Dynamic human erythropoiesis in a three-dimensional perfusion bone marrow biomimicry

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\textbf{ABSTRACT}

Traditional culture systems for human erythropoiesis lack microenvironmental niches, spatial marrow gradients and dense cellularity rendering them incapable of effectively translating marrow physiology \textit{ex vivo}. Herein, a bio-inspired three-dimensional (3D) perfusion bioreactor was engineered and inoculated with unselected single donor umbilical cord blood mononuclear cells (CBMNCs). Functional stromal and hematopoietic environments supporting long-term erythropoiesis were generated using defined medium supplemented only with stem cell factor (SCF) and erythropoietin (EPO) at near physiological concentrations. Quantitative 3D image analyses spatiotemporally mapped 21 multi-lineal cell distributions and interactions within multiple microenvironments that secreted extracellular matrix proteins and at least 16 endogenous hematopoietic and stromal growth factors. Tissue-like culture densities (\geq \text{2.10}^{10} \text{cells/mL}) and abnormally high cyto- sin of EPO, towards marrow sinuses for reticulocyte egress

1. Introduction

Clinical translation of \textit{ex vivo} human erythropoietic models has been limited. Until now, current \textit{in vitro} approaches fail to conserve \textit{in vivo} microenvironments present in human bone marrow (hBM) such as: multiple hematopoietic lineages with dynamic maturation, supportive stroma producing extracellular matrix proteins and cytokines, and gradients of oxygen, paracrine and endocrine factors \cite{1}. Current human erythroid massive amplification (HEMA) cultures expand \text{10}^{4–6} reticulocytes per hematopoietic stem/progenitor cell (HSPC) suitable for human transfection \cite{2,3} and have been adapted to produce reticulocytes from immortalised erythroblast cell lines \cite{4}. However, non-physiological low cell densities (\leq \text{10}^{9}/\text{mL}) and abnormally high cytokine concentrations inflate HEMA reticulocyte production costs 1- to 2-orders of magnitude higher compared to donor-based blood trans- fusion \cite{5}.

In animal models, erythropoiesis is a spatially distributed process in heterogeneous marrow microenvironments. These models demonstrate microenvironments with HSPCs and erythroid cells distributed within unique oxygen gradients \cite{6–9} which interact with neighbouring niche cells, including macrophages, mesenchymal, and osteoelastic cells, that secrete supportive growth factors and extracellular matrix (ECM) pro- teins \cite{10–12}. Erythroid progenitors migrate as they mature, in the presence of EPO, towards marrow sinuses for reticulocyte egress \cite{13–16}. Alas, animal models are limited in accurately representing human erythropoiesis at phenotypic and genotypic levels \cite{17}.

Static 3D culture systems, using porous scaffold materials, operate at increased cell densities (\geq \text{10}^{7} \text{cells/mL}) \cite{18,19}, which may facilitate red blood cell (RBC) maturation \cite{20}. Stroma and multi-lineal 3D co-cultures \cite{21–26} enable a growth environment similar to that found in marrow by augmenting stroma-hematopoietic cell interactions. Perfused 3D systems can further increase culture density (\geq \text{10}^{8} \text{cells/mL}) by supplying medium through hollow channels \cite{27,28}, imparting biochemical diffusion gradients which may structure cell distribution more akin to that found \textit{in vivo}. Furthermore, certain 3D systems can support cytokine-free culture of human cord blood mononuclear cells \cite{29}. Limitations to be overcome include use of serum and/ or abnor- mally high concentrations of exogenous cytokines and xeno- or allo- geneic co-cultures.

Herein, we have developed an \textit{ex vivo} 3D perfusion model of erythropoiesis, inoculated with unselected single-donor CBMNCs and perfused with serum-free medium supplemented with near-physiologic...
concentrations of SCF and EPO only. With minimal handling, the 3D hollow fibre reactor (3DHFR) achieved human bone marrow-like cell densities ($10^8−9$ MNCs/mL) [30], maintained immature hematopoietic populations for the duration of culture (28 days), expanded stromal and osteogenic cell types, generated ECM proteins, secreted hematopoietic and stromal factors, and produced enucleated RBCs that were continuously harvested through hollow fibres. Quantitative image analysis [31] revealed multilinear dynamics with spatio-temporal distribution of cells and secreted proteins at unique distances from hollow fibre medium perfusion. The 3DHFR represents a physiologically-relevant system of erythropoiesis, which addresses many limitations of current in vitro and in vivo models.

2. Materials and methods

2.1. Study approval

CB units were freshly acquired after collection from term deliveries and processed in accordance with the Declaration of Helsinki, having received the required ethics and local research approvals (London-Harrow NRES Committee, UK; reference 05/Q0405/20).

2.2. Fabrication of hollow fibres

Ceramic hollow fibres were fabricated by the dissolusion, milling, and degassing of 58.6 wt% 1 μm aluminium oxide powder (VWR, Lutterworth, UK) with 1.3 wt% Arlacel P135 (Sigma-Aldrich, Dorset, UK) in N-methylpyrrolidione (Sigma-Aldrich) solution, followed by extrusion through a tube-in-orifice spinneret prior to sintering with a temperature profile rising to 1350°C. Hollow fibres were air-dried and hollow fibre porosity was assessed by mercury intrusion porosimetry.

2.3. Hollow fibre permeability analysis

The analysis of hollow fibre permeability to nutrients/metabolites and proteins, and the hollow fibre egress of enucleated and nucleated cells through hollow fibre pores was performed as previously described [32]. Briefly, four hollow fibres were adhered within a polyfluoroalkoxy fine thread flake tee (Swagelok, London, UK) applying a quick drying two-component resin (Araldite, Basel, Switzerland) on both the inlet or outlet of the reactor. A suspension of $3×10^7$ CB cells/mL comprised of 30% enucleated cells and 70% MNCs in cell culture medium was seeded into the polyfluoroalkoxy tee around hollow fibres. Culture medium was perfused at 460 mL/day through the hollow fibres. Perfused medium and medium remaining in the extraluminal space were analysed at multiple time points for 24 h for glucose and lactate using the corresponding assay kits as per the manufacturer’s instructions (Universal Biologicals, Cambridge, UK), and for bicarbonate acid assay to measure bovine serum albumin (Thermo Fisher Scientific, Loughborough, UK). Viable cells were counted using the trypan blue and methylene blue dye-exclusion methods (StemCell Technologies, Grenoble France).

2.4. Fabrication of the 3DHFR

The polyfluoroalkoxy tee affixed with four hollow fibres was filled with a 5 mL solution of 5% (w/v) polyurethane (Noveon, Brussels, Belgium) dissolved in 1,4-dioxane at 60°C (Sigma-Aldrich) and then transferred to a −80°C freezer for 2 h. The solidified 1,4-dioxane was selectively sublimed through thermally induced phase separation by applying a vacuum pressure of 0.01 mbar at −15°C to leave a porous polyurethane scaffold. The porosity of the resultant 3DHFR was assessed by mercury intrusion porosimetry, scanning electron microscopy (SEM), and micro-scale computerised tomography. The porosity of scaffolding was analysed by mercury intrusion porosimetry separately from the reactor hollow fibres by pouring 60 mL of polyurethane-dioxane solution into a glass petri dish prior to applying thermally induced phase separation. The 3DHFR was further prepared by thoroughly immersing and mixing scaffold and fibre components with a series of solutions: first PBS, 70% (v/v) ethanol, 62.5 μg/mL bovine collagen type 1 in PBS at pH 7.0 (Sigma-Aldrich), finishing with PBS. The 3DHFR was sterilized through a series of washes with 70% (v/v) ethanol and PBS followed by UV sterilisation. Subsequently, the 3DHFR was conditioned for 3 days with cell culture medium and assembled into a perfusion platform consisting of silicon tubing, oxygen permeable tubing, polycarbonate adapters (all from Cole Parmer, Hanwell, UK), 100 mL and 500 mL bottle reservoirs (VWR), stopcock adapters for sampling (Smith’s Medical Watford, UK), and peristaltic pump tubing and pumps (Instech Technologies, Plymouth Meeting, USA).

2.5. Cell culture

CB MNCs were isolated using standard Ficoll-Paque separation (Sigma-Aldrich). A suspension of $10^6$ or $2.5×10^7$ CB MNCs in StemSpan SFEM (StemCell Technologies) supplemented with 1% (v/v) penicillin-streptomycin (ATCC) and 50 ng/mL SCF (R&D Systems) was seeded into the 3DHFRs and incubated for 1 h without perfusion, then perfused with 30 mL of recycled StemSpan SFEM with 1% penicillin-streptomycin and 50 ng/mL SCF at a rate of 460 mL/day. On D1, an additional 30 mL of StemSpan SFEM with 1% penicillin-streptomycin and 50 ng/mL SCF was added. From D2, the 60 mL recycled reservoir was gradually replenished with fresh StemSpan SFEM supplemented with 1% penicillin-streptomycin and 0.5 IU/mL EPO (R&D Systems) at incremental perfusion rates: 20 mL/day from D2-D7, 21.5 mL/day from D7-D14, and 36 mL/day from D14-D28, resulting in relatively constant concentrations of 0.05 ng/mL SCF and 0.25 IU/mL EPO perfused through 3DHFRs towards the latter half of the culture (D16 to D28). Perfused medium was sampled every 2 days for extracellular metabolic and protein analysis, and at D14 and D28 for cell analysis. Hollow fibres were not perfused individually; the entire bioreactor inlet enaspering 4 hollow fibres was perfused from one channel. Cultures were performed within a humidified incubator at 37°C, 20% O2, and 5% CO2. Wall shear rate inside the perfused hollow fibre adluminal surfaces could be estimated by simplifying the Navier-Stokes equation for a Newtonian fluid flowing inside a pipe as $\gamma = 4Q/r^2$, where Q represents volumetric flow rate and r represents hollow fibre inner radius. Consequently, hollow fibre wall shear stress is approximately 100 s⁻¹, similar to murine marrow vasculature [33].

2.6. Extracellular nutrient, metabolite and growth factor analysis

During culture, medium collected directly at the 3DHFR inlet and outlet was evaluated for nutrients, metabolites, pH, and dissolved gases using a Bioprofile 400 (Nova Biomedical, Runcon, UK). The outlet samples were additionally analysed for a panel of 27 growth factors by bead capture assay and enzyme-linked immunosorbent assay according to manufacturer’s instructions (Biologend, R&D Systems), as described in supplemental Table 1. Growth factors reported were detected in N ≥ 3 replicates at concentrations 30% above detection limit.

2.7. Cell counts, morphology, proliferation, and viability

Filtered cells were collected from perfused medium during 3DHFR culture at days 14 and 28. Total cells, viable cells, and nucleated cells were counted using trypan blue and methylene blue dye-stains (StemCell Technologies) using a standard haemocytometer. Cell morphology was assessed by cytospin and by smear of aspirated and filtered cell types, or by 3DHFR section imprint onto poly-l-lysine slides (Thermo Fisher Scientific), stained with May Grünwald, Giemsa, and Modified Wright Giemsa stains (Sigma-Aldrich), mounted with DPX (Sigma-Aldrich) and imaged on a BX-51 Olympus microscope utilizing 20x (dry, 0.5 numerical aperture, UPLFL), 50x (oil-immersion, 0.9 numerical aperture,
The 3DHFR was cryopreserved in liquid nitrogen. Thin axial cross sections were manually cut from random positions (“3DHFR sections”; 9 mm diameter, 0.5 mm thick) of the frozen 3DHFR. Cells were aspirated from 3DHFR sections after 0, 14, and 28 days of 3DHFR culture.

The 3DHFR sections cut from the inlet, centre, and outlet of the 3DHFR were immediately transferred into 3D Cell Titre Glo assay solution (Promega, Southampton, UK), mixed on an orbital shaker for 1 h, and analysed on a GloMax Promega spectrophotometer (Promega) for adenosine triphosphate content. The 3DHFR sections cut from the centre 50% of the frozen 3DHFR were immediately incubated in a viability staining solution comprised of 4 μM ethidium homodimer and 2 μM calcein acetoxyethyl in culture medium for 1 h. 3DHFR sections were washes thrice with PBS and imaged on a Leica SP5 upright confocal microscope with Leica LAS AF software (Leica, Milton Keynes, UK).

2.8. Flow cytometry

Cells (2–10^5) were stained with Calcein Violet Acetoxyethyl (Thermo Fisher Scientific) for 45 min at room temperature, washed with cell staining buffer (PBS with 1% bovine serum albumin and 0.01% sodium azide, all components from Sigma-Aldrich), then stained with mouse anti-human antibodies (all from Becton Dickinson, Oxford, UK) in cell staining buffer with 10% fetal bovine serum (Life Technologies, Paisley, UK) for 1 h at 4 °C. The following mouse anti-human conjugated monoclonal antibodies were used: CD45-FITC (clone HI30), CD235a-PE (clone GA-R2), CD71-PECy5 (clone M-A712), CD36-APC (clone CB38), IgG1-FITC (clone MOPC-21), IgG2b-PE (clone 27–35), IgG2a-PECy5 (clone G155-178), and IgM-APC (clone GA-R2). Cells were analysed in 4 combinations: positive sample, fluorescence-minus-one (lacking CD235a-PE), isotype control, and Calcein Violet Acetoxyethyl-only control. Shaded regions in (F–H) represent standard error about the mean. (I) Simulated cross-sectional organizational maps of MNC distribution along the axial 3DHFR length at D28. Independent replicates: N = 4 (D0), N = 3 (D14) and N = 4 (D28).

2.9. Scanning electron microscopy

The 3DHFR sections (discussed above) were immediately fixed with a cold 3% (wt/v) glutaraldehyde solution (Sigma-Aldrich) in Sorenson’s buffer for at least 12 h. They were subsequently post-fixed for 1 h,
dehydrated in a 4-step ethanol gradient, dehydrated in a 3-step hexamethylsilazane (Sigma-Aldrich) gradient, and then air-dried overnight. Post-fixative solution consisted of 1% (w/v) OsO₄ (Sigma-Aldrich) in Sorenson’s buffer. Each step was separated by multiple 15 min washing steps in appropriate buffer (Sorenson’s, pure ethanol, or pure hexamethylsilazane). After solvent evaporation, sections were adhered to SEM stubs with carbon tape (Elektron Technology, Stansted, UK), sputter-coated with gold (20 mA, 30 s), prior to imaging on a JSM-6010LA SEM with InTouchScope 1.05 software (JOEL, Watchmead, UK).

### 2.10. Fixed confocal microscopy

The 3DHF cultures were perfused for 1 h with 0.65 mg/mL Pimondiazole-HCl in culture medium (Hypoxyprobe, Inc., Burlington, USA) prior to cryopreservation and sectioning. 3DHF sections cut from the centre 50% of the frozen 3DHF (as discussed above) were immediately transferred into cold 4% paraformaldehyde (Sigma-Aldrich) in PBS overnight.

Sections were subsequently permeabilized for 2 h, blocked for 4 h then stained with primary antibodies overnight. Thereafter, sections were stained with secondary antibodies for 8 h, counterstained overnight, and imaged as previously described [31]. Wash buffer contained 1% (wt/v) BSA, 0.5% (v/v) Tween-20, 0.01% (wt/v) NaNO₃ in PBS and was supplemented with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for permeabilization or with 10% (v/v) donkey serum (AbCam, Cambridge, UK) for blocking. Supplemental Table 2 describes concentrations and product names for all primary and secondary antibody stains applied in wash buffer. Counterstains included 1:200 DAPI (Life Technologies), 1:1000 cell membrane (CellMask, Life Technologies), and/or 1:200 rhodamine B isothiocyanate (Sigma-Aldrich) in PBS. Storage buffer contained 0.01% (wt/v) NaNO₃ in PBS. Each step was separated by multiple 15 min washing steps, and was performed at 4 °C from the blocking step onwards.

Confocal microscopy images of 3DHF sections in PBS at room temperature utilized 10x (dry, 0.4 numerical aperture, EC Plan-Neofluar) or 20x objectives (dry, 0.7 numerical aperture, Plan-Apochromat) (Carl Zeiss, Jena, Germany) and a Leica SP5 inverted confocal microscope and Leica LAS software (Leica, Milton Keynes, UK). 3DHF sections were imaged in one sequence with 405 nm UV-diode and 543 nm helium-neon lasers where emission light was collected at 420–520 nm (DAPI) and 560–650 nm (Alexa Fluor 555, ethidium homodimer-1, or rhodamine B isothiocyanate), and in a second sequence by 458 nm and 488 nm argon and 633 nm helium-neon lasers where reflected light was collected at 450–470 nm (3DHF structure) and emission light was collected at 500–550 nm (Alexa Fluor 488 or calcein acetoxyethyl) and 655–700 nm (Alexa Fluor 647 or CellMask). Primary and secondary antibody and stain specifications can be found in supplemental Table 1.

Images were not manipulated for computational analysis of cell identification, location, or distribution. Confocal microscopy images were captured at 512 × 512 XY resolution utilizing a 10x (3.03 μm/pixel) and 20x (1.51 μm/pixel) lens at multiple z-distances (5 μm/pixel) rendered in 3D using the “3D Stack” plugin of ImageJ 1.49i software (U.S. National Institute of Health, Bethesda, USA). Resultant 3D images were manipulated within all figures by adjusting only brightness and contrast equally across the entire image and was applied identically to both sample and negative (isotype) controls which are presented alongside sample images for all figures. Detailed image processing methods have been previously described [31].

### 2.11. Computational analysis of fixed confocal microscopy results

Fluorescent confocal images of 3DHF axial cross-sections (discussed above) were computationally analysed as previously described [31]. Briefly, once the full image was prepared, the centre-point of each
(caption on next page)
individual fluorescence stain was identified. Each MNC was characterised by the identification of DAPI and was considered to express other stains if they existed within a threshold distance of the nucleus of interest. As hematopoietic, erythroid, mesenchymal, and osteogenic cells vary in size, different threshold “stain-to-DAPI” distances were chosen based on maximum cell sizes imaged during confocal and SEM within this study which are in agreement with prior studies [34] and detailed for each stain in supplemental Table 3. Enucleate cells were considered as CD235a or CD71 stains further than a 40μm distance from any DAPI stain and labelled as “eCD235a” or “eCD71”, respectively.

Once cells were located and analysed for stain expression, two distribution metrics were assessed: (1) the 2D distribution of cells from the abluminal wall of hollow fibres and, (2) the 3D distribution of cells away from each other. These metrics were calculated as cell density (cells/mL) at consecutive distance intervals, fitted by continuous kernel density estimations for the mean and standard error of the measurements.

The confocal microscope was unable to image through the polyurethane scaffold. Therefore, an aminogroup stain, rhodamine B isothiocyanate, was used to visualise the polyurethane scaffold and measure the distance of cell marker stains from the scaffold surface. Higher-magnification images (20x magnification, 1024 × 1024 resolution) enabled manual measurement of 218 cell nuclei-to-scale distances, where virtually all (96%) cell nuclei were within 13μm of the scaffold surface (supplemental Fig. 1). Nonetheless, confocal microscopy computational analysis demonstrated an accurate representation of in situ cell content and distribution, as found in vivo [35].

2.12. Computational simulation of 3DHFR organizational maps

To illustrate the utility of quantitative confocal computational analysis of cell distributions, a mathematical simulation was constructed which describes the a) average quantity of cells at different abluminal distances and, b) average frequency that these different cells associate with one another across independent biological replicates. This model can be described by a single equation:

$$\lambda(x, C_j, C_k) = N(x, C_j) \times (1 - \mu(x, C_j)) + \mu(x, C_k) \sum_{d(C_j, C_k) \leq 100\mu m} \frac{P(x, C_j, C_k) N(x, C_j)}{\sum P(x, C_j, C_k) N(x, C_k)}$$

Where the probability, $\lambda$, of a cell type $C_j$ existing within distance $x$ of an hollow fibre abluminal surface and co-localising within 100μm of cell $C_k$ is proportional to the quantity of cell type $C_j$ imaged at abluminal distance $x$ as $N(x, C_j)$. The likelihood of cell type $C_j$ associating within 100μm of cell type $C_k$ is dependent on the frequency with which cell type $C_j$ was imaged in association with MNCs at abluminal distance $x$ as $\mu(x, C_j)$ and, when associating with a MNC, the probability that $C_j$ will uniquely associate within a 100μm distance of $C_k$ versus all other $i$ cell types imaged.

To simulate this mathematical formula, the full imaged abluminal distance (1900μm) was discretised into 100μm bins, and the number of cells $C_j$ to generate within bin $x$ is equal to the average cell density calculated as $N(x, C_j)$ for a 1900 × 1000 × 13μm “confocal image” volume. Each $C_j$ generated is placed within 100μm of other MNCs at interval $x$ with frequency $\mu(x, C_j)$, or specifically placed within 100μm of cell type $C_j$ with probability $P(x, C_j, C_k)$. Simulations were processed in R-Project (R Foundation for Statistical Computing, Vienna, Austria).

2.13. Statistical analysis

Error bars in bar and line graphs represent the standard deviation about the mean of 3–4 independent biological replicates, except for spatial cell distribution metrics, which are presented with shaded regions representing standard error about the mean of 3–4 independent biological replicates. Standard error better emphasizes differences across independent biological replicates without exaggerating the error contributed by outlier data points [36]. Specifically, standard error is appropriate within imaging metrics as outlying cell positions frequently exist. Analysis of cytokine profiles was performed by one-way analysis of variance using GraphPad Prism 7 (La Jolla, USA) and all other p-values were generated as two-tailed comparisons between either paired replicates or replicates of equal variance (with significance given by p-values: *p ≤ 0.05; **p ≤ 0.01) as reported within figure legends.

3. Results

3.1. Establishment of cellular hypoxic and proliferative microenvironments

The 3DHFR system, which consisted of a collagen-coated polyurethane scaffold (87.4% porous, 180μm average pore diameter) embedded with four ceramic hollow fibres (61.7% porous, 0.2μm average pore diameter; Fig. 1A–C and supplemental Fig. 2A–D), was inoculated with 2.10⁷ CBMNCs/mL and cultured for 4 weeks. The hollow fibres allowed unrestricted transport of metabolites and proteins as well as enhanced egress of enucleated cells through the pores, similar to the transluminal migration observed in murine marrow sinusoids (supplemental Fig. 2E–G) [8]. The system (Fig. 1D) was perfused with recycled serum-free medium at a velocity of 1.1 cm/s (460 mL/day), supplemented only with 50 ng/mL of SCF (added in the first two days of culture) and 0.5 IU/mL EPO (added from day 2 onwards; supplemental Fig. 2H–J). Cell viability remained above 80% throughout culture; inlet and outlet nutrient/metabolite concentrations were similar (< 5% difference), suggestive of equilibrium environmental conditions (pseudo steady-state) achieved within the 3DHFR (Fig. 1D).

Perfusion appeared to structure the distribution of cellular microenvironments during culture. Quantitative confocal microscopy analysis of 3DHFR cross-sections (radial distribution; Fig. 1E) demonstrated that inoculated CBMNCs were near uniformly distributed throughout the 3DHFR establishing dense “clusters” of cells with inter- and/or nuclear distances of 100μm (Fig. 1F and G). By D14, more cells (2-fold increase, supplemental Fig. 2K) were observed within 500μm of the hollow fibre abluminal surfaces, wherein 100μm clusters became more prominent (1.7-fold increase). These clusters, which peaked at D14, exhibited a 5-fold higher cell density compared with that of the bulk 3DHFR (supplemental Fig. 2L). Axial cell distribution showed that by
D28 cells accumulated at the 3DHFR outlet (Fig. 1H and I and supplemental Fig. 2M). Together, these data formed a bioreactor-wide map of dynamic microenvironment evolution.

Cellular hypoxia was dependent on distance from hollow fibres and local cell density. At D0, HIF-1α expressing cells distributed evenly throughout the 3DHFR (<6% average variance; Fig. 2), possibly...
attributed to the shock experienced by the cells following processing and inoculation. In contrast, at D28 HIF-1α was predominately observed in regions distal to the hollow fibres (Fig. 2B and C). Throughout the culture, increased HIF-1α expression was observed within the 100 μm clusters (supplemental Fig. 2N), which was further supported by the enhanced pimonidiazole (Pimo) expression observed at D28 within the clusters (Fig. 2C and supplemental Fig. 2O). These results suggest the creation of oxygen gradients within the 3DHFR as well as within the clustered cellular microenvironments. Interestingly, cellular proliferation (Ki-67 expression) was pronounced at D28 and was
localised near the hollow fibres (Fig. 2A,C), whereas it was sparsely detected at other days of culture, indicating the remodelling of the cellular microenvironments correlated to proliferation and differentiation, as discussed below. Specifically, the first 14 days represent culture adaptation followed by formation of the functional biomimicry.

3.2. Establishment of interactive stromal-hematopoietic microenvironments

Large, adherent, cell morphology and stromal phenotypes were observed in the 3DHFR throughout the culture period (Fig. 3A and B). These stromal cells expanded from less than 10% at D0 to greater than 35% at D28 and included mesenchymal cells (Stro-1; 4% at D0 to 14% at D28), pre-osteoblasts (OSSx; 4% at D0 to 27% at D28), and mature osteoblasts (OPN; 0% at D0 to 7% at D28), as shown in Fig. 3C and supplemental Fig. 3A. Distribution of Stro-1+ and OSSx+ cells was relatively uniform throughout, whereas differentiated OPN+ osteoblasts localised distal (1 mm) to hollow fibres (Fig. 3C and D and supplemental Fig. 3B). Stro-1+ and OSSx+ cells were the most prominent cell type found within cell clusters at D14 and D28, most notably found together in high cell density clusters (Fig. 3D and supplemental Fig. 3C-E). In addition, cells exhibiting spheroid and elongated morphologies expressing CD34 and VCAM-1 were observed at D28, suggestive of endothelial progenitors (Fig. 3E). CD31 and nestin expression was absent throughout the culture. Within the hematopoietic cell compartment, differences in culture dynamics could be distinguished between the “adaptive” (D0-D14) and “functional” (D15-D28) periods. CD45+ cells were evenly distributed throughout the 3DHFR with cell proportions fluctuating from 22% at D0 to 8% at D14 and recovering to 23% by D28 (Fig. 3F-H). Furthermore, C-KIT+, CD34+, and VCAM-1+ cells were observed throughout the culture period (Fig. 3E-H).

The established stroma produced ECM proteins, including collagen type 1, fibronectin, and laminin-2; stroma-derived factor 1 (SDF-1; CXCL12) expression was observed on large, stretched cell membranes of nucleated cells (Fig. 4A and B). Whereas human collagen type-I expression decreased with culture time, SDF-1 expression increased predominately distal to the hollow fibres (≥400 μm; Fig. 4C and D) and was co-localised within clusters of CD45+ and OSSx+ cells at D28 (Fig. 4E and supplemental Fig. 3F and G). Endogenously produced growth factors were detected in the perfusate (supplemental Table 3) including: a) factors that primarily act on stroma such as platelet-derived growth factors-alpha and -beta (PDGF-αα and PDGF-ββ), epidermal growth factor (EGF), fibroblast growth factor-beta (FGF-ββ), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and angiopoietin 2 (Ang-2); and b) factors that primarily act on hematopoietic and immune cells such as granulocyte-macrophage colony stimulating factor (GM-CSF), M-CSF, G-CSF, IL-6, IL-21, transforming growth factor-alpha (TGF-αα), interferon-gamma (IFN-γ), and tumour necrosis factor-alpha (TNF-αα), as shown in Fig. 4F-J.

Despite cord-to-cord variability, patterns related to culture phase were identified. The main immune-associated cytokines IL-21, IFN-γ and G-CSF (Fig. 4F,G,J) were present throughout the culture time, with a clear decline in expression from the “adaptive” to the “functional” culture periods for IL-21 (D4 vs D16-24, p < 0.05) and IFN-γ (D2 vs D16-24, p < 0.05). IL-6 was highly expressed early in the “adaptive” culture period and waned as culture time progressed (D4 vs D8-12, p < 0.05), with inconsistent expression in some of the cultures during the “functional” period after D15 (Fig. 4F). In addition to IL-6, there was consistent expression during the first 4 days of culture of IL-10, IL-22, GM-CSF, IL-2, IL-13, Ang2, FGF-β, HGF, VEGF, PDGF-αα, PDGF-ββ, EGF and TNF-α (Fig. 4F-J). Interestingly, the mostly stroma-acting factors GM-CSF, M-CSF, Ang-2, FGF-β, HGF, VEGF, PDGF-αα, PDGF-ββ and EGF were not detected at D16 of the “functional” phase, which suggested increased consumption, representing a critical time-point in the culture; most of these growth factors recovered thereafter. Shed EPO-receptor (EPO-R) was never detected.

Together, these data indicated that 3DHFR microenvironmental remodelling and kinetics occurred in roughly two phases: (1) the “adaptive” phase (D0-D14) was characterised by an early decline of CD45+ hematopoietic cells with increasing stromal elements and initially high expression of both stroma- and hematopoietic-acting cytokines, particularly notable for IL-6 and, (2) the “functional” (D15-D28) phase where stroma-acting factors were consumed early but recovered later and resulted in SDF-1 expressing stromal cells and hypoxic regions formed distal to the hollow fibres with fibre-proximal increases in highly proliferating hematopoietic cells. We next sought to identify the erythropoietic microenvironments and kinetics supported by the 3DHFR, in particular in the phase of the “functional” biomimicry.

3.3. Erythropoiesis and erythrocyte harvest

The system supported erythroid differentiation to maturity in response to near physiological EPO supplementation only. A 3D growth environment sustained uniform high cell density facilitating cell to cell interactions; erythroid precursors were concentrated distal to the hollow fibres whereas mature erythrocytes were observed throughout (Fig. 5A). Specifically, nucleated erythroid cells expressing EPO-R, CD71, or CD235a, and enucleated erythrocytes expressing CD71 or CD235a (referred to as eCD71 or eCD235a, respectively) increased from D0 to D28 (Fig. 5B and C and supplemental Fig. 4A). By the end of the “adaptive” period at D14, erythropoiesis became established and appeared to be spatially restricted in an area distal to the hollow fibres (≥250 μm; Fig. 5B and C). Interestingly, in the “functional” period at D28, EPO-R+ erythrocytes were found primarily clustered with OSSx+ cells and enucleated CD235a+, when the formation of CD71+ clusters was also observed (supplemental Fig. 4B-D), suggesting formation of specialised erythroid niches. Specifically, erythroblasts and enucleated cells were observed attached to central macrophages (Fig. 5D and E), indicative of erythroid islands. Enucleated erythrocytes egressed through the hollow fibres and were collected within the perfusate (Fig. 5F and G); egressed cells were enriched for mature enucleated red blood cell phenotypes compared with that of the inoculum (from 3% to 23% CD235a+ cells) and reduced non-erythroid hematopoietic cells (from 55% to 7% CD45+ cells; Fig. 5H and supplemental Fig. 4E and F). Viable egressed cells negative for CD45, CD36, CD71, and CD235a may comprise CD45-low hematopoietic cells (supplemental Fig. 4E and F) or CP platelets able to transport through hollow fibre pores [32].
3.4. High cell density alters the microenvironment and improves erythropoiesis

In order to determine whether cell density impacted upon erythropoietic activity, we compared two different inoculation densities from the same cord: 5 × 10^8 CBMNCs/mL (2.5 × 10^9 CBMNCs per 3DHFR) and 2 × 10^7 CBMNCs/mL to simulate erythropoiesis in conditions that resemble marrow normocellularity (40–70% cellularity; high density, HD) [30] and hypocellularity (< 10% cellularity; low density, LD) [37]. At D28, the HD 3DHFR resulted in 125-fold higher extraction of cells.
Fig. 6. Increased cell density enhances RBC production. Confocal images of calcein acetoxymethyl (Live) and ethidium homodimer-1 (Dead) cell distribution at D28 of (A) low density (LD) and (B) high density (HD) 3DHFR cultures. Scale bar = 100 μm, 10x objective. Confocal images of DAPI and cell membrane (Memb; CellMask red) at D28 of (C) LD and (D) HD cultures. Scale bar = 100 μm, 10x objective. Confocal images of DAPI, CD235a, C-KIT, EPO-R, CD45, and OSx at D28 of (E) LD and (F) HD cultures. Inset represents isotype control. Scale bar = 50 μm, 10x objective. (G) Quantitative image analysis representing cellular distribution (abluminal to hollow fibres) of DAPI at D28 for LD and HD cultures. Shaded areas represent standard error about the mean from n = 9 imaged sections of one independent replicate. Organizational maps illustrating distribution (abluminal to hollow fibres) and intercellular associations for simulated 3DHFR cross-section confocal images of DAPI at D28 for (H) LD and (I) HD cultures. (J) Quantitative image analysis representing cellular distribution (abluminal to hollow fibres) of CD235a, CD45, OSx, and SDF-1 at D28 for LD and HD cultures. Organizational maps illustrating distribution (abluminal to hollow fibres) and intercellular associations for simulated 3DHFR cross-section confocal images of CD235a, CD45, OSx, and SDF-1 at D28 for (K) LD and (L) HD conditions. Endogenous growth factors detected in HD cultures at concentrations 30% above detection limit including (M) IL-10, IL-21, IL-22, and IL-2, (N) G-CSF, GM-CSF, and M-CSF, (O) IL-13, IL-17A, IL-2, TGF-α, and TNF-α, (P) Ang-2, FGF-β, HGF, and VEGF, (Q) IFN-γ, PDGF-αa, and PDGF-βb. (R) CD71 and CD235a flow cytometry plots of filtered cells at D28 gated on calcein acetoxymethyl for LD and HD cultures with isotypes presented in supplemental Fig. 5F. N = 1.

(1.1·10^10 total cells) and 25-fold higher filtration of cells (3.4·10^7 total cells) with high viability (98%) when compared to the LD 3DHFR (Fig. 6A–F and supplemental Fig. 5A). The HD 3DHFR peaked in MNC density at 550 μm distally from hollow fibres, 66% of which were CD45^+ cells, whereas the LD 3DHFR density was highest along hollow fibre surfaces (Fig. 6G–I). Despite differences in total culture density and MNC distribution relative to hollow fibres, local MNC association densities were similar for HD and LD 3DHFRs (supplemental Fig. 5B), which appeared confluent. In situ, the HD 3DHFR had D28 expression levels greater than that of the LD 3DHFR for CD45, SDF-1, fibronectin, and CD235a, similar expression levels of OSx and OPN, but lower expression of CD34, Str-1, collagen type 1, EPO-R, CD71, and enucleated CD235a (Fig. 6E,F,J–L and supplemental Fig. 5C and D). CD235a+E.MNCs were present at a 10-fold higher density within 500 μm of HD culture hollow fibres versus LD hollow fibres (Fig. 6J–L).

Endogenous growth factor profiles were distinctly different in HD cultures. In the “adaptive” phase, SCF consumption was more rapid and increased concentration of M-CSF in the absence of TNF-α and IL-10 was observed in comparison to LD cultures (Fig. 6M–Q and supplemental Fig. 5E). Interestingly, similar to the LD cultures, IL-6 concentration was high early on (until D12) and IL-21 was present throughout. A significantly increased proportion of mature red blood cells (almost 300% higher) expressing CD235a and CD71 egressed from the HD 3DHFR compared to the LD 3DHFR (Fig. 6R and supplemental Fig. 5F). This increased erythroid cell egress corresponded to the reduction of maturing erythroid markers (EPO-R and CD71) expressed in situ (supplemental Fig. 5D) and the increased CD235a+E.MNCs found nearer the hollow fibre in HD compared to LD cultures (Fig. 6J), indicating a migratory predilection of more mature erythroid cells towards the perfused hollow fibre in HD 3DHFR. In addition, similar EPO consumption profiles (supplemental Fig. 5E) suggested that cell density, which modulated endogenous growth factor production, affected the differentiation and kinetics of erythropoiesis, rather than the amount of EPO added to the cultures. In summary, increasing 3DHFR density changed the cellular and inductive microenvironment resulting in more efficient erythropoiesis and egress of mature erythroid cells into the perfusate, as well as greater culture expansion reaching viable densities 1000-fold higher than current liquid suspension systems.

4. Discussion

We have engineered a perfused human ex vivo bone marrow model which formed dynamic autologous stromal-hematopoietic microenvironmental niches. As in normal human bone marrow, the 3DHFR established gradients of cellular hypoxic and proliferative microenvironments as well as endogenously-produced growth factors in dynamic phases over time: (1) the “adaptive” phase (D0–14) which corresponded to the time needed for the cells to adapt and produce the stromal and inductive microenvironments and, (2) the “functional” phase (D15–28) where hematopoietic cells were generated more robustly. In response to only physiologic supplementation of SCF and EPO, we achieved continuous erythropoiesis and erythrocyte harvest through the hollow fibres, especially noted in the functional phase of culture, and enhanced when higher cell densities were used. Due to the unique 3D perfusion features, including the capacity to support high cell densities akin to normal human bone marrow, we propose that this 3DHFR system is ideally-suited for the study of erythropoiesis and as a platform to produce cell products and drug testing for translational use in future.

Normal hematopoiesis relies on spatially-structured cellular and inductive microenvironments which are dynamic over time and responsive to various stimulatory and inhibitory factors. These include oxygen [6,7] as well as endogenous factors associated with trabecular bone (e.g. OPN) [10] and stromal-hematopoietic niches (e.g. SDF-1) [9–11]. Typically, these occur in gradients within the marrow and help to form the maturing spatial niches that eventually lead to egress of functional cells into circulation. Recently, non-hematopoietic marrow components have been mapped for whole mouse femurs using confocal microscopy computational analyses, demonstrating hematopoietic interactions on the basis of marker co-localisation [12,35]. In order to recapitulate the human bone marrow ex vivo, the model system would need to sustainably support both hematopoietic [7,9,10,38] and non-hematopoietic [11,12,39] stromal elements in a dynamic long-term culture, produce endogenous growth factors [12,39], achieve a cell density of 10^8–9 MNC/mL [30], enable cell harvest of differentiated cell types [8], maintain perfusion homeostasis [10], and be able to represent the organ in both normal and abnormal physiological states.

Erythropoiesis is comprised of multiple spatially-structured [14–16] differentiation stages captured in this model, including erythroid commitment and survival by exogenous EPO supply and endogenous stromal factors (EPO-R placement and OSx interactions) [13], maturation within erythroblastic islands (CD71, CD235a, and CD68 interactions) [24], and continuous egress of enucleated erythrocytes (CD235a^+DAPI^-) through sinusoidal pores (represented by egress through the hollow fibres). By D28, erythrocytes were found to associate with macrophages prior to egress and harvest in the perfused medium. Interestingly, erythropoiesis and erythrocyte egress appeared to be influenced by cell density and endogenously-produced factors rather than by the presence of exogenously-supplemented EPO alone, suggestive of the physiology observed in the less cellular marrow of the older individual [40], or in patients with aplastic anaemia [41] or hypocellular myelodysplastic syndrome [42]. In such cases, hypocellular (10^6–8 MNC/mL) [37] marrow erythropoiesis features elevated TNF-α, IFN-γ, and fibroconnect [41–43] with incomplete erythropoiesis (accumulation of progenitors) and decreased erythrocyte egress [37], compared with that of normocellular (10^6–8 MNC/mL) [30] marrow. These phenomena would be unfeasible in current 2D (10^5 MNC/mL) [5] and 3D (10^6–8 MNC/mL) [27] cultures.

Two-dimensional flask cultures, even with stromal co-culture [44] or in perfusion bioreactors [45], have limited usefulness for the study and production of hematopoietic cells in normal and abnormal physiological states. Three-dimensional scaffold systems provide marrow-mimetic structure [22,23,28], increased cell culture densities [27], stromal co-culture including growth factor and ECM protein production [21,22,24], and long-term propagation of normal and abnormal hematopoiesis [19,29]. Recently, 3D scaffolds have been developed to
provide continuous erythroblast egress (10^6 cells in 28 days) [18]. However, current 2D and 3D ex vivo systems typically start with enriched cell types, such as HSPCs [20–23,25–27] and mesenchymal cells [21–23,25,26], are limited to abnormally low cell densities (at best 10-fold [27] and typically 1000-fold [5] less than physiological), and are supplemented with a cytokine cocktail of abnormally high concentration (10–100 fold higher than physiological) [2–4,18,21,22,24,25,28], dexamethasone [22,23], animal serum [20,23–26,29], or co-culture from an allogeneic or xenogeneic source [21,22,24–26,28].

Patient-derived xenograft (PDX) models provide animal marrow surrogates to propagate human blood disease and monitor drug response [46,47]. PDX models have additionally utilized “humanised niche” implants (human mesenchymal cells and/or HSPCs injected into gelatin-based [48] or polycaprolactone-tricalcium phosphate [49] porous scaffolds, biphasic calcium phosphate particles [50,51], or an ECM protein gelatin solution [52–54]) with human ECM protein and growth factor deposition [49,53] and sustainable engraftment over 3 months [48]. Although PDX models have been useful to elucidate stromal-hematopoietic cell interactions [23,53], monitor cancer cell invasion and metastasis [50], and study the effect of hypoxia and metabolism [51,54], they are influenced by the murine host and deviate from human physiology.

The advantage of the 3DHFR is that it establishes dynamic, sustainable and structured ex vivo marrow-mimicking microenvironments, which include: 1) autologous stromal-hematopoietic components with endogenous growth factor production, 2) long-term culture at tissue-like cell densities, 3) 3D erythropoiesis with continuous erythrocyte harvest, 4) serum-free cultures supplemented with only EPO and SCF at near-physiological concentrations, and 5) enhanced mass transport exchange that maintains metabolic homeostasis by providing nutrients and removing metabolites through the perfused hollow fibres. Use of unselected CBMNCS at high cell densities is unique, practical and critical to developing a model whereby both hematopoietic and stromal elements were from a single donor. Specifically, this autologous and functional stromal component was developed without exogenous factors and included Strom-1 mesenchymal cells, OSEa preosteoblasts, OPN* osteoblasts, and elongated CD34* endothelial cells, which are known to augment hematopoiesis in vivo [35,39] and are not represented in other ex vivo culture models [21,22,24–26,28]. The initial 2-week “adaptive” phase of the 3DHFR model was required for the production of stromal and inductive microenvironments, featuring hematopoietic decline, high concentrations of pro-inflammatory IL-6, IFN-γ, and G-CSF typical of pre-engraftment syndrome [55], cytokine release syndrome [56], and hypocellular marrow states [43]. These early inflammatory features subsided by D16 with an abrupt consumption of immune- (IL-21 and G-CSF) [57] and stroma-related factors (M-CSF, GM-CSF, PDGF-αα, FGF-β, Ang-2, HGF) [58] commensurate with features of engraftment [57]. A further 2-week “functional” phase reconstructed hematopoiesis within growth factor-producing stromal “clusters” along a hypoxic gradient from hollow fibres.

The above-mentioned unique features render the 3DHFR suitable and cost-effective for ex vivo erythropoiesis since it significantly reduces the production costs (approximately 45-fold less per generated erythrocyte) when compared with the most proliferative, serum-free 2D HEMA cultures [3,59], consuming 63-fold less medium and 410-fold less EPO per produced erythrocyte. Although other 3D systems that support high cell density exist [18,26], hollow fibre bioreactors overcome mass transport limitations and support larger operational volumes [27]; our 3DHFR operates at 10-fold higher cell density, in defined medium, compared to traditional hollow fibre systems (supplemental Table 4). Specifically, 2D cultures achieve 80–100% CD235a⁺ cells and 80–90% erythroid cell enucleation of the 10^7–10^8 total cells generated in multi-litre cultures [2,3] whereas 3D cultures attain 77% CD235a⁺ cells and 22% enucleation of 1.6·10^5 total cells inside an 8 mL hollow fibre bioreactor [27]. In contrast, the 3DHFR achieved 40% CD235a⁺ cells and 27% enucleation of the 3.4·10^7 egressed cells and 1.1·10^6 cells remaining inside the 5 mL 3DHFR with biconave morphology comparable to circulating RBCs when compared to 2D-produced cells which mostly resemble reticulocytes [2]. Nonetheless, the RBCs generated in the 3DHFR require further characterisation by cell volume [3,60], protein content [61], metabolism [62], oxygen uptake, deformability and in vivo maturation and survival [2]. The 3DHFR platform is disadvantaged by a lengthy fabrication process [63], a high inoculum requirement (2.5·10^5 CBMNCS), and a low erythroid cell differentiation efficiency. Recent immortalised erythroblast cell lines may mitigate cord blood source limitations [4]. Current methods of ex vivo erythropoiesis remain inefficient compared with human marrow (1.4·10^9/day·mL, [64]) and still cannot compete with donated blood unit costs (2.10^12 RBCs/unit, $225/unit, [65]). Improving hollow fibre porosity and the supportive microenvironment in the 3DHFR would enrich RBC harvest and extend culture longevity achieving a self-sustaining, long-term, continuous harvest, cost-effective system.

The 3DHFR platform has been used to model leukemic hematopoiesis as demonstrated by the successful culture of human primary mixed-phenotype acute leukemia, acute myeloid leukemia, and chronic lymphocytic leukemia for 4–8-weeks [66–68]; differences in response to chemotherapy were observed between 2D and 3D cultures highlighting the physiological relevance of the 3D system [67]. We propose that the 3DHFR is uniquely suitable for the study of normal and abnormal hematopoiesis as well as for the design of cell expansion protocols and physiologically-relevant disease modelling and drug testing.

5. Conclusion

Human erythropoiesis is not accurately captured by in vitro systems or animal models, which deviate from human physiology. There is immense need in understanding human hematopoiesis and the role of bone marrow architecture and functionality. A perfused, 3D human biomimicry has been developed, which spatiotemporally recapitulates hematopoietic and stromal function in long-term serum-free culture supplemented only with near physiological EPO and SCF. Tissue-like cellularity was achieved with continuous release of cells enriched for enucleated erythrocytes. We propose that this platform is uniquely suitable for the study of normal and abnormal hematopoietic processes, such as erythropoiesis, as well as the design of cell expansion protocols, physiologically-relevant disease modelling and for drug testing and/or discovery aimed at clinical translation.

Declaration of interest

None.

Data availability

The complete raw/processed data required to reproduce these findings cannot be shared at this time due to technical limitations.

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