapidly re-populating cells and promoting T-cell and inhibiting B-cell development (8). This is the probable explanation for the Delta1 pre-cultured cells showing a sustained expression of erythroid markers and small cells between the two differentiation events (i.e., a delay in differentiation of some of the committed progenitors through the third week of culture). It would also explain any proliferative benefit of the Delta1 pre-culture protocol.

The different differentiation profiles of the directly isolated or Delta1 ligand-treated CD34$^+$ cells suggest different approaches to harvest of mature product. The continual maintenance of high Hb expression over the final week of Delta1 cell culture suggests multiple high-yield harvest points could be carried out over the period to take advantage of the increased proliferation or delayed differentiation of the early maturing cells, relative to two batch harvest points in the directly isolated CD34$^+$ cells. However, the harvest frequency and consequent yield implications cannot be fully specified without further understanding the longevity of mature cells in the bioreactor environment and the effect of maturing cells on the immature proliferating population.

Figure 4. To characterize further the link between the differentiation cycles and the metabolic behavior of the cells, we withdrew Dex and SCF supplementation after 7 days of culture (triplicate bioreactors individually shown). This had the effect of changing the rate of early differentiation measured by CD235a (A) or Hb (B) with respect to proliferation at approximately 12 ACPD (circled). Removal of Dex and SCF and more rapid differentiation reduced the first peak in glutamine consumption (C) and ammonia production (D) per cell. (E) There was a linear relationship between growth rate and glutamine use while SCF and Dex were present; this relationship was non-linear when growth factors were removed, suggesting a change in metabolic demand relative to activity.
An accurate measure of functional position in the blood production process is critical for timely process control, such as cytokine supplementation changes, or product harvest and purification. In a large-scale system, such measurements dictate bio-process interventions, downstream processing requirements and potentially upstream initiation of further batches. Mapping of variable process inputs onto consistent process outputs is enabled through incorporation of process analytic technology and implementation of control loops, an approach increasingly favored by regulatory agencies (16).

Informed choice of markers and associated measurement systems for process analytic technology and process control require knowledge of the sensitivity of the relationship between process function and various process markers. Definition of stem cell populations as categorically positive or negative for surface and intracellular markers is still widely applied to assess process quality (17); this is partly due to uncertainty regarding the functional significance of higher resolution measurements and the challenge of standardizing among laboratories. However, these metrics can reduce focus on lower expressed markers that provide equally significant but lower magnitude shifts in expression with less categorical distinction between populations. A categorical, two-population classification inevitably has maximum sensitivity when the population distribution is centered at the level of the population divide; this is arbitrary, and there is no evidence to suggest this maximum sensitivity is aligned to maximum functional effect, where measurement sensitivity needs to be greatest. Markers that are functionally informative for the final product are not always informative for earlier process control. The predictive utility of CD45 demonstrated here is a good example; it is a low expressed non-erythrocyte marker that nonetheless provides the best functional tracking of cell population proliferative state for the erythrocyte production process.

The risk of higher resolution process measurement is identification of phenomena that are difficult to interpret from a product quality perspective. Although erythrocyte markers such as Hb and CD235a responded in a qualitatively similar manner to process changes, quantitative responses were of different relative magnitude. When such differences are observed, a product developer may have the challenge of explaining the functional and safety implications and incorporating necessary changes into process development and validation.

Defining a stem cell population as a series of discrete compartments that evolve on a probabilistic basis has been used to develop population balance equations for stem cell differentiation that can be combined with temporal profiles of the culture environment to generate predictive models (18). Such models, if sufficiently broad and predictive, have evident value for controlling a manufacturing process; the model complexity required to achieve this value would depend primarily on the number of significantly functionally distinct cell compartments and their sensitivities to the process environment.

A small panel of predictor markers would define a limited bio-process operating space. A smaller panel requires greater a priori knowledge of process operation and incurs the risk of being out of an expected process space. Conversely, an increasing number of markers, or strong mechanistic basis, would allow the prediction of process performance over a wider process space, although for both diminishing return in precision and increasing cost. This is a cost-to-risk/benefit decision required to determine monitoring requirements of a stem cell product manufacturing process. The process reported covered a broad range of operating conditions, including tests against different input cells, to increase confidence in robustness of observed relationships.

There is a mechanistic argument that CD45 expression would be associated with proliferative rate; CD45 expression decreases as the highly erythropoietin sensitive and proliferative burst-forming unit erythroid cell population changes to a colony-forming unit erythroid cell population and through normoblast toward terminal maturation. This finding provides mechanistic confidence in the statistical relationship observed. It is possible, particularly later in the process, that an attribute of a small proportion of the population is predicting the behavior of the bulk population. The robustness of any such predictive relationship would be limited to a restricted set of operating conditions; if a greater operating space is anticipated, or this process function needs to be predicted with greater precision to control quality, additional cell or process predictors would need to be developed.

Different functional outputs would require different levels of complexity and precision in model terms based on control requirements (process tolerances) and control capability (capability to maintain in given operating space)—hence the disparity between the ability to predict proliferation from markers compared with metabolic state. There is early recognition that homeostatic metabolic pathways could play key roles in differentiation and by implication be key control parameters in ex vivo.
demands higher measurement frequency to de
as the cells differentiate. This situation potentially
changing their relationship with metabolic behavior
terminants of metabolism, such as growth rate,
periods, (ii) insuf
observed over the process bridging measurement
and rapid shifts in marker phenotype and metabolism
robustly from cell markers is due to (i) the substantial
this inability to predict directly metabolic state

ammonia production periods are excluded (where
very low production per cell occurs across a relatively
wide range of CD235a expression). We suggest that
this inability to predict directly metabolic state
robustly from cell markers is due to (i) the substantial
and rapid shifts in marker phenotype and metabolism
observed over the process bridging measurement
periods, (ii) insufficient definition of cell population
to classify metabolic state fully and (iii) key de
terminants of metabolism, such as growth rate,
changing their relationship with metabolic behavior
as the cells differentiate. This situation potentially
demands higher measurement frequency to define
relationships in periods of critical change; the data
presented here indicate target areas where measure
ment and monitoring resolution could be economi
cally focused. It appears likely that a relatively
high-frequency and complex definition of population
differentiation state and proliferative state will be
necessary to elucidate these relationships fully.

In conclusion, models that can accurately predict
bio-process behavior from simple measurements are
necessary to monitor and control production of
complex cellular therapies. These models may
become a regulatory necessity or at least greatly
facilitate development, but they can be challenging to
identify. A red blood cell product is likely to be
particularly challenging because of the production
efficiency required for economic manufacture and
the complex population dynamics that underlie
aggregate process behavior. CD45 is identified as a
marker to monitor progress through an erythroid
expansion and differentiation process and generate
quantitative predictions of process function, with
ACPDS as a high-value secondary measure if marker
data are unavailable. We have also identified com
plex cyclic behavior of cells with regard to functional

phenotype; such behaviors would need to be
accounted for and monitored in a final optimized
manufacturing system.

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