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Generation of red blood cells in vitro: monitoring the process for improved efficiency

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In this issue of *Cytotherapy* (*Cytotherapy* 2013), Glen and colleagues describe a simple approach to monitor and improve the complex process of red blood cell (RBC) production in vitro from primary cord blood (CB)-derived hematopoietic stem cells (HSCs). They also discuss the implications of their findings for manufacturing of a blood product.

Improving efficiency is an essential part of any successful manufacturing operation. Businesses must dependably monitor manufacturing processes and implement appropriate systems of improvement to remain competitive. This fundamental business concept is also central to the manufacturing development of a highly scalable and economically viable in vitro process of RBC production for clinical applications. Therapeutic generation of RBCs in vitro using biotechnologies could alleviate the shortcomings of the current system based on voluntary blood donations, including chronic shortages for rare blood groups, sporadic restrictions in association with natural or man-made disasters, increasing unit rejections due to positivity for new or emerging transmissible pathogens, a predicted insufficient blood supply by 2050 due to increased usage by an aging population, and the detrimental effects of storage on RBCs.

Successful protocols to produce late-stage maturity RBCs have been developed by promoting erythroid differentiation of primary HSCs derived from CB units [1,2,3], mobilized apheresis products [4], or cell fractions discarded during the leukoreduction process of adult blood donations [5]. In a “first-in-human” clinical trial, autologous RBCs generated from mobilized CD34+ cells using this technology survived in vivo as long as their natural counterparts [4]. While these advances intensified enthusiasm about the potential for therapeutic production of RBCs in vitro, they also highlighted the immense costs and technical challenges to generate sufficient RBCs for routine clinical applications using current protocols. Given their unlimited proliferation potential, pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are also investigated as an alternative stem cell source for industrial production of RBCs [6,7,8]. However, current technologies based on PSCs favor production of fetal-like RBCs that are less efficient oxygen carriers than RBCs expressing adult hemoglobin. PSC-derived

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RBCs may also carry risks of immunogenicity and, importantly, their expansion potential from PSC-derived HSCs is 1 to 2 logs lower compared to RBCs generated from primary CB-derived HSCs. Thus, significant and costly expansion would be required for efficient manufacturing irrespective of the stem cell source utilized for RBC production.

In this study (Cytotherapy 2013), CD34+ progenitor cells directly isolated from CB specimens or pre-expanded for 7 days by induction of Notch signaling with Delta-1 ligand [9] were coaxed to generate RBCs in a microscale stirred tank bioreactor under standard erythroid differentiation conditions over a period of 4 weeks. Aliquots of cells were collected every 2 to 3 days to monitor the differentiation process. Both populations of CD34+ cells showed a similar bimodal differentiation profile with a first peak in RBC production at day 13, and a second peak of more mature erythroid cells at day 22 of culture. This pattern may be explained by the known heterogeneity within the initial CD34+ cell population composed of both progenitors already committed to the erythroid lineage resulting in the first wave of RBCs, and more immature stem/progenitor cells that require longer time in culture before giving rise to the second erythroid differentiation peak. Manipulation of the culture conditions through withdrawal of SCF and dexamethasone, a previously documented approach to enhance the pace of late stage erythroid differentiation [10], enhanced generation of RBCs from both populations.

Interestingly, differentiation of the Delta-1 pre-expanded CD34+ cells favored the second more mature RBC peak, perhaps owing to the presence of more immature progenitors in culture after Delta-1 expansion. Pre-expanded CD34+ cells also improved erythroid production between each differentiation peak which may allow more frequent harvest points and therefore higher RBC production yields. Taken together, these results define Delta-1 expanded CB-derived CD34+ cells as a possibly superior starting population for RBC generation compared to directly isolated CD34+ cells. However, pre-expansion does not appear to address the issue of great variability in the number of RBCs generated from CD34+ cells isolated from different individuals [11]. Understanding the factors underlying this variability, such as intrinsic differences in HSC content and in proliferation ability of hematopoietic progenitors, will aid in optimizing selection of candidate donors and overall RBC yield from primary HSCs. Moreover, delta-1 expansion of CD34+ cells derived from clinically more abundant adult sources, such as mobilized apheresis products, has not been demonstrated [9]. The inability to efficiently expand HSCs in vitro has plagued this field for decades and, while Delta-1 ligand used in this study is perhaps the most clinically mature approach, the technological advances in recent years will undoubtedly bring forth novel avenues for efficient stem cell expansion. The study of Glen and colleagues (Cytotherapy 2013) serves as an important proof-of concept that pre-expansion of CD34+ cells can be of benefit to improve RBC generation from primary HSCs.

The authors emphasize the importance of meticulous monitoring and characterization of the RBC differentiation process to enable data-driven optimization of current protocols for eventual cost-effective industrial scale-up. Parameters effective at predicting the functional state of a cell population in a bio-manufacture process were measured throughout the course of in vitro differentiation, including growth rate, phenotype, and metabolic activity. By exposing two distinct input cell sources (freshly isolated or pre-expanded) to a wide array of

differentiation conditions in vitro (variations in pH, stir rate, oxygen concentration, temperature, cytokines), multiple data points could be generated throughout the differentiation process, allowing identification of possible relationships between these parameters.

The influence of the different culture conditions on cell growth rate was found to be highly predicted by the adjusted cumulative population doublings (ACPD), a value calculated by adding population doublings and adjusting for cell death. However, calculation of ACPD requires knowledge of cell numbers at the start and throughout the culture period and is thus an impractical parameter to predict culture behavior. Instead, the authors identified an easily measurable cell surface marker, CD45, as an effective independent predictor of ACPD. Paradoxically, CD45 is a leukocyte marker that fared better than tested RBC markers (CD235a and CD71) as an alternative indicator of growth rate during the erythroid differentiation process. Downregulation of CD45 expression known to take place during erythroid maturation provides a mechanistic rationale for this unexpected statistical relationship.

The influence of metabolic activity on the regulation of cellular differentiation has recently been proposed in eukaryotic organisms [12,13]. In this study (Cytotherapy 2013), metabolic activity, as measured by ammonia production and glutamine/glucose production, was used as a predictor of the functional state of the cell population during ex vivo differentiation. Even in the setting of high proliferation, low metabolic activity was recorded when cells entered the high differentiation state associated with the second peak of erythroid differentiation. However, the cyclical variations in metabolic activity could not be reliably predicted by CD45 expression which declined linearly throughout the process of differentiation. Even markers of late stage erythroid differentiation (hemoglobin and CD235a) could not predict metabolic state robustly across the entire process.

This study provides the foundation for a rational monitoring of ex vivo erythroid differentiation using a simple surface marker (CD45) to enable quantitative predictions and timely process control, such as optimal timing of medium changes and RBC harvests/purification. The development of such systems for analyzing and controlling manufacturing through the measurement of critical quality parameters during production, referred to as process analytical technology (PAT), is one of the objectives contained in the Initiative for Pharmaceutical Current Good Manufacturing Products (cGMP) for the 21st Century published by the Food and Drug Administration (FDA) [14]. The measurements performed on in process materials as utilized in this study bring important advantages for industrial ex vivo production of RBCs but also for the manufacturing of other cell types with therapeutic potential. Cost control resulting from more efficient production processes and minimization of the necessity of final batch discard, is an important incentive. Perhaps most removed but of great potential, continuous monitoring could facilitate the development of new and improved methods of production by allowing a finer control of interim production steps. Importantly, the addition of monitoring during production could enhance the likelihood of catching aberrant situations and increasing patient safety, an important consideration in a world in which financial issues and cost control have become tightly entangled with patient treatment and cure.

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