Suitability of small diagnostic peripheral-blood samples for cell-therapy studies

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Abstract

Background aims. Primary hematopoietic stem and progenitor cells (HSPCs) are key components of cell-based therapies for blood disorders and are thus the authentic substrate for related research. We propose that ubiquitous small-volume diagnostic samples represent a readily available and as yet untapped resource of primary patient-derived cells for cell- and gene-therapy studies. Methods. In the present study we compare isolation and storage methods for HSPCs from normal and thalassemic small-volume blood samples, considering genotype, density-gradient versus lysis-based cell isolation and cryostorage media with different serum contents. Downstream analyses include viability, recovery, differentiation in semisolid media and performance in liquid cultures and viral transductions. Results. We demonstrate that HSPCs isolated either by ammonium-chloride potassium (ACK)-based lysis or by gradient isolation are suitable for functional analyses in clonogenic assays, high-level HSPC expansion and efficient lentiviral transduction. For cryostorage of cells, gradient isolation is superior to ACK lysis, and cryostorage in freezing media containing 50% fetal bovine serum demonstrated good results across all tested criteria. For assays on freshly isolated cells, ACK lysis performed similar to, and for thalassemic samples better than, gradient isolation, at a fraction of the cost and hands-on time. All isolation and storage methods show considerable variation within sample groups, but this is particularly acute for density gradient isolation of thalassemic samples. Discussion. This study demonstrates the suitability of small-volume blood samples for storage and preclinical studies, opening up the research field of HSPC and gene therapy to any blood diagnostic laboratory with corresponding bioethics approval for experimental use of surplus material.

Key Words: β-thalassemia, colony-forming cell assay, cryopreservation, density-gradient separation, erythrocyte lysis by ACK buffer, hematopoietic stem and progenitor cell, in vitro expansion, lentiviral transduction

Introduction

Utility and availability of hematopoietic stem and progenitor cells

Hematopoietic stem and progenitor cells (HSPCs) are the most extensively studied tissue-specific stem cells, with direct clinical application in the treatment of blood-related genetic diseases, immunodeficiencies and hematologic malignancies using HSPC transplantation and cell-replacement therapy protocols [1]. Common sources of HSPCs include extra-embryonic tissues (e.g., umbilical cord blood) and adult tissues (e.g., bone marrow, unmobilized peripheral blood [PB] and mobilized PB [mPB] after chemical mobilization of HSPCs from the BM) [2]. With PB as the most widely available, cheapest and most accessible of the HSPC sources, several reports have documented the presence of distinct multipotent progenitors in the PB mononuclear cell (PBMC) fraction that have the potential to differentiate into mature functional cell types [3]. Extraction, culture and storage techniques that sustain the multipotentiality of HSPCs in leukocytes (white blood cells, WBCs) from whole blood would thus be a key source of HSPCs for research and therapy.

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development. Toward this goal a recent study reported the production of a large number of functional red blood cells (RBCs) from buffy coat, a byproduct of standard blood donations, for normal and rare anemia blood samples by established in vitro culture systems, demonstrating the preservation of HSPC multi-differentiation potential after cryopreservation of PBMCs [4]. Patients with disorders of the hematopoietic system, however, are typically not blood donors, so that similar surplus material is usually unavailable for corresponding studies.

The rationale of HPSC isolation from small-volume blood samples

The present study investigates the suitability of small-volume pre-transfusion blood samples (1.5–3 mL) from patients with β-thalassemia to create repositories of WBC samples as a source of hematopoietic progenitors, because collection of large-volume blood samples from patients is an exception. Cell repositories from surplus material of small diagnostic samples, with associated hematological and molecular data, could comprehensively cover all patients of a given center and facilitate genotype-phenotype analyses, patient stratification and the development of novel therapies, for example. Moreover, routine sampling of small blood volumes is a standard component of modern disease management for hereditary anemias, allowing pooling of several same-patient samples over time for larger scale experiments.

Investigating isolation and storage methods for HSPCs

The choice of HSPC isolation protocol affects WBC yield and composition, contributing to differences in downstream applications and their clinical performance [5,6]. In practice, the choice of cell isolation and storage protocols is also determined by cost, hands-on time, manageability and thus reproducibility. Toward the use of routine diagnostic blood samples for functional assays on HSPCs and pooling of cryopreserved same-patient cells, we therefore sought to determine the most suitable procedures for whole-blood WBC isolation and cryostorage. We performed a side-by-side comparison of two common cell extraction protocols: (i) a density-gradient technique using commercial Ficoll-Paque PLUS (Ficoll-Paque) or AccuPrep Lymphocytes (Lymphoprep) media and (ii) a whole-blood lysis method using a basic ammonium-chloride potassium (ACK) lysis buffer comparable to commercially available buffers [7]. The isolated WBC fractions were frozen in culture media supplemented with 10% dimethyl sulfoxide [8] and different concentrations of fetal bovine serum (FBS). The major focus of this study was to establish whether the cryopreserved samples maintained key characteristics of fresh samples, including yield, viability and hematopoietic potential, and whether any combination of methods would interfere with functional analyses or transduction by lentiviral vectors. Reports to date include parallel comparisons of commercially available whole-blood lysis buffers [7,9–11] or density-gradient media [5,6,12,13], as well as comparisons between the two cell extraction methods [14–17]. These studies mainly used flow cytometry to characterize the extracted cell populations. To our knowledge, no other study has compared cell extraction methods or cell cryopreservation for small-volume samples or for patients of hemoglobinopathies. This is also the first study to analyze the resulting HSPCs for their clonogenic potential or their suitability for lentiviral transduction.

Methods

Study samples

Small-volume pre-transfusion blood samples (1.5–3 mL) from β-thalassemia patients (n = 90) were obtained from the Thalassaemia Centre, Nicosia, Cyprus, and normal samples (n = 3) were collected from healthy volunteers at the Cyprus Institute of Neurology and Genetics. All thalassemic blood samples were taken as routine diagnostic samples from thalassemia patients just before transfusion and were used for diagnostic procedures, with surplus material used in this study. Same-patient comparisons of methods for thalassemic samples relied on samples collected on different days. Normal samples, by contrast, were collected as multiple small-volume samples on the same day to reduce intra-group variation owing to sample handling. Throughout, sample allocation for each processing protocol was randomized to avoid confounding of sample characteristics and protocol choice. The blood was collected into ethylenediaminetetraacetic acid–anticoagulated tubes (Greiner Bio-One; 455036), stored at room temperature and processed 24–30 h after collection. Most selected samples harbored the HBB mutation IVSI-110(G > A) (HGVS ID HBB:c.93-21G>A), and the genotypes covered were β+IVSI-110/β+IVSI-110 (66 samples), β+IVSI-110/β−IVSI-1 (HBB:c.92 + 1G>A) (14 samples), β+IVSI-110/β+“IVSI-6 (HBB:c.92 + 6T>C) (six samples), β+IVSI-110/β−CD39 (HBB:c.118C>T) (three samples) and β−IVSI-110/β−86 (HBB:c.-136C>A) (one sample).

All experimental protocols for human cell material and the corresponding research programs were approved by the Cyprus National Bioethics Committee (Applications EEBK/EEI/2012/02 “Advancing Gene Therapy Vectors for Thalassaemia” and EEBK/EEI/2013/23 “ThalaMoSS”). In line with this and in addition to routine diagnostic procedures in place between the Thalassaemia Centre and the Cyprus Institute of Neurology and Genetics for the national
thalassaemia prevention program, explicit written informed consent for the research use of the blood samples was received from all participants of this study. Processed cell material was discarded after our analyses, and outcomes of this study were not communicated to and did not affect the treatment of patients; thus, no approval for clinical study was required for our research.

Common parameters

All incubation and centrifugation steps are at room temperature, unless otherwise indicated. All live cell counts are based on microscopic analysis using a hemocytometer and trypan blue exclusion of dead cells.

Isolation of mononuclear cells by density-gradient centrifugation

Peripheral blood was re-suspended in 1.5 volumes phosphate-buffered saline (PBS; without Ca²⁺/Mg²⁺), to reduce RBC clumping, and gently layered onto 1.25 blood volumes of Picoll-Paque PLUS (GE Healthcare Life Sciences) or Lymphoprep (Axis-Shield) media. Preparations were centrifuged using 900g for 35 min and brakeless deceleration. The PBMC layer was carefully removed by pipetting and washed twice in PBS with centrifugation for 5 min at 300g, followed by a third wash with centrifugation at 200g to eliminate platelet contamination. The resulting pellet consisted of the whole-blood mononuclear cell (MNC) fraction.

Isolation of nucleated cells by ACK lysis

PB was mixed with hypotonic ACK solution (0.15 mol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 7.2) at 1:2 volume ratio by gentle inversion, incubated for 10 min and centrifuged at 200g for 10 min. The cell pellet was resuspended in 1 blood volume ACK solution and centrifuged at 200g for 10 min. The resulting pellet consisted of the nucleated cell (NC) fraction of whole-blood.

Cryopreservation and thawing

MNC and NC pellets were resuspended in 1 mL freezing medium, distributed to chilled cryovials on ice and transferred to a cotton-wool-insulated precooled box at −80°C for storage. The freezing medium was freshly made of 10% dimethyl sulfoxide in Iscove’s Modified Dulbecco’s Medium before incubation for 5 min, collection by centrifugation at 200g for 10 min and resuspension in prewarmed culture medium for post-thaw analyses.

Expansion of blood-derived leukocytes

WBCs from fresh or frozen preparations (1 × 10⁶ live cells/mL) were cultured in expansion medium consisting of StemSpan SFEM II (Stemcell Technologies, 09650) supplemented with 1 × CC-100 (Stemcell Technologies, 02690), 2 U/mL erythropoietin (Sandoz, Binocrit 4000 IU/0.4 mL), 10⁻⁶ mol/L dexamethasone (Sigma-Aldrich, D4902) and 1 × penicillin/streptomycin (Corning-Cellogro, 30-002-CI), in line with published procedures but omitting CD34⁺ cell selection [18]. Total and live cell counts were determined at the end of expansion phase.

Transduction of blood-derived leukocytes

Expansion-phase WBCs (2 × 10⁶) were suspended in 0.3 mL expansion medium supplemented with 8 μg/mL polybrene in sterile 1.5-mL microcentrifuge tubes, and mixed with a lentiviral vector (LV) harboring a phosphoglycerate kinase (PGK) promoter to express green fluorescent protein (GFP), with a titre of >1 × 10⁶ TU/mL at a multiplicity of infection (MOI) of 10 and 50, respectively, in line with reagents and procedures described elsewhere [19]. The cell-virus mixture was incubated for 6 h at 37°C with hourly agitation by pipetting, before cells were collected by centrifugation at 200g for 5 min.

Colony-forming cell assay using methylcellulose-based media

Blood-derived MNCs and NCs were counted and the cell volume was adjusted to cell plating concentrations of 1.5 × 10⁵ cells per 35-mm plate (Corning, 430588) in 1.1–1.5 mL methylcellulose complete media (R&D Systems, HSC003) according to manufacturer’s instructions. Sample plates were incubated for 14 days at 37°C in 5% CO₂ humidified atmosphere, followed by colony scoring using an AxioVert 200 inverted microscope (Carl Zeiss) to quantify multipotent and single-lineage hematopoietic progenitors based on their morphological characteristics. Herein, the term “CFC” (colony-forming cells) refers to blood-derived progenitor cells capable to develop into colonies, and “CFU” (colony-forming units) refers to the resulting colonies of any type.

Calculations

WBC counts were determined on an XT-2000i (Sysmex) hematology analyzer before processing. Before freezing and after thawing, total and live cell
counts were determined. Yield raw data are presented as cell counts per mL (of PB) instead of total cell counts to normalize for differences in initial sample volumes, as received from the referring clinicians and in the range of 1.5–3 mL. Viability (the percentage of live cells in the analyzed fraction) was calculated by dividing the number of live cells per milliliter by the number of all cells (alive and dead) per milliliter at the end of blood processing. Recovery (relative yield of viable cells) was calculated by dividing the number of live cells in the isolated cell fraction by the total number of WBCs in unprocessed blood. CFC progenitor frequencies (per mL) were calculated by dividing the average colony number by the blood-volume equivalent of the number of live cells that were initially plated, that is, by (number of cells plated/total number of cells) × milliliter of original blood volume.

Statistical analysis

Statistical significance was tested by unpaired t-test and analysis of variance (one-way for Figures 2–4 and supplementary Figures S1–S3, and two-way for Figures 5 and supplementary Figure S4) using Prism 5.0 (GraphPad Software Inc.) and an alpha level of 0.05. All data are given as median to reduce the impact of outliers. Where appropriate, box-and-whisker plots are used to illustrate data, showing median (as a line across boxes), mean (as a “+” in the middle of the boxes), 25%–75% interquartile range (IQR, as central boxes), and maximum and minimum values (as extreme horizontal lines either end of each box). For Figures 2–5 and supplementary Figures S1–S4, statistically different comparisons (P < 0.05) are indicated by horizontal brackets.

Results

Experimental setup

The effects of two density-gradient media (Lymphoprep and Ficoll-Paque) and one RBC lysing solution (ACK) on PB processing in combination with freezing and/or lentiviral transduction was analyzed as depicted in Figure 1. The effect of cryostorage media on WBC parameters of human PB-derived CD34+ cells was investigated for the frequently used concentrations of 90% FBS [20], the similarly established concentration of 50% FBS [21] and the substantially cheaper concentration of 30% FBS. The parameters analyzed were WBC yield, cell recovery and viability, CFC frequency and differentiation for both, normal control samples and thalassemic samples, and HSPC expansion, transducibility and differentiation potential for thalassemic samples.

Isolation of WBCs from fresh PB with density-gradient centrifugation did not give significantly different yield compared to the ACK lysis method for thalassemic samples (P = 0.68; Figure 2A,E) or normal samples (P = 0.074; supplementary Figure S1A,E). Processing using Lymphoprep and Ficoll-Paque had a median count of 16.60 × 10^5 (n = 5) and 15.15 × 10^5 (n = 8) MNCs/mL (P = 0.65), respectively, for thalassemic samples (Figure 2A) and of 35.60 × 10^5 MNCs/mL and 35.20 × 10^5 MNCs/mL (P = 0.57), respectively, for normal samples (supplementary Figure S1A), indicating that the number of viable cells per milliliter was not influenced by the type of density-gradient media used, in line with published results [13].

Processing using ACK lysis gave a 30%–35% increase in WBC yield compared with density-gradient methods for thalassemic (24.60 × 10^5 NCs/mL; n = 7; Figure 2A) and normal samples (49.70 × 10^5 NCs/mL; supplementary Figure S1A) alike. The observed trend was expected because the ACK-isolated NC fraction comprises MNCs and polymorphonuclear WBCs, whereas density-gradient centrifugation enriches MNCs [22]. Assuming an approximate ratio of 2:5 for MNCs to total nucleated PB cells [23], ACK

![Figure 1](image)

Figure 1. Schematic giving the processing paths of this study. Processing paths (A–C) are represented by differently colored arrows. For paths A and C, the whole blood sample was processed using one extraction protocol at a time. The final product was either used fresh for immediate application (A) or cryopreserved (B) after extraction. For assessment of lentiviral transduction (C), blood samples designated for fresh use were split in half for same-sample assessment of ACK lysis and Lymphoprep extraction. The key parameters compared were cell isolation (by ACK lysis, Lymphoprep, Ficoll-Paque) and freezing medium (30%, 50%, 90% FBS). Parameters for the evaluation of processing methods were cell yield, recovery and viability, CFU score and lineage distribution, and lentiviral transduction efficiency. Not all indicated alternative freezing media and cell extraction methods were tested for each processing path (see main text).

Leukocyte yield

Isolation of WBCs from fresh PB with density-gradient centrifugation did not give significantly different yield compared to the ACK lysis method for thalassemic samples (P = 0.68; Figure 2A,E) or normal samples (P = 0.074; supplementary Figure S1A,E). Processing using Lymphoprep and Ficoll-Paque had a median count of 16.60 × 10^5 (n = 5) and 15.15 × 10^5 (n = 8) MNCs/mL (P = 0.65), respectively, for thalassemic samples (Figure 2A) and of 35.60 × 10^5 MNCs/mL and 35.20 × 10^5 MNCs/mL (P = 0.57), respectively, for normal samples (supplementary Figure S1A), indicating that the number of viable cells per milliliter was not influenced by the type of density-gradient media used, in line with published results [13]. Processing using ACK lysis gave a 30%–35% increase in WBC yield compared with density-gradient methods for thalassemic (24.60 × 10^5 NCs/mL; n = 7; Figure 2A) and normal samples (49.70 × 10^5 NCs/mL; supplementary Figure S1A) alike. The observed trend was expected because the ACK-isolated NC fraction comprises MNCs and polymorphonuclear WBCs, whereas density-gradient centrifugation enriches MNCs [22]. Assuming an approximate ratio of 2:5 for MNCs to total nucleated PB cells [23], ACK
sis performed comparably to the density-gradient methods in isolating WBC fractions from fresh PB. Comparison of the leukocyte yield in freshly isolated cells from normal and thalassemic PB demonstrated a higher yield of approximately 50% with normal PB using either of the three leukocyte extraction methods, significant for ACK isolation \( (P < 0.0001) \), and with much lower sample-to-sample variation among normal compared with thalassemic samples \([24]\), whereas higher yields for normal samples are most likely caused by their swifter processing compared with thalassemic samples, which could be processed only 24–30 h after collection. Storage of PB samples 24 h or more after collection is known to reduce leukocyte yield significantly \([25]\).

For frozen samples, the viable WBC count did not differ significantly between MNC and NC fractions after freezing for either normal \( (P = 0.685) \) or thalassemic samples \( (P = 0.18) \) \((\text{Figure 2E and supplementary Figure S1E})\). Likewise, thalassemic and normal samples gave similar viable WBC counts after freezing across all three extraction methods \( (P = 0.786) \). Expectedly, cell numbers after cryopreservation fell for all three isolation methods compared with fresh preparations, down by 60%–80% and with extreme significance for normal samples, regardless of extraction protocol and freezing medium \( (P < 0.003) \) \((\text{supplementary Figure S1B–D})\). A similar trend was observed for the albeit more heterogeneous thalassemic samples, with significance in the reduction of viable cells only for the ACK-processed samples \( (P = 0.0014) \) and with overall progressive reduction for increasing FBS concentration in freezing medium, irrespective of cell isolation method \((\text{Figure 2B,D})\). Against the general assumption that cells are preserved better in higher serum concentrations, our own data in accordance with the work of others \([26–28]\) showed that freezing media containing 30% FBS performed at least

![Figure 2. Blood processing conditions and their effect on leukocyte counts. Yield is compared for extraction methods for fresh thalassemic samples (A) and for fresh samples against cells isolated using different cryostorage media for each extraction method, as indicated (B–D). Additionally, median, interquartile range (IQR) and the number of analyzed samples per treatment (n) are given in tabular form (E). Processing conditions performing significantly better among fresh or frozen samples are indicated by brackets. Equivalent data for normal samples are shown in supplementary Figure S1.](image)

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comparably to media with a higher FBS concentration in terms of WBC per milliliter.

Cell recovery and viability

Comparing the effect of isolation principle on recovery, total recovery of PB WBCs by density-gradient centrifugation was comparable to that for ACK lysis of thalassemic and normal samples, for both freshly isolated cells and frozen preparations (Figures 3A,C,E and supplementary Figure S2A,C,E, respectively). Comparing the effect of cryostorage on recovery, median recovery of ACK-isolated NCs was significantly decreased from between 64.12% for thalassemic (P < 0.0001) and 38.00% for normal fresh samples (P < 0.0023) to below 10% after cryostorage (Figure 3A and supplementary Figure S2A). Similarly, Lymphoprep-isolated MNCs had significantly decreased post-thaw recovery from 73.54% to below 12% for thalassemic samples (P = 0.0002) and from 20.28% to below 8% for normal samples (P = 0.018) (Figures 3C and supplementary Figure S2C), whereas for Ficoll-Paque-isolated MNCs, a drop of post-thaw recovery from an already low 20.29% to below 14% for thalassemic samples (P = 0.2708) and from 18.25% to below 9% for normal samples (P = 0.0191) was detected (Figure 3E and supplementary Figure S2E).

Comparing the effect of isolation principle on viability, density-gradient isolation resulted in significantly higher viability for thalassemic samples (P = 0.0008) compared with ACK lysis and in comparable numbers for normal samples (P = 0.46) (Figures 3B,D,F,G and supplementary Figure S2B,D,F,G) curiously, cryostorage significantly reduced the viability of ACK-isolated normal cells (P = 0.006) (supplementary Figure S2B), but not of thalassemic cells (P = 0.498) (Figure 3B), whereas it reduced the viability of gradient-isolated thalassemic (P < 0.01 compared with either gradient medium; Figure 3D,F), but not of normal cells (P > 0.299; supplementary Figure S2B).

In summary, of the three isolation methods tested, recovery for fresh cells in general was lowest for Ficoll-Paque gradient centrifugation, whereas viability was lowest for ACK lysis and highest for Ficoll-Paque gradient centrifugation. Overall, freezing media with 30%, 50% and 90% FBS performed comparably for the parameters of recovery and viability. However, freezing in general decreased recovery and significantly reduced viability of ACK-lysis-isolated normal cells and also of gradient-isolated thalassemic cells.

For practical purposes, the derived measure obtained by multiplying recovery with viability may be a key criterion for the choice of extraction and storage method because it calculates the percentage of viable cells relative to the number of cells in the initially processed cell population. Analysis of this parameter for subsets of samples gave no statistically significant difference by post hoc test, between any of the fresh sample preparations, among any of the thalassemic or the normal frozen sample preparations or between equivalent sample preparations for thalassemic and normal samples (not shown). A comparison across all samples (including both disease states and all isolation and storage conditions) detected significant differences only between fresh and frozen samples (Figure 4), indicating that for this criterion, all freezing media and isolation methods performed comparably, independent of disease state. For the same criterion, freezing in 50% FBS gave 1.12 × the average achieved for all three freezing media (with 0.97 × for 30% FBS and 0.91 × for 90% FBS) across all isolation methods and disease states, albeit without statistical significance. Likewise, across all storage methods and disease states, Lymphoprep gave 1.17 × the average achieved for all isolation methods (with 0.87 × for ACK lysis and 0.96 × for Ficoll-Paque gradient), thereby suggesting isolation by Lymphoprep gradient and freezing medium with 50% FBS as marginally preferable choices for the parameter of viable cells relative to the initial cell number.

CFC frequencies

The effect of different WBC extraction protocols and freezing conditions on the frequencies of hematopoietic progenitors, termed colony-forming cells (CFCs), was assessed using methylcellulose culture medium assays, followed by colony scoring and quantification based on colony-forming unit (CFU) size, morphology and cellular composition. The number of colonies provided a retrospective measure of the number and type of viable and functional CFCs in the samples.

In line with published observations [29] and despite its procedure-related longer storage time pre-processing, thalassemic PB (fresh and frozen preparations) had higher CFC frequencies than normal (by an average more than 113% across all test groups, comparing Figures 5E and supplementary Figure S3E). Analysis of freshly isolated normal cells showed a trend toward greater CFC frequencies using density-gradient centrifugation compared with ACK lysis, whereas the opposite held for thalassemic cells, albeit without overall significance for either observation (P = 0.11 and 0.587, respectively). Given the elevated number of progenitor cells in thalassemic PB, this resulted in comparable numbers of normal and thalassemic CFCs for Lymphoprep (870.90/mL, n = 3 vs. 901.3/mL, n = 4) and Ficoll-Paque density gradients (899.60/mL, n = 3 vs. 727.0/mL, n = 8), and a
Figure 3. Blood processing conditions and their effect on leukocyte recovery and viability. Median and interquartile range (IQR) of recovery and viability are shown for cells isolated from thalassemic samples using ACK RBC lysing buffer (A, B), Lymphoprep density-gradient (C, D) and Ficoll-Paque density-gradient (E, F). Median, IQR and the number of analyzed samples per treatment (n) are given in tabular form (G). Processing conditions giving significantly higher viability among fresh or frozen samples are indicated by brackets. Equivalent data for normal samples are shown in supplementary Figure S2.
striking difference for normal and thalassemic CFCs with ACK lysis (470.70/mL, n = 3 vs. 1231/mL, n = 6) (Figure 5A,E; supplementary Figure S3A,E).

Cryostorage markedly reduced CFC frequency for all sample groups, with significant reductions fresh to frozen for ACK-lysis-separated thalassemic samples (P = 0.0036; Figure 5A) and for all normal samples (P = 0.0003 or lower for each extraction method) (supplementary Figure S3). For gradient-isolated thalassemic samples, high variability interfered with detection of reductions in CFC numbers with statistical significance, including a 71% decrease from fresh...
to frozen for Lymphoprep-isolated cells (901.3 mL\(^{-1}, n = 4\) vs. 259.9 mL\(^{-1}, n = 7\)) for cryostorage in 30% FBS, and a 96.5% decrease from fresh to frozen for Ficoll-Paque-isolated cells (727.00 mL\(^{-1}, n = 8\) vs. 24.84 mL\(^{-1}, n = 12\)) for cryostorage in 90% FBS (Figure 5D). The choice of freezing medium had no significant effect on CFC frequencies, in contrast to the choice of isolation method. Reduction of CFC frequencies from fresh to frozen was greatest for ACK-lysis-isolated cells throughout, both for normal cells (to 13% from 470.7 mL\(^{-1}, n = 3\) to on average 59.4 mL\(^{-1}; n = 9\) (supplementary Figure S3) and for thalassemic cells (to 9%, from 1231 mL\(^{-1}, n = 6\) to on average 115.1 mL\(^{-1}, n = 25\) (Figure 5). Overall,
cryostorage of ACK-lysis-isolated thalassemic and normal samples gave only 21% and 27%, respectively, of the CFC frequencies achieved with density-gradient isolation. These findings showed that the cryostorage of ACK-isolated NCs had a detrimental effect on CFC survival, rendering the lysis method unsuitable for WBC cryostorage, at least without post-thaw expansion culture.

In summary, freezing media had negligible effects on CFC frequencies after cryostorage. Thalassemic samples had higher CFC frequencies than normal samples, and CFC frequencies were exceptionally and consistently high in fresh ACK-lysis-isolated thalassemic samples. However, for fresh normal samples and in particular for frozen samples of any type, density-gradient-isolated cells gave several-fold higher CFC frequencies than equivalent samples derived by ACK-lysis isolation.

**CFC differentiation**

The effect of different extraction and freezing protocols on the differentiation potential of extracted CFUs was assessed in CFC assays for enumerating multi-potential (e.g., CFU granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM)) and lineage-committed CFU granulocyte, macrophage, granulocyte/ macrophage (CFU-G/M/GM) and burst-forming unit erythroid (BFU-E) hematopoietic progenitor cells (Figure 6 and supplementary Figure S4).

Overall and in line with expectations for progenitor cells in unmobilized PB, BFU-E greatly outnumbered other colony types ($P < 0.0001$), with a rare observation of CFU-GEMM colonies (Figure 6A and supplementary Figure S4) [30], which were represented only in a fraction of samples and with only one or few colonies per sample. Thalassemic PB had a higher percentage of CFU-G/M/GM and a lower percentage of BFU-E than normal PB ($P = 0.0379$), as also shown by others for mixed $\beta$-thalassemia patient populations [31]. Across all samples, extraction method subtly but significantly changed CFU composition ($P < 0.0001$). Post hoc tests attributed this observation to the effect of few extraction and storage combinations, such as an elevated CFU-G/M/GM percentage, first for ACK-lysis-derived cells frozen in 50% FBS to all other preparations for both, thalassemic (Figure 6E) and normal cells (supplementary Figure S4E) and, second, for fresh normal cells isolated by Ficoll-Paque compared to ACK-lysis (supplementary Figure S4E).

Overall, relative frequencies of BFU-E, CFU-G/M/GM and CFU-GEMM were comparable across almost all combinations of extraction methods and post-extraction storage conditions, despite some statistically significant differences between individual combinations.

**HSPC expansion and differentiation potential**

The effect of blood processing (WBC extraction and/or freezing) on HSPC proliferative capacity and suitability to lentiviral transduction was assessed in thalassemic WBC samples cultured in expansion media designed to support CD34$^+$ primary cell growth. To avoid an increase of possible experimental permutations for these analyses, a choice of isolation methods and freezing media was made. Accordingly, small-volume blood samples were processed using ACK lysis or Lymphoprep density gradient, to juxtapose lysis- and gradient-based isolation methods, and this isolation was followed by immediate culture or by cryopreservation with 50% FBS-based freezing medium before culture. The choice of 50% FBS was in line with our own favorable results for viable cells after freezing as a proportion of the initial cell number (see Figure 4) and moreover complied with standard procedures [21,32] adopted for comparability of lentiviral work across a network of collaborators.

Owing to sample size, the present investigation avoided immunoaffinity-based CD34$^+$ cell isolation, application of which to small-volume blood samples would render the approach impractical. Instead and in line with existing work [33], the expansion protocol served to enrich and proliferate HSPCs from an initially heterogeneous cell population. In the process, a total of three samples were used for each patient, the first for cryostorage after extraction with ACK lysis, the second for cryostorage after extraction with Lymphoprep and the third divided in half for analysis of fresh samples after extraction of cells by ACK lysis and Lymphoprep, respectively. After 48 h in culture, WBCs were transduced, if applicable, and plated in methylcellulose cultures to measure the effect of blood processing on their clonogenic capacity and suitability for LV transduction.

Results for untransduced cells showed a reduction of cell numbers during initial expansion for all combinations of processing conditions, down to an average of 13% of initial cell numbers after 7 days, likely reflecting the depletion of terminally differentiated cells from all samples. Further extending the culture period over 11 days, however, resulted in subsequent rapid cell growth toward a more homogeneous cell population, as assessed by microscopy, in turn likely reflecting the efficient expansion of HSPCs after their enrichment during the first culture period (Figure 7). For cells frozen after isolation by ACK lysis and in line with the corresponding low CFC numbers (Figure 5 and supplementary Figure S3), expansion was inferior to that observed for cryopreserved gradient-isolated cell samples. Overall, the CFC assay demonstrated the ability of the expansion protocol to maintain the primitiveness of HSPCs in nucleated and
mononuclear cell fractions and to support their continued proliferation on day 11 to at least 5–50 times the progenitor cell numbers reported for unprocessed PB (assuming a range of 1000–9000 CD34+ mL⁻¹) [34], with continuous culture of cells expected to give additional cell growth.

### HSPC transducibility

Cryostorage of cells from small blood samples would allow laboratories without access to larger or stem-cell rich samples the collection over time of sufficient same-patient material to perform comprehensive diagnostic blood samples for cell-therapy studies
analyses of therapeutic approaches. To demonstrate this option we tested lentiviral transduction of thalassemic samples as an exemplary procedure that scales reliably for different cell numbers as starting material, facilitates scoring of the outcome in combination with reporter proteins and is currently applied in clinical trials for thalassaemia [2]. Where extracted cells were transduced using a GFP-encoding lentiviral vector (PGK-GFP), each of four cell preparations per patient were subdivided into three samples of equal cell number for mock treatment and transduction with PGK-GFP at a MOI of 10 and 50, respectively. Clonogenic activities of HSPCs after LV transduction were assessed as described earlier, and LV-mediated gene transfer in CFCs was confirmed by scoring CFUs for GFP fluorescence. Although only one sample was analyzed per processing condition, these data would serve to demonstrate the suitability of small-volume samples for lentiviral transduction and subsequent functional analyses. Overall, cell numbers were elevated in 48-h expansion cultures with all combinations of processing conditions, and with Lymphoprep density gradient isolation giving high CFC and GFP counts, irrespective of the MOI employed (Table I).

Taken together, our findings demonstrate the suitability of cell isolation from diagnostic PB samples by density gradient or ACK lysis and of the resulting cells for erythroid liquid cultures, transduction and functional analyses.

**Discussion**

**Overview**

This study investigated the feasibility of isolating and cryopreserving normal and thalassemic WBCs from small-volume PB samples for the functional analysis of HSPCs and the establishment of a biorepository. It investigated parameters important to stem cell and gene-therapy research after sample processing by cell
Thalassemic cells were processed as indicated, expanded for 48 h in liquid culture and transduced as specified. Values are given as absolute numbers for the initial inoculum, as relative fold expansion compared with the initial inoculum and as CFC progenitors per milliliter of initial blood volume used, respectively. GFP+ (%) are positive colonies as a percentage of all colonies grown in semisolid media after transduction or mock transduction.

**Sample condition**

In line with routine diagnostic procedures and the known variation between peripheral blood of different thalassemics [24], thalassemic samples varied in blood volume, WBC counts and the day of collection and processing. By contrast, normal samples were processed the same day shortly after sampling and therefore had small intra-group variation, owing to both their physiological state and the uniformity of their handling. Storage time and conditions are critical for cell viability and other factors [35], including post-thaw survival, with one study showing that blood processing 24 h after collection would result in decreased PBMC viability after thawing (~86%–92%), compared with processing within 8 h (≥94%) [8]. Factors aggravating intra-group variability for thalassemic samples include elevated numbers of progenitor cells varying with differences in disease management [29,31] and elevated numbers of nucleated RBCs, which also vary among thalassemia patients and may be miscounted as WBCs [36]. These factors particularly affect gradient-isolated thalassemic samples because nucleated RBCs are partially susceptible to cell lysis [37], while they are retained in the WBC fraction of gradient-based separation methods [38,39]. Although such differences inherent to thalassemic samples are unavoidable, immediate sample processing after collection might already substantially reduce intra-group variability and potentially achieve superior results for thalassemic samples for many or all of the parameters investigated.

**Application of density-gradient media and ACK lysis**

Density-gradient centrifugation remains the most commonly used procedure for initial separation of stem cells from whole blood. The density media used here for PBMC separation were the widely applied Ficoll-Paque and Lymphoprep, for which variation in cell-processing protocols [5,6] or other factors [13,40] has at times led to conflicting outcomes after HSPC isolation. Indeed, the density-gradient technique is highly skill-dependent and thus prone to inter-operator variability for purities and yields achieved [38]. Moreover, the method is labor-intensive, requiring approximately 50–60 min for each sample, which renders the simultaneous and standardized processing of many small samples impractical. This further implies that daily processing of numerous diagnostic samples could not simply be added onto existing staff duties but would require allocation of substantial dedicated staff time. Of note for this study, gradient media carry a marked cost for general processing of high numbers of diagnostic samples. Likewise and owing to the nature of the gradient method, the cell loss of 15%–30% of the initial content that is observed for standard samples [5,41], in addition to the depletion of specific sub-populations [42], will be exacerbated for small sample volumes. Despite these actual and potential shortcomings and in line with the general uniformity in sample handling, quantity and quality of gradient-isolated HSPCs in this study were high and, except in combination with specific freezing conditions, without marked differences between both gradient media.

ACK lysis is a fast and inexpensive method for the removal of RBC contaminations from PBWBCs. Importantly, the lysis protocol is straightforward and completed within 30 min for whole batches of samples. On the downside, RBC lysis affects different WBC properties [11], although this appears to reduce WBC viability only slightly [10], with ACK comparing favorably with alternative lysis buffers [7] and with hypotonic shock and ammonium chloride solutions reportedly retaining cellular viability and membrane integrity [43,44].

### Table I. Cell expansion and transduction efficiency after HSPC lysis and gradient isolation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACK (fresh)</th>
<th>ACK+ (50% FBS)</th>
<th>Lymphoprep (fresh)</th>
<th>Lymphoprep+ (50% FBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting cell number (×10⁶)</td>
<td>1.43</td>
<td>0.55</td>
<td>1.40</td>
<td>1.30</td>
</tr>
<tr>
<td>Fold expansion</td>
<td>0 h</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.56</td>
<td>1.49</td>
<td>0.78</td>
</tr>
<tr>
<td>CFC progenitors (×10⁶)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>352.62</td>
<td>353.62</td>
<td>454.64</td>
<td>530.16</td>
</tr>
<tr>
<td>MOI 10</td>
<td>203.63</td>
<td>206.28</td>
<td>546.88</td>
<td>393.68</td>
</tr>
<tr>
<td>MOI 50</td>
<td>357.59</td>
<td>134.71</td>
<td>724.79</td>
<td>440.93</td>
</tr>
<tr>
<td>GFP+ (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 10</td>
<td>9.76</td>
<td>42.86</td>
<td>53.01</td>
<td>57.33</td>
</tr>
<tr>
<td>MOI 50</td>
<td>27.78</td>
<td>81.25</td>
<td>60.91</td>
<td>65.48</td>
</tr>
</tbody>
</table>
Here, ACK lysis performed comparably for the criteria of WBC yield, recovery, viability, CFC frequency and differentiation potential in the isolation of viable WBCs for downstream application without freezing. For both isolation principles, cell viability after thawing was decreased compared with that of fresh samples. This was expected, with previous studies demonstrating the impact of freezing on PBMC viability, the extent of which was shown to vary with the method of cryopreservation [45]. In contrast to observations for fresh samples, however, cryopreserved ACK-lysis-derived samples compared unfavorably to gradient-isolated samples for CFC frequencies, cellular expansion in liquid media and other parameters, possibly indicating a sensitivity of progenitor cells to cell freezing after ACK lysis. Whatever the cause, a selection against progenitors by freezing after ACK lysis is an unequivocal result of this study. Applications, however, that include cell culture steps after thawing before functional analyses may overcome corresponding reduction of cell numbers and sensitization of cells to experimental procedures, with ACK-isolated samples after thawing and expansion performing well in CFC assays (see Figure 7 for 11-day and Table I for 48-h expansion) and giving a high level of transducibility (Table I). Similarly, a recovery period in culture before freezing of NCs obtained by ACK lysis may improve post-thaw parameters throughout, but this procedure would be impractical and costly for the processing of many samples and has was therefore not tested in this study.

Choice of sample processing and storage conditions

Extraction by any of the methods tested gave similar results for fresh samples, allowing the inference that none of the extraction methods in itself introduced a bias toward particular lineages. For any application of HSPCs from fresh samples, speed, economy and reproducibility therefore put forward ACK lysis as the isolation method of choice, in particular for small and numerous PB samples or where an expansion phase after freeze-thawing precedes analyses. However, for sample freezing generally, density-gradient isolation is preferred, without clear difference between the Lymphoprep and Ficoll-Paque media and with good performance and economy of freezing media containing either 30% or 50% FBS. In particular, freezing in 50% FBS gave favorable CFC progenitor frequencies (Figures 5 and supplementary Figure S3) and combined viability and recovery (Figure 4), albeit in the absence of statistical significance, in combination with different isolation protocols and for most samples, making this the general FBS concentration of choice.

Suitability for expansion and transduction

We showed that the PB WBC fractions comprise HSPCs that can be expanded in vitro from small-volume samples without prior enrichment for CD34+ HSPCs, tying in with a previous study that moreover demonstrated the substantial contribution of PB CD34+ cells to erythroid expansion in liquid cultures [33]. Overall, there are low numbers of circulating HSPCs in unmobilized PB (<0.01% of PB cells), at only 1% of the frequency found in bone marrow or cord blood samples [46], so that avoidance of further cell loss through selection procedures is of additional advantage for small sample volumes.

Finally, we investigated the transducibility of fresh and frozen cells, using 50% FBS for cell freezing and comparing gene transfer efficiency for a lentiviral PGK-GFP vector between WBC fractions of Lymphoprep and ACK-lysis extractions. Of note, the PGK promoter is subject to position effects and silencing over time [47,48], and cells with multiple integration sites are scored as single-positives for the GFP reporter assay used, so that the efficiencies reported here underestimate molecular events. Fresh and frozen samples of both cell-processing protocols after an expansion phase of only 48 h were permissive to viral transduction and analyses in clonogenic assays, with no adverse effect of freeze-thawing on cell viability and transduction efficiency (see Table I) and without change in progenitor frequencies.

Conclusions

Surplus small-volume blood samples are a resource for HSPC research that is readily available to many laboratories. This study showed that density-gradient isolation of HSPC-containing cell populations with either Ficoll-Paque or Lymphoprep equally allows the isolation and downstream experimentation based on these samples, including clonogenic assays, cell expansion and lentiviral transduction. Gradient isolation was suitable for direct cryostorage of isolated cells, preferably in freezing media containing 50% FBS, with good recovery, viability, transducibility, proliferation and without lineage bias of thawed samples. The alternative, fast and inexpensive isolation of HSPC-containing cell populations by ACK lysis, allows similar analyses at a fraction of the processing cost and time but is only suitable for analyses of freshly isolated cells or when allowing for cellular expansion after freeze-thawing.

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Appendix: Supplementary material
Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.11.007.