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Short communication

Measuring dissolved oxygen to track erythroid differentiation of hematopoietic progenitor cells in culture

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ABSTRACT

As stem cell technologies move from the developmental to the commercial stage strategies must be developed to monitor culture operations. These will ensure consistency of differentiation programs and maintenance of optimum cell viability during production runs. Due to the sensitivity of stem cells to their environment, and their variability in response to external stimuli, accurate monitoring of in vitro conditions will be crucial for effective large-scale culturing of therapeutic stem cells. Here we describe a simple method to monitor the expansion and maturation of adult human haematopoietic stem/progenitor cells into red blood cells in vitro by measuring the oxygen consumption rate of cultures. Cell cultures followed a characteristic pattern of oxygen consumption that is reflective of in vivo erythroid maturation. This method could be easily developed as an online system to map erythroid differentiation and maturation of cultured cells as effectively as the more time consuming process of flow cytometric analysis of surface marker expression patterns.

The past number of decades has seen an explosion in stem cell research, work now culminating in the transfer to mainstream therapeutic application. With this comes the need to adapt to large-scale, GMP grade, tightly regulated processes. However, stem cell culture is more complex than that of traditional biopharmaceuticals, as the cell is the final product rather than a production host. Stem cells are notoriously sensitive to environmental cues (Kinney et al., 2011) and bioreactor conditions, such as shear stress, can alter differentiation (Shafa et al., 2011; Taiani et al., 2010) leading the European Medicines Agency to issue manufacturing guidelines for stem cell derived products. These will ensure process reproducibility and effective differentiation, as persistence of undifferentiated cells could lead to tumourigenicity or ectopic tissue formation in vivo (EMA, 2011).

HSPCs (haematopoietic stem/progenitor cells) can be differentiated into all blood cell types in vitro and researchers worldwide are developing systems to produce blood cells for transfusion from HSPCs from sources including peripheral blood, cord blood, embryonic, and induced pluripotent stem cells (Timmins and Nielsen, 2009). The highest clinical demand is for RBCs (red blood cells) and, although roughly 92 million donations are collected per year worldwide (WHO, 2011), shortages are common particularly in developing countries and for rare types.

Generation of RBCs from HSPCs is feasible, if not quite practical economically. Expansion rates are low relative to the market requirements (10^{12} cells per donation unit), yet proof of principle studies have demonstrated the efficacy of these cells in vitro and in vivo (Giarratana et al., 2005, 2011); and meeting the transfusion requirements of patients with rare blood types or haemoglobinopathies in the medium-term is conceivable.

Our group has developed a system to generate RBCs from peripheral blood HSPCs isolated from buffy coats – a by-product of RBC donation (Boehm et al., 2009). This process is highly reproducible, yet sensitive to environmental changes, and relatively small differences in parameters such as pH can affect differentiation. Thus, a simple and effective method to ensure integrity of differentiation and maturation is required.

Erythroid cells follow a distinct pattern of differentiation characterised by progressive changes in surface marker expression, size, metabolic profile, and packaging and extrusion of intracellular organelles. Here we show they also exhibit a characteristic pattern of oxygen consumption as they mature.

HSPCs were differentiated into reticulocytes in a 21-day culture split into 3 phases based on cytokine supplementation and cell phenotype. In Phase 1 (days 0–11) quiescent HSPCs emerged from a lag phase (days 0–4) and proliferated at a high rate until day

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11 (average specific growth rate 0.046 h^{-1}), hereafter they committed to erythroid differentiation. This led to a sharp decrease in growth rate as cells cycled through erythroid precursor stages and accumulated haemoglobin (Fig. 1). From day 15 (Phase 3) growth ceased as cells entered terminal maturation, giving a final population of approximately 40% enucleated reticulocytes with the remainder orthochromatic erythroblasts (data not shown) (Boehm et al., 2009). Overall expansion of cells was 2×10^5 fold occurring mainly in Phase 1 (Fig. 1).

Fig. 2 shows the changing cell phenotype as defined by expression of cell surface markers. HSPCs are characterised by high CD34 expression which decreases as cells commit to the erythroid lineage. By day 7 CD34 expression was low, and by day 9 absent with cells expressing the immature erythroid marker CD71 and a low level of Glycophorin A (GpA), a committed erythroblast marker. At day 12 all cells expressed CD71 and GpA, and from days 15 to 19 CD71 expression decreased as cells matured, while GpA was maintained at a high level. CD71 was not completely lost indicating cells did not fully mature.

Dissolved O_2 concentration ($[dO_2]$) was measured automatically every 30 min over the course of culture using a commercial O_2 measuring system. The overall oxygen consumption rate (OCR) was calculated from these measurements using a model developed by Guarino et al. (2004) to measure OCR in an open system, which gives values comparable to closed systems (Guarino et al., 2004; Mamchaoui and Saumon, 2000). OCR was calculated from the localised dO_2 level at the bottom of the micro-well plate where cells had settled to form a monolayer culture. This calculation takes into account the diffusion rate of O_2 through culture medium at equilibrium under static conditions, and uses the following equation:

$$\mathrm{OCR} = \frac{DSLa\Delta p}{h}$$

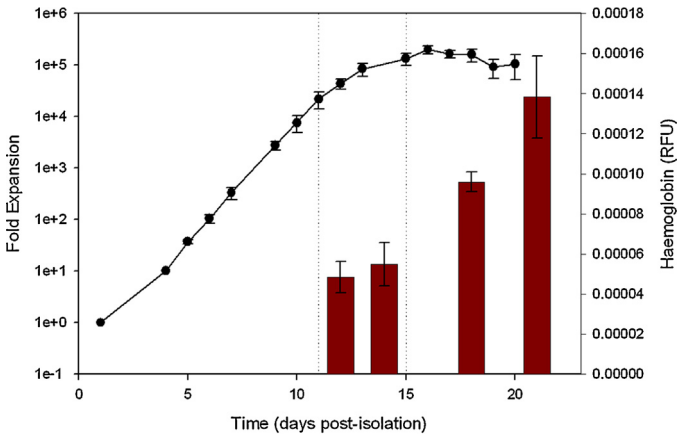

Fig. 1. Peripheral blood buffy coats from healthy donors were obtained from the Irish Blood Transfusion Service (Dublin, Ireland). Mononuclear cells were separated by density-gradient centrifugation and $CD34^+$ HSPCs were isolated using anti-CD34 magnetic microbeads (Miltenyi Biotech, Surrey, UK). HSPCs differentiated into RBCs in erythroid differentiation medium at 37 °C, 5% CO_2 in a humidified atmosphere in a 3-phase culture system as per Boehm et al. (2009). Phase changes are marked with vertical dotted lines. In Phase 1 (days 0–11) medium was further supplemented with stem cell factor (100 ng/ml, Millipore, Cork, Ireland), erythropoietin (3 U/ml, Janssen–Cilag, Dublin, Ireland), interleukin-3 (5 ng/ml, R&D Systems, MN, USA), and hydrocortisone (10^{-6} M, Sigma–Aldrich, Poole, UK); in Phase 2 (days 11–15) medium was supplemented with erythropoietin only; in Phase 3 (day 15 onwards) there was no cytokine supplementation. Fold expansion of viable cells (line) was determined by trypan blue exclusion. Haemoglobin accumulation (bars) in cell lysates was determined from the colour change of OPD reagent mix (0.5 mg/ml o-phenyldiamine and 0.03% H_2O_2 in 0.1 M citrate/phosphate buffer) allowed to develop for in the dark for 10 min, reaction stopped 2.5 M H_2SO_4, and absorbance read at 492 nm.

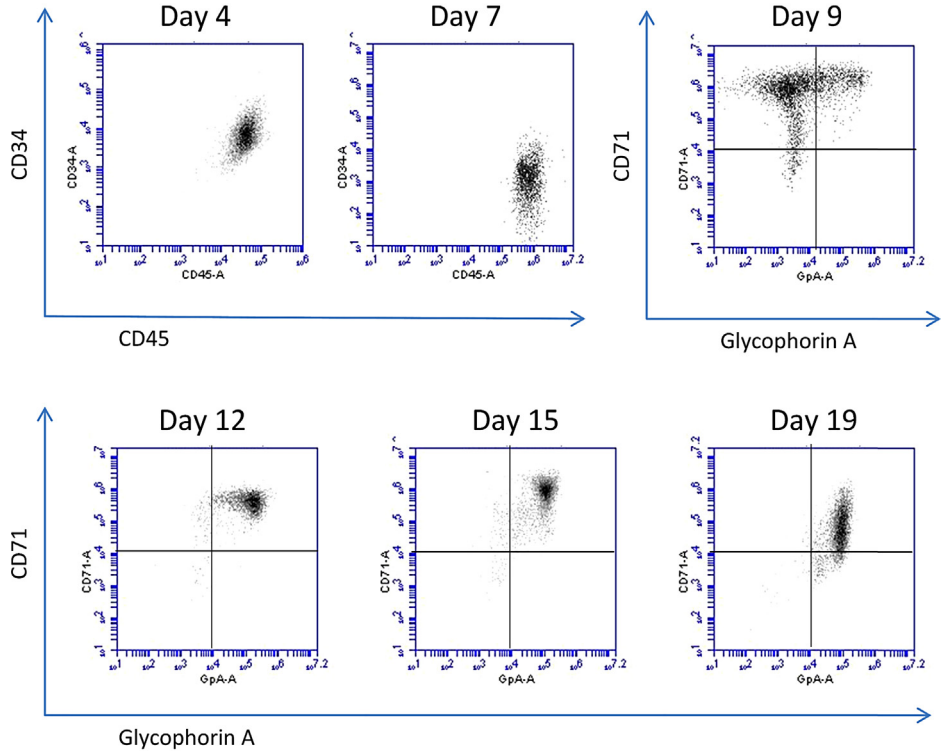

Fig. 2. For flow cytometric analysis of cell surface marker expression over the course of erythroid differentiation cells were stained with mouse anti-human CD34-R-PE and CD45-FITC at days 4 and 7, and mouse anti-human CD71-R-PE and Glycophorin A-FITC at days 9, 12, 15, and 19 (all antibodies obtained from Caltag, Buckingham, UK). Stained cells were analysed on the Accuri C6 flow cytometer with 488 nm laser excitation and the following filter sets: 533/30BP for FITC fluorescence and 585/40BP for R-PE fluorescence. Gates for cells stained with Glycophorin A-FITC and CD71-R-PE define positivity of cells for these surface markers i.e. lower left quadrant: $CD71^-$ Glycophorin A^-; upper left quadrant: $CD71^+$ Glycophorin A^-; upper right quadrant: $CD71^+$ Glycophorin A^+; and lower right quadrant: $CD71^-$ Glycophorin A^+.